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**Host Elicited Germination and Mechanisms of
Penetration in Broomrape (*Orobanche* spp.)**

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Host Elicited Germination and Mechanisms of Penetration in
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HOST ELICITED GERMINATION AND MECHANISMS OF PENETRATION IN BROOMRAPE (*OROBANCHE* spp.)

BARD project NO. IS - 2170 - 92RC

Final Report

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Abstract

Orobanch is an important parasitic weed. For developing novel methods for its control a thorough understanding of crucial stages of its development is needed. Therefore the objectives of this project were characterization of *Orobanch* germination stimulants, analysis of mechanisms of haustorial penetration, and characterization and isolation of penetration enzymes.

The first highly potent natural germination stimulant for *Orobanch* was isolated from sunflower and identified by high-field 1D (^1H and ^{13}C), 2D (^1H - ^1H COSY, HMQC, HMBC)-NMR, GC/FT-IR, and GC/MS as costuslactone, a guaiane type sesquiterpene lactone that resembles strigol only in possessing a lactone moiety that is required for activity.

The first direct *in situ* evidence for the enzymatic nature of the infection process of a parasitic angiosperm was established. Pectin deesterification and depletion of pectins in host cell walls were shown adjacent to haustorial cells. Pectin methyl esterase and polygalacturonase were immunocytochemically detected in intrusive cells and in adjacent host apoplast. *Orobanch* tissues contain inhibitors of PGase activity. PME and three PGases were isolated from *Orobanch* calli. PME was characterized and purified, and antibodies were prepared against it.

This study presents novel findings regarding parasitism in *Orobanch*, which may help to open up new approaches for controlling broomrapes.

Objectives of the original research proposal

- (a) Characterization of *Orobanch* germination stimulants.
- (b) Histological and immunocytochemical analysis of mechanisms involved in penetration of host root tissues by intrusive cells of *Orobanch* haustoria.
- (c) Characterization and isolation of penetration enzymes.

**Isolation and Structure-Activity Study of Dehydrocostus Lactone: A
Natural Host Germination Stimulant for *Orobanche cumana* Wallr.
Produced by Cultivar Sunflowers(*Helianthus annuus* L.)**

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INTRODUCTION

Parasitic weeds of the genera *Striga* (Scrophulariaceae) and *Orobanche* (Orobanchaceae) cause severe reductions of the yields of graminaceous and leguminous crops in tropical and semitropical areas of the world (Musselman, 1987; Parker, 1986; Ramaiah, 1987). *Orobanche* spp., or "broomrapes", affect broad-leaf crops, e.g. sunflower, tomato, tobacco, lentils, broad beans and many other crops in temperate and semiarid regions. Seeds of these parasitic weeds generally remain dormant in the soil until germination is stimulated by the release of a chemical signal from a host plant (Brown, 1965; Riopel, 1983; Shaw et al., 1962; Whitney, 1978) or by suitable germination stimulants such as (+)-strigol (fig. 3). Unfortunately, the chemical signal is often produced by the root of a developing crop plant. As a result the parasite germinates and generally is in an excellent position to attach itself to the host root and extracts nutrients and water.

The chemical nature of natural germination stimulants occurring in the root exudates of host plants is of great interest as these may form a lead in designing selective germinating agents. Much attention has been focused on the isolation and identification of such germination stimulants. The first *Striga* germination stimulant identified, strigol (Cook et al., 1966, 1972), a sesquiterpene lactone, was isolated from the roots of cotton (*Gossypium hirsutum* L.), a non-host plant for *Striga*. Through the synthesis and evaluation of numerous analogs (GR-compounds) and precursors of strigol, many chemicals effective in stimulation of seeds of witchweed and broomrape have been discovered (Hassanali, 1984; Johnson and

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Rosebery, 1977; Johnson et al., 1976; Johnson et al., 1981; Pepperman et al., 1982; Saghir, 1979; Vail et al., 1985; Zwanenburg, 1986). There are several reviews on chemical germination stimulants for parasitic angiosperms (Musselman, 1980; Worsham, 1987). However, the first host-derived *Striga* germination stimulant identified was sorgoleone, from sorghum [*Sorghum bicolor* (L.) Moench] roots hair exudates (Chang et al., 1986). Subsequently several *Striga* germination stimulants viz. sorgolactone (Hanck et al., 1992), alectrol (Muller et al., 1992), strigol (Butler et al., 1993) have been isolated from host plants. Furthermore several natural and synthetic sesquiterpene lactones which share structural features of the lactone rings of (+)-strigol have also been evaluated as witchweed germination stimulants (Fisher et al., 1990). Different host plants produce different mixtures of stimulants, as demonstrated by chromatographic investigation (Visser, 1974, 1975).

A number of species of *Orobanche* have considerable economic importance in that they damage a variety of crops, particularly in Mediterranean, in S. E. Europe, the Middle East and some other areas. Many species of *Orobanche* have a very wide host range, some are more restricted, and strains with narrow host specificity may exist within a species. For example, *O. cumana*, sometimes regarded as a subspecies of *O. cornua* Loefl., appears to attack only sunflower and related Compositae (Joel and Jacobsohn, 1988). Although the literature on *Orobanche* is extensive, host specificity aspects of this parasitic weed has received little attention during recent years. A few articles have been reported in the literature on *Orobanche cumana* Wallr. (e.g. Barcinskij, 1934, 1935; Steffens et al., 1991) and *Orobanche minor* Sm. (Brown et al., 1951a, 1952), respectively. Germination stimulants for *Orobanche* species have not been reported from the root exudates of host plants. The objective of the present study was to isolate the active germination stimulant for *Orobanche cumana* Wallr. from cultivated sunflowers (*Helianthus annuus* L.), to elucidate its chemical structure and to identify critical structural features necessary for activity.

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MATERIALS AND METHODS

Abbreviations

NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum correlation; HMBC, heteronuclear multiple bond correlation; GC/FT-IR, gas chromatography/Fourier transform-infrared; GC/MS, gas chromatography/mass spectrometer; UV, ultraviolet; Si gel, silica gel; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; STL, sesquiterpene lactone

Organisms and compounds

Seeds of *O. cumana* and *O. aegyptiaca* were collected in Lower Galilee, Israel in 1994, from sunflower and tomato respectively. In virulence tests, *O. aegyptiaca* from this seed lot was able to parasitize tomato, potato, tobacco, eggplant, peanut, faba bean, pea, vetch, chickpea, carrot, parsley, celery, cauliflower, broccoli, radish, cabbage, rape, mustard, lettuce and sunflower; *O. cumana* only attacked sunflower and related Compositae. Seeds of *O. cumana* tested under the same conditions were distinguished from *O. cumans* by their wider host range and by the morphological characters described by Joel (1987).

Sunflower, *Helianthus annuus* L., was from local market, Ithaca, NY. Samples of GR-7 (Johnson et al., 1981, compound 7) and GR-24 (Johnson et al., 1981, compound 17, $R^1 = R^2 = H$) were a generous gift from R. E. Eplee (USDA-APHIS, Plant Protection and Quarantine Programs).

Reagents

Si-gel chromatography supplies from E. Merck. All other chemicals or solvents were reagent grade.

HPLC and TLC

HPLC was performed with a Beckman System Gold instrument with UV absorbance monitor and a Waters Radial-Pack C-18 column, 0.8 x 10 cm. Samples were purified by injection of 1mg/100 ml (5-100 mg/50 ml, for checking purity of the sample and other experiments) solutions in MeOH/H₂O (8:2) and eluted with a 30-min linear gradient of 50% MeOH in H₂O to 100% MeOH at 1ml/min in order to elute all injected compounds. UV

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absorption of the column effluents was monitored at 210 nm.

TLC was carried out on Merck aluminum-backed TLC sheets (si gel F₂₅₄), with visualization using vanilline-sulfuric acid spraying reagent (1% vanilline-0.5% H₂SO₄ in EtOH). Preparative TLC was performed on si gel plates (10 cm x 10 cm, 0.25 mm layer thickness) and developed in CHCl₃.

Germination assay

*Orobanch*e seeds were surface disinfested in bags sewn from 74 mm mesh nylon monofilament cloth, closed with a dialysis-bag clip, by treatment with 3% NaClO, 0.02% Photo-Flo 200 for 10 min and thorough rinsing.

Germination assays were performed on glass-fiber filters (Whatman GF/A). For preconditioning, each sample of 30 to 200 seeds was pipetted in a few drops of water onto a 21-mm filter resting on a 55-mm filter in a 60-mm plastic Petri dish, moistened with 1.4 ml sterile glass-distilled H₂O. The dish was sealed with Parafilm and incubated inverted (i.e. filter side up, so that most of the moisture remained in the filter instead of condensing on the lid) in a closed container in a dark incubator at 25°C. Preconditioning was complete in 7 days for both *O. aegyptiaca* and *O. cumana* (Matthews et al., 1991). After preconditioning, each 21-mm filter was transferred to a separate Petri dish containing a 55-mm filter in which first test sample solutions (in *n*-hexane, 300 ml) was poured, evaporated to dryness completely in a hood and then 1 ml of sterile glass-distilled H₂O was added, and incubated further under the condition described above. Except as noted otherwise, assay solutions were prepared by dissolving germination stimulant with ice-cold *n*-hexane. Germination was normally assessed after 5 days incubation in the presence of stimulant.

In the figures, % germination values with error bars indicate the mean $\pm$ SD of two replicate filters in the same experiment. G₅₀ values, calculated as described in Results, are reported as the mean $\pm$ SD of duplicate G₅₀ interpolations from paired concentration series in the same experiment. G₅₀ values for the sunflower stimulant were measured with *O. cumana*.

Preparation and purification of germination stimulants

To prepare root exudates, 200 shelled sunflower seeds were surface-disinfested in

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0.525% NaClO, 0.02% Photo-Flo 200 for 10 min, rinsed, and spread over a layer of cheesecloth that was stretched across the surface of 1800 ml H₂O in a 17-cm-diameter crystallizing dish. After 6 to 7 days at room temperature in the dark, at which time the accumulation of germination stimulant activity had leveled off, the seedlings were discarded and the liquid("exudate") stored at -20⁰ C or processed immediately.

Subsequent purification steps differed somewhat from preparation to preparation, but generally involved solid-phase extraction with Amberlite XAD-2 resin, organic solvent extraction, si-gel column chromatography and reverse-phase HPLC. The procedure was as follows. Sixty seven ml(settled volume) of Amberlite XAD-2, 20-50 mesh, was prepared by stirring with 200 ml 95% EtOH, then decanting and washing three times with 200 ml H₂O. The exudate from two sunflower preparations, 3500 ml, was filtered through Whatman # 1 and stirred with the Amberlite resin for 2 hr. The liquid was discarded and the resin washed twice with H₂O, then eluted by washing three times with 100 ml MeOH. The eluate was rotary-evaporated at 30⁰ C under vacuum to dryness and taken up in 4 ml EtOH. The material was concentrated to an oil by centrifugal evaporation, suspended in 2 ml H₂O, and extracted twice with 2 ml CHCl₃. The organic fraction was concentrated to dryness under N₂. Stimulant preparation at this or the previous stage of purification, stored at -20⁰ C, were used most of the experiments.

The yield of stimulants obtained from the above root exudates is insufficient for proceeding further purifications. For making a large quatity of stimulants in efficient ways, we developed the following method. To prepare stimulant extract, approx. 4000(308 g) shelled sunflower seeds were surface-disinfested as mentioned above for root exudates, spread on layers of moist paper towels placing on aluminum foils(each aluminum foil contained five moist paper towels, spreaded 80 100 conditioned seeds, paper towels and aluminum foils were sterilized before uses), rolled the foils, and put the rolls vertically in a sterile plastic beaker, covered with aluminum foil to germinate for 5-6 days, at room temperature.

After 5-6 days, the seedlings were removed gently from paper towels, and surface extracted with pet. ether. Generally, 650-700 g fresh mass of intact plant parts (In bioassay for *O.cumana*, we found that the germination stimulating agent was present in more or less amounts in all parts of the seedlings) were extracted by immersion in 1.5 L of pet. ether for 5

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min at room temperature with intermittent agitation. The crude extract was filtered on glass wool and the filtrate then concentrated to minimal volume on a rotatory evaporator under vacuum at 30° C. The oil-like dark-brown residue was stored with N₂ at -20° C until further experiments.

A glass column (38 cm x 3.5 cm) was filled with si-gel (150 g), 70-230 mesh and equilibrated with *n*-hexane. The crude pet. ether extract (15 g) was dissolved in 3 ml of *n*-hexane, chromatographed on si-gel and successively eluted with 300 ml of *n*-hexane, 200 ml of *n*-hexane/CHCl₃ (3:1), 300 ml of *n*-hexane/CHCl₃ (1:1), 400 ml of CHCl₃, 100 ml of CHCl₃/MeOH (99:1) and 200 ml of MeOH. Subsequent bioassay indicated that the residue obtained from CHCl₃ eluting fractions retained the original biological activity.

Further purification of the stimulants was done by preparative TLC of CHCl₃ fractions (0.6 g) followed by reversed-phase HPLC. Prep. TLC fraction (16 mg) of the stimulants was purified on HPLC. Fractions were collected in test tubes, evaporated the solvents to dryness, and then bioassayed. The most active fraction (1.5 mg) which appeared at the retention time of 22.3 min, was used for bioassay, identification, chemical reaction and other experiments. Purity was assessed by TLC and HPLC (fig. 1)

NaBH₄ Reduction of 1.

Dehydrocostus lactone (1 mg), dissolved in MeOH (0.3 ml) was treated with NaBH₄ (0.4 mg) in installments during 10 min and the reaction mixture kept at room temperature for 1.5 hours. After dilution with 3 N HCl, it was extracted with CHCl₃. The CHCl₃ layer was washed with H₂O and dried. Removal of CHCl₃ layer gave a liquid product (1.2 mg) which was purified by HPLC. The purified product 2 (fig. 2) was identified by ¹H-NMR and GC/MS as a mixture of an equal amount of dehydrodihydrocostus lactone (mokkalactone) and its C₁₁ epimer.

General Identification Methods

A Varian Unity 500 (500.618 MHz) was used for ¹H-NMR(including HMQC and HMBC experiments), Varian XL-200 (200 MHz) was used for ¹H-NMR and ¹³C-NMR was on a VXR- 400 S at 125.893 MHz; with stimulant dissolved in CDCl₃. GC/FT-IR analysis was performed on a Hewlett packard 5890 gas chromatograph coupled with a Hewlett packard 5965A infrared

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detector, using a HP-5 stationary phase coated capillary column (25 m x 0.32 mm ID, 0.52 mm film thickness, Hewlett packard). GC/MS analysis for the stimulant (1) and NaBH₄ product 2 was performed on a Hewlett packard 5890 Series II gas chromatograph coupled with a Hewlett packard 5970 Series mass spectrometer, EI mode at 70 eV, using a DB-5 stationary phase coated capillary column (30 m x 0.25 mm ID, 0.25 mm film thickness; J & W Scientific, California). For GC/FT-IR and GC/MS analysis oven temperature program 1 min at 60° C then 60° C to 250° C at the rate of 15° C/min and finally 15 min at 250° C was employed for both analysis.

RESULTS

On the basis of the bioassay results (Table 1), active compounds from plant root exudates were effectively partitioned into petroleum ether from the aqueous phase. Petroleum ether extraction of the root exudates, defatted with methanol, showed a stimulant activity profile

Table 1. Purification of *O. cumana* germination stimulant from cultivated sunflowers.

Isolates	Total Volume (L)	Total weight (mg)	1G ₅₀ (µg/ml)	Total G ₅₀ units	G ₅₀ u/mg
Root exudates	3.48	65.25	30	2,175	33.3
Root exudates trapped with amberlite resin	3.48	3.46	0.8	4,319	1,248
Defated pet. ether extract of root exudates	3.48	2.11	0.8	2,643	1,253
Dehydrocostus lactone		0.016	0.006	2,668	166,667

(G₅₀ units) very comparable to that observed in root exudates before the extraction. Collection of root exudates by adsorption on XAD-4 resin has been shown to be very effective (Young et al.,1982; Visser et al., 1987). Root exudates trapped with Amberlite XAD-2, and extracted with petroleum ether, showed comparable G₅₀ units with that of root exudates. Again, the recovery of total G₅₀ units in germination stimulant, dehydrocostus lactone (1) [Fig. 3] is very

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similar to that of root exudates before and after the extraction with petroleum ether. The amount of germination stimulant recovered from root exudates either by trapping with Amberlite XAD-2 or by partitioning with petroleum ether is not enough for further purification. The HPLC profiles (Fig. 1) showed the germination stimulant, dehydrocostus lactone (1) is present (solid black peaks) in petroleum ether extract of the roots of sunflower seedlings and the root exudates, respectively. The retention time (Rt) of this stimulant, eluted on HPLC was 22.3 min.

The active stimulant is also present in stems and leaves of the seedlings, cultivated in water as well as in moist paper towels (Table 2). Greater amounts of germination stimulant (1) and G_{50} units were found in defatted petroleum ether extracts of the exudates when seedlings were germinated in water. Table. 2 shows that the amounts and activity (G_{50} units) of the germination stimulant 1 (seedlings were germinated in water) was high in fresh stems. However, when the seedlings were cultivated in moist paper towels, fresh leaves possess the germination stimulant in greater quantity and higher G_{50} units than roots and stems.

Table 2. Quantification of dehydrocostus lactone(1) in root exudates, roots, stems and leaves of cultivated sunflowers by HPLC technique.

Plant's organ	Lactone 1 (mg) in defated Pet.ether ext.	Lactone 1 (G_{50} units) in defated Pet. ether ext.	Lactone 1 (μ g)/g fresh tissue.	Lactone 1 G_{50} units/g fresh tissue
Root exudate ^a	0.0159	2,650	0.0046 μ g/ml	00.77 G_{50} /ml
Root ^a	0.0064	1,067	0.317	52.82
Stem ^a	0.0091	1,517	0.758	126.42
Leaf ^a	0.0111	1,850	0.241	40.22
Root ^b	0.0531	8,850	0.900	150.00
Stem ^b	0.0232	3,867	1.137	189.56
Leaf ^b	0.3479	57,983	2.172	361.94

a. Sunflower seeds germinated in water, leaves have chlorophyll.

b. Sunflower seeds germinated in moist paper towels, leaves don't have chlorophyll.

1 G_{50} = amount giving half-maximal germination of *O.cumana* Wallr. in a 1-ml assay.

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Fig. 1. HPLC profiles demonstrating the presence of the germination stimulant, dehydrocostus lactone (1, retention time 22 min) in sunflower root extracts and root exudates, respectively.

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O. cumana is stimulated to germinate only by exudates from its host, sunflower (*Helianthus annuus* L.), while *O. aegyptiaca* Pers. was more sensitive to exudate from flax (*Linum usitatissimum* L.) [Steffens et al., 1991]. This specificity is mediated by different germination stimulants in the exudates rather than by different species-specific germination inhibitors. Fig. 2 shows that the % germination rates of two *Orobanche* species were different in response to the presence of inducer, dehydrocostus lactone (1). *O. cumana* and *O. aegyptiaca* germinated 54.5% and 23.4%, respectively at 4.3×10^{-7} M/L and 2.2×10^{-5} M/L dehydrocostus lactone (1).

Fig. 2. Relative response of *O. cumana* (filled circles) and *O. aegyptiaca* (open squares) to varying concentrations of dehydrocostus lactone.

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In order to prepare more germination stimulant, sunflower seedlings were germinated in moist paper towels and surface extracted with petroleum ether for 5 min. The petroleum ether extract was subsequently purified on Si gel column chromatography followed by preparative TLC and finally on HPLC. Fractions were collected based on UV absorption peaks at 210 nm and bioassayed each fractions at 1×10^{-6} mg/ml to 5×10^{-3} mg/ml concentrations with *O. cumana*. The most active and pure fraction was eluted at 22.3 min (Fig.1), induced half-maximal germination of seeds of *O. cumana* at 0.2608×10^{-7} M/L. Table 1 showed the total G_{50} units and G_{50} units/mg of 1.

The germination stimulant 1, a guaiane skeletal sesquiterpene lactone for the seeds of *O. cumana* was first isolated from costus root oil (petroleum ether extract of *Saussurea lappa* Clarke [Compositae]) [Ukita, 1939]. Later it was found in different organs of plants, e.g. roots of *Brachylaena transvaalensis* (Bohlmann et al., 1982), leaves and trunk bark of *Magnolia watsonii* (Kobayashi et al., 1984). After isolation of lactone 1 by Ukita, it was subsequently investigated by Crabalona, 1948; Naves, 1948; and Romanuk et al., 1956. The structure of dehydrocostus lactone (1) has been deduced from spectral and chemical evidence (Bhattacharyya et al., 1965). The chemical structure of 1 was elucidated by high-field ^1H , ^{13}C including two-dimensional (^1H - ^1H COSY, HMQC and HMBC)-NMR, GC/FT-IR, GC/MS as well as chemical analysis. Dehydrocostus lactone (1) was isolated as colourless oil. GC/MS showed a m/z (% intensity) 230 (M^+ , 55) in accord with a molecular formula of $\text{C}_{15}\text{H}_{18}\text{O}_2$. Its GC/FT-IR spectrum showed bands assignable to γ -lactone (1799 cm^{-1}) and vinylidene group (3100 , 1641 and 902 cm^{-1}). The unambiguous assignments of ^1H and ^{13}C -NMR data (Table 3) were established based on two-dimensional correlation spectra viz. ^1H - ^1H COSY, HMQC and HMBC. No unambiguous ^{13}C -NMR data of dehydrocostus lactone (1) has been available in the literature so far. The IR, ^1H and ^{13}C -NMR (Table 3) data are very similar to those previously reported for 1 (Bhattacharyya et al., 1965; Hikino et al., 1964; Kobayashi et al., 1984).

Structure-activity relationship studies strongly suggest that the C/D ring moieties of strigol (Fig. 3) are primarily responsible for bioactivity (Hassanali, 1984; Mangnus and Zwaneburg, 1991). It has been reported that the double bond is essential for structural

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Table 3. ^1H and ^{13}C -NMR data of Dehydrocostus lactone (1)

C	δ (ppm)	H	δ (ppm) *
1	47.85	1	2.94
2	30.53	2a	1.95
		2b	1.89
3	32.82	3a	2.53
		3b	2.53
4	151.43		
5	52.27	5	2.88
6	85.46	6	3.98, <i>t</i> (9.3)
7	45.36	7	2.89
8	31.16	8a	2.25
		8b	2.17
9	36.48	9a	2.50
		9b	2.50
10	149.45		
11	139.98		
12	170.49		
13	120.40	13a	6.23, <i>d</i> (3.4)
		13b	5.50, <i>d</i> (2.9)
14	112.85	14a	4.91, <i>brs</i>
		14b	4.83, <i>brs</i>
15	109.86	15a	5.28, <i>brd</i> (2.4)
		15b	5.08, <i>brd</i> (1.9)

Run at 500 MHz for ^1H and 400 MHz for ^{13}C in CDCl_3

* ^1H signals were multiplets, where coupling is first order; J is given in (Hz).

All protons assigned with the aid of ^1H - ^1H COSY and HMQC spectra.

All carbons assigned with the aid of HMQC and HMBC spectra.

activity of 'reduced' analogue of GR-24 (Zwanenburg et al., 1992). Based on above information, we prepared a reduced analogue 2 (Fig. 3) by NaBH_4 reduction of dehydrocostus lactone (1) having structural features of the lactone ring (C) of strigol (Fig. 3). Reduced product 2 was characterized as a mixture of an equal amount of dehydrodihydrocostus lactone (mokkalactone) and its C_{11} epimer by GC/MS [m/z (% intensity) 232 (M^+ , 18)] and ^1H -NMR (CDCl_3): d 5.18 and 5.04 (two *d*, $J = 2.2$ Hz, H-15a, b), 4.87 and 4.77 (two *brs*, H-14a, b), 3.91 (*t*, $J = 9$ Hz, H-6), 1.24 (*d*, $J = 6.9$ Hz, Me-13 β for mokkolactone), 1.23 (*dd*, $J = 6.9, 1.2$ Hz, Me-13a). The ^1H -NMR data of 2 are very similar to those previously reported for dehydrodihydrocostus lactone or mokkolactone and the NaBH_4 reduction product of dehydrocostus lactone (1) [Hikino et al., 1964; Bhattacharyya et al., 1965]. The bioactivity

Fig. 3. Structural formulae of (1) dehydrocostus lactone, (2) NaBH₄ reduction product of dehydrocostus lactone (dehydrodihydro costuslactone [mokkolactone]), and strigol.

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results of lactone 1 and its reduced product 2 is shown in Fig. 4. Germination stimulant activity in dehydrocostus lactone (1) was found much greater relative to 2 for the parasitic seeds *O. cumana* Wallr. Fig. 4 showed that the lactone 1 and its reduced product 2 was germinated 52.6% and 20%, respectively at 2.17×10^{-6} M/L and 4.31×10^{-5} M/L of their respective concentrations.

Fig. 4. Relative *O. cumana* germination activity with dehydrocostus lactone (1, open squares) and dehydrodihydro costuslactone [mokkolactone] (2, closed squares).

DISCUSSION

Germination of the seeds of parasitic flowering plants is triggered by highly specific germination stimulants. Host plants produce a mixture of different compounds which stimulate the germination of their respective parasitic flowering plants (Sunderland 1960, Visser et al., 1974). The most active germination stimulant for *Orobancha cumana* in the root exudates of cultivated sunflowers (*Helianthus annuus* L.) is a guaiane skeletal sesquiterpene lactone (STL), dehydrocostus lactone (1). Although lactone 1 was first isolated from a medicinal plant, *Saussurea lappa* Clarke (Ukita, 1939), the biological properties of it have not been evaluated.

STLs are characteristic secondary metabolites of the Compositae (Hegnauer, 1964; Herout, 1971; Bohlman, 1980). However much less is known about the biosynthesis, function and storage of STL. In the sunflower (*Helianthus annuus* L.) several STLs have been reported (Spring et al., 1981, 1982a). The function of these substances have been postulated to be chemical defenses of the plants against microbial infection and animal feeding. A few of these lactones have a strong cytotoxic and plant-growth inhibitory effects (Spring et al., 1982b; Spring and Hager, 1982). In cultivated sunflowers, the major STLs are of the germacranolide skeletal type esterified with angelic acid and containing a five-membered α -methylene- γ -lactone ring. These STL-angelates are strong antifeedants for Western corn root worm (Mullin et al., 1991). However, only two STLs were analyzed among the eight plus STLs, now known from cultivated sunflower (Melek et al., 1985; Spring et al., 1989; Mullin et al., 1991). STL having guaiane skeleton have not been reported from cultivated sunflower (*Helianthus annuus* L.).

Leaves and flower heads were the major tissues in sunflower accumulating STLs, while the roots, stems, cotyledons, pollen, and achenes did not contain detectable STL. In a recent study, it has been found that the STLs of sunflower are localized within the cuticular globe of a special type of glandular hair at the leaf surface (Spring et al., 1987, 1987). In present investigation, we found by HPLC that the germination stimulant for *O. cumana* is distributed in different amounts in all parts of cultivated sunflowers (Table 2). This was corroborated by

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the previous investigations by Barcinskij, 1935. The biosynthesis of STL in Compositae seems to be influenced by environmental condition (Shibaoka, 1961; Jamieson et al., 1976; Gershenzon and Mabry, 1984). Thus Table 2 showed that etiolated leaves contained greater amounts of germination stimulant 1 compared to leaves having chlorophyll.

Germination stimulant activity of dehydrocostus lactone (1) was found to be very comparable with that of root exudates (Table 1). The recovery of germination stimulant activity (G_{50} units) from root exudates by partition with petroleum ether, indicated the inducer for *O. cumana* is highly hydrophobic. This was also supported by its retention time (Rt), 22.3 min in the HPLC profile (Fig. 1). It was known that the compound to be tested as germination stimulant was hydrophobic and solutions of known concentrations would be difficult to obtain without the use of a carrier. The purpose of the carrier is to obtain solution of the compound prior to dilution with water. Carriers were used in closely related work, but the carrier often caused significantly higher germination even after the samples were well diluted with water (Pepperman et al., 1982). We consider it more useful to test all samples in absence of a carrier in water solution. In our assay, we used *n*-hexane as a carrier for preparing test sample solutions with known concentrations. After pouring the test sample solution onto a glass-fiber-filter, *n*-hexane was evaporated completely to dryness and then 1 ml of water was added onto the same filter having test compound. Using of *n*-hexane in our assay method, any germination effect on the parasitic seeds, *O. cumana* has not been observed.

Germination stimulant 1 for *O. cumana* was isolated as colorless oil. Its molecular formula was established as $C_{15}H_{18}O_2$ by GC/MS: m/z (%) 230 (M^+ , 55). GC/FT-IR spectrum of 1 showed the presence of γ -lactone (1800 cm^{-1}) and vinylidene (3100 , 1641 and 902 cm^{-1}) groups. The ^1H -NMR spectrum (Table 3) exhibited signals typical of three exocyclic methylene double bonds (1H, *brs*, δ 4.83; 1H, *brs*, δ 4.91; 1H, *brd*, $J=1.9\text{ Hz}$, δ 5.08; 1H, *brd*, $J=2.4\text{ Hz}$, δ 5.28; 1H, *d*, $J=2.9\text{ Hz}$, δ 5.50; 1H, *d*, $J=3.4\text{ Hz}$, δ 6.23) at C-14, C-15 and C-13, and a 1H as a triplet at δ 3.98 ($J=9.3\text{ Hz}$) for the proton attached to the carbon bearing the lactone function at C-6 (δ 85.45 ppm). The attachment of the γ -lactone moiety between C-6 and C-7, and one of the exocyclic methylene double bonds conjugated to γ -lactone in 1 was supported by ^1H - ^1H COSY, HMBC spectra and NaBH_4 reduction. COSY experiments confirmed the relationship between H-6 (δ 3.98, *t*) and H-5 (δ 2.88, *m*), as well as between H-6 and H-7 (δ 2.89, *m*). In addition, a

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weak 4-bond correlations (W-type) also shown between H-7 and H-13a (δ 6.23, d , $J=3.4$ Hz) and H-13b (δ 5.50, d , $J=2.9$ Hz), respectively. In the HMBC spectrum of **1**, the carbon signals (C-7) at δ 45.36 and (C-12) at δ 170.4 showed correlations with the proton signals (H-13a and H-13b) at δ 6.23 and 5.50, respectively. The ^1H -NMR spectrum of NaBH_4 reduction product **2** resembled that of **1**, except for the presence of α - and β -methyl signals (δ 1.23, dd , $J=6.9$ and 1.2 Hz and δ 1.24, d , $J=6.9$ Hz) in place of a α -methylene double bond in γ -lactone function. The stereochemistry of the γ -lactone function at C-6 was determined as α on the basis of the coupling constant of H-6. Thus H-6 signal shows the trans-diaxial disposition of the protons at C-5 (α), C-6 (β) and C-7 (α).

The remaining two exocyclic methylene double bonds in **1** were located at C-4 and C-10, supporting by ^1H - ^1H COSY and HMBC spectra. In COSY spectrum of **1**, the proton signals (H-3) at δ 2.53 and (H-5) at δ 2.88 showed 4-bond correlations (W-type) with the proton signals (H-15a and H-15b) at δ 5.28 and 5.08, respectively. In HMBC experiments, the carbon signals (C-3) at δ 32.82 and (C-5) at δ 52.27 showed correlations with the proton signals (H-15a and H-15b) at δ 5.28 and 5.08, respectively. Again, the carbon signals (C-1) at δ 47.85 and (C-9) at δ 36.48 showed correlations with the proton signals (H-14a and H-14b) at δ 4.91 and 4.83, respectively. Other ^{13}C - ^1H long-range correlations were also shown in **1** between C-1 and H-2a, H-2b, H-9a, H-9b; C-4 and H-2a, H-2b; C-5 and H-1, H-2a, H-2b; C-6 and H-1, H-5; C-7 and H-8a, H-8b, H-9a, H-9b; C-10 and H-1, H-2a, H-2b, H-8a, H-8b.

The ^{13}C -NMR spectrum of **1** had not previously been reported unambiguously. We rigorously assigned the ^1H - and ^{13}C -NMR spectra (Table 3) with the aid of ^1H - ^1H COSY, HMQC and HMBC experiments. The ^{13}C -NMR spectrum of **1** (Table 3) exhibited signals for seven methylene, four methine and four non-protonated carbons further confirming its molecular formula of $\text{C}_{15}\text{H}_{18}\text{O}_2$. The presence of a α -methylene- γ -lactone moiety in **1** was also corroborated by the carbon resonances, appeared at δ 45.36 (C-7), 85.46 (C-6), 120.40 (C-13), 139.98 (C-11) and 170.49 (C-12). Again, the downfield shifts (ca δ 7.6 and 10.5 ppm) of C-13 signal (δ 120.40) relative to C-14 (δ 112.85) and C-15 (δ 109.86) and upfield shifts (ca δ 11.5 and 9.5 ppm) of C-11 (δ 139.98) relative to C-4 (δ 151.43) and C-10 (δ 149.45), indicating the exocyclic methylene (C-13) conjugated to γ -lactone moiety in **1**. Analyses of 1D- and 2D-NMR spectra including COSY, HMQC and HMBC, led to the conclusion that **1** was a

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(1a, 5a, 6a)-form dehydrocostus lactone.

The biogenesis of dehydrocostus lactone (1) may proceed through a germacranolide type STL precursor (Yoshioka et al., 1973). This supported by the recent isolation of seven germacranolide-type STLs from cultivated sunflower (*Helianthus annuus* L.) which contain a similar five-membered α -methylene- γ -lactone ring [Mullin et al., 1993].

In recent studies, several natural and synthetic germacranolides, eudesmanolides and pseudoguaianolides have been evaluated as witchweed germination stimulants (Fisher et al., 1990). The findings indicated that it is the spatial arrangement of the STL skeleton in conjunction with the five-membered lactone ring which is important in the promotion of witchweed germination, rather than the presence of any specific functional group on the molecule. The structure of these STLs is similar to portions of the active molecule (+)-strigol (Fig. 3). Analysis of the bioactivity of (+)-strigol analogues showed that the CD part of these molecules is primarily responsible for germination stimulation of seeds of parasitic weed. It has also been noticed that the double bond in enol ether is essential for stimulatory activity in reduced analogue of GR-24 (Zwanenburg et al., 1992). Again, α -methylene- γ -lactone has also been reported a major functional group for biological activity in diverse STLs (Rodriguez et al., 1976).

For studying the structure- germination stimulant activity of 1 and based on the above information, we prepared a reduced analogue 2 (Fig. 3) by NaBH_4 with germination stimulant, dehydrocostus lactone (1) which possessing the structural features of the lactonering (C) of strigol (Fig. 3, shown with dotted square). Reduced product 2 was characterized by GC/MS and $^1\text{H-NMR}$ as an equal amount of dehydrodihydrocostus lactone or mokolactone and its epimer at C_{11} . The germination stimulant activity results of the reduced product 2 (Fig. 4), certainly indicated that the most active site of the germination stimulant 1 for *O.cumana* is an exocyclic methylene double bond conjugated with γ -lactone function. This finding was also corroborated by the stimulatory activity for reduced analog of GR-24, in which the enol ether carbon double bond has been replaced by a carbon single bond (Zwanenburg et al., 1992). Hence the molecular mechanism for germination stimulation could involved addition of a nucleophilic species at the conjugated exocyclic methylene double bond in γ -lactone moiety of 1, in a Michael addition fashion (Zwanenburg et al., 1992).

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Penetration Mechanism of *Orobanche* Haustorium.

1. Host Root Penetration by *Orobanche*: Ultrastructure of Host and Parasite Cell Walls in the Infection Site.

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INTRODUCTION

All parasitic plants develop a haustorium, which is a multicellular organ that penetrates the host tissues and serves as a physiological bridge between their vascular system and that of the host (Kuijt 1969, Musselman and Dickison 1975, Stewart and Press 1990, Press and Graves, 1995, Riopel and Timko 1995). Crucial steps in the penetration of intrusive cells of the parasite into host tissues are not well understood and need further investigation.

Some work has previously been done on the development of the haustorium in *Orobanche*, mainly in the studies of Krenner (1958), Dörr and Kollmann (1974, 1975, 1976), Attawi and Weber (1980) and Aber (1984), nevertheless the mode of penetration remained unclear. The question commonly addressed in this context is whether penetration is *mechanical* or *enzymatic*, i.e. whether the intrusive cells use mechanical pressure to force their way through host tissues, or use enzymes to facilitate a smoother penetration. As discussed in the previous section of the report, a third possible mechanism also exists, namely *coordinated* penetration. Recently, we documented the release of pectin methylesterase from seedlings and infective calli of *Orobanche* (Ben-Hod et al. 1993) that indirectly support the possibility that enzymes are involved in penetration, but there was so far no direct evidence for any involvement of wall degrading enzymes in the penetration process itself.

The following models predict possible symptoms of *mechanical*, *enzymatic* and *coordinated* penetration mechanisms of digitate intrusive cells.

In all cases, symptoms of penetration should be classified in two groups: primary symptoms,

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which are caused by the primary agent of penetration on its action along the penetration axis, and secondary symptoms that are caused after the primary agent has facilitated the first steps of penetration in any given site along its route toward the vascular system of the host. In the following we list the possible primary symptoms.

Mechanical penetration: when intrusive cells apply mechanical pressure to force their way through host tissues.

This pressure can be applied in various directions:

A. When an intrusive cell advances between host cells (Fig. 1), and applies pressure that separates cells by rupture of the middle lamella, the remnants of the middle lamella may remain attached to the separated walls (Fig. 1A). in case that the middle lamella is tightly bound to the cell walls, the compound wall (composed of the middle lamella and the two

Figure 1. Penetration of digitate intrusive cell between host cells. Models of the resulting symptoms of possible mechanisms. A-B: mechanical penetration. C-D: enzymatic penetration. Above: host and parasite cells before penetration. P = parasite cell, H = host cell. See text for details

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neighboring walls) may split in an irregular way, and wall remnants may attached to both walls (Fig. 1B).

B. When pressure is directed perpendicular to a host cell wall (Fig 2), three different symptoms may arise:

(a) the wall may rupture, facilitating penetration of intrusive cell into host cell (Fig 2A). In this case living parasite cells may be seen inside dead host cells.

(b) host wall may stretch and consequently become thinner, facilitating penetration of intrusive cell into host cell without causing direct damage to host cell (Fig 2A). In this case stretched host cell walls will cling to the intrusive cell walls (Fig. 2B), and living parasite cells will be seen inside living host cells..

(c) host cell may crush. In this latter possibility a whole host cell corner may be pushed inwards (Fig. 2C) so that the host cell wall may fold while maintaining its original thickness, and changing the shape of host cell. In extreme cases parts of cells or whole cells may flatten

Figure 2. Penetration of intrusive cells *perpendicular* to host cells. Models of the resulting symptoms of possible mechanisms. A-C: mechanical penetration. D: enzymatic penetration. Above: host and parasite cells before penetration. P = parasite cell, H = host cell. See text for details

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between other cells, only their walls remaining attached to the walls of neighbouring cells.

Detection of such walls will be possible due to triangular intercellular cell corners within the walls (cf. Joel and Fahn 1980).

Enzymatic penetration: when intrusive cell releases enzymes to facilitate a smooth penetration.

The primary symptoms of this type of penetration are changes in wall composition. Enzymes either change the composition of middle lamella and/or host cell walls, or degrade them.

A. When the intrusive cells advance between host cells.

(a) degradation of pectins may occur. In this case neighboring host cell walls may remain intact while the middle lamella may dissolved (Fig. 1C). This degradation would not cause significant changes in the overall shape of host cells unless other agents, mechanical or/and coordinated are accompanied with it, allowing the intrusive cells to occupy space that was previously occupied by neighbouring host cells.

(b) wall components like cellulose and hemicelluloses may enzymatically be degraded. In this case wall portions of host cells would disappear and host cell walls would either become thinner, or disintegrate, or disappear (Fig. 2D) in contact areas with intrusive cells. Another possibility is that wall components may change their chemical composition rather than disintegrate, leading to changes in wall texture and reducing resistance to parasite advance. In this case we may see swellings of portions of host cell walls.

B. When the intrusive cell advances in a direction perpendicular to a host cell wall, wall dissolution would facilitate penetration of the intrusive cell into host cell (Fig. 1D). In this case the cytoplasm of the host cell may remain viable, and living parasite cells would be seen inside living host cells.

Coordinated penetration: when intrusive cells stimulate changes in the host that facilitate its intrusion. Growth of parasite cells may then be coordinated with that of neighboring host cells, with no damage to cells and cell walls of host cells. In this case redifferentiation, growth and division of host cells would be expected to open schizogenous gaps that facilitate parasite penetration.

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Summary of possible penetration mechanisms:

Mechanism	Expected Symptoms in host
1. Mechanical penetration	no changes in chemical composition of the wall.
A. between host cells	cell separation, intercellular rupture, wall remnants.
B. perpendicular to cell wall	(a) wall ruptured. Parasite cells inside dead host cell. (b) wall stretched, cling to intrusive cell. Parasite cell inside live host cell. (c) cells crushed, changes in cell form and dimensions. Folded cell walls. Intercellular cell corners within walls.
2. Enzymatic penetration:	changes in host cell wall composition.
A. between host cells	(a) degraded middle lamella. (b) wall thinning, degradation or disappearance. (c) change wall texture, swellings.
B. perpendicular to cell wall	(a) wall dissolves. Parasite cell inside living host cell.
3. Coordinated penetration:	(a) no chemical changes in wall composition. (b) no breakdown, rupture or collapse of host cells. (c) redifferentiation, growth and division of host cells.

MATERIAL AND METHODS

Plant material

Orobancha cumana Wallr. was grown on sunflower (*Helianthus annuus* L.); *O. aegyptiaca* Pers. was grown on tomato (*Lycopersicon esculentum* Mill.). The plants were grown in three different systems:

- (a) In pots, using the "sandwich method": surface-sterilized *Orobancha* seeds were spread between two layers of glass-fiber paper (Whatman GFA) placed in sterile sand. Young host plants were then planted in the pots. Some of their roots grew into the space between the two GFA layers, reached the *Orobancha* seeds, stimulated their germination, and were eventually infected by the young seedlings of the parasite.
- (b) *In vitro* in aseptic transparent bags, modified from Parker and Dixon (1983) as

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follows: surface-sterilized and antibiotics-treated *Orobanch*e seeds (Losner-Goshen and Joel, 1996) were preconditioned for germination for 10 d in Petri dishes on sterile agar medium (0.8% agar in water), and transferred inside a sterile "Biohazard" bag containing glass-fiber paper (Whatman GF/A) on which a host plant has grown aseptically and developed a root system. Sterile Haugland solution was added to the bottom of the bag. The *Orobanch*e seeds were placed adjacent to young developing host roots and were allowed to germinate and infect the roots. The whole system was strictly kept sealed under sterile conditions, in a growth chamber.

Preparation of plant material for microscopy

Infected roots were fixed either in glutaraldehyde followed by OsO_4 , dehydrated and embedded in either AGAR 100 resin or LR. White. 2-4 μm sections were prepared using Ralph glass knives on a rotary microtome. The sections were stained with toluidine blue and basic fuchsin and examined under the light microscope. For electron microscopy, ultrathin sections were prepared with a diamond knife and mounted on nickel or gold grids. Some grids were stained with uranyl acetate and lead citrate, other grids were either stained for carbohydrates using PATAg (periodic acid-thiocarbohydrazide-Ag proteinate, Roland and Vain 1993).

RESULTS

Young intrusive cells penetrate host tissues while elongating without dividing. Each intrusive cell extends from the outer haustorium, outside the host root, to the vascular cylinder of the host root (Fig 3). The number of primary intruding cells varies. In some cases only few digitate cells intrude the host separately and penetration changes the shape of a few host cells, but does not cause any real destruction in host tissues. In other cases a group of cells form an intrusive body. This organ pushes forward, crushing cortex cells of the host root.

An osmiophilic secretion filled the narrow gaps between the cell walls of the intrusive cells and that of neighboring epidermal cells (Fig 3C). A continuous layer of secretion was also found along neighbouring free surfaces of the parasite's radicle, outside host root

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Fig. 3. Intruding cells of *Orobanche* in host tissues. A. Light micrograph giving a general view of young infection site, with intrusive cells (P) of *O. cumana* reaching the vascular cylinder (V) of sunflower root. x400. B-C Young infection site, with intrusive cells (P) of *O. aegyptiaca* in tomato root (P). Osmiophilic material (arrows) at interface between parasite and host cells in the epidermal and outer cortex regions of host root. B. Light micrograph showing the osmiophilic material also coating the parasite outside host tissues (arrowheads). x400. C. Electron micrograph of the area framed in Fig. 3B, showing the osmiophilic material at the interface between parasite cell and epidermal cell. x16 700.

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(Fig. 3B), indicating that the secretion originates from the parasite. The contact between parasite cells and inner root tissues of susceptible hosts is not always tight, and rarely includes any secreted substance. In incompatible systems, i.e. where *Orobanch*e attacked non-host plants with a hypersensitive reaction (material kindly provided by Y. Goldwasser), the osmiophilic secretion coated the whole interphase between host and parasite that extended from the epidermis to the vascular cylinder. Hand-cut sections of fresh material revealed that this osmiophilic material is a brown-yellow. It occurred in intercellular space and in vascular cavities few vessel elements: it fills the whole cross section of a few (or single) vessels, or it coats the inner side of the vessel cell wall. In longitudinal sections we saw that the portion of the vessel which was filled with the substance is limited in length, normally occupying only a single vessel element. There has always been a continuity between the substance in xylem elements and that coating the haustorium. In fact, the highest concentration of this substance was in host-parasite interface, judging both by density (looks red-brown-to almost black, according to section thickness) and by section area occupied by the substance.

In no host root did we find any sign of host cell proliferation that was induced by the invasion of the parasite. The increase in the diameter of host root at the site of infection was found to be due to haustorial growth inside the root.

Ultrastructural analysis of specific sites along the penetration route revealed the nature of the cell walls at the interface between host and parasite tissues. Symptoms of *mechanical* penetration, similar to those demonstrated in Fig 2C were found in all internal tissues of host roots. Condensed stacks of walls of crushed cortex cells often occurred in the immediate vicinity of intrusive cells. In other cases cell walls of cortex cells became considerably thinner and cling to the intrusive cell walls, as predicted in the model (Fig. 2B). Some other host cells at the periphery of the haustorium were at least partly crushed.

In host epidermis the wall of intrusive cells was tightly bound to the walls of the neighboring epidermal cells (Fig 3BC), Host epidermis cell walls remained intact with unchanged thickness compared to non-infected regions. In the endodermis, intrusive cells

Fig. 5. A-B. Intrusive cells inside host endodermis. A. General view showing an intrusive cell crushing an endodermal cell (a), whereas another endodermal cell (b) is crushed between two intrusive cells. B. Part of A. Intact Casparian is seen strip between the two endodermal cells (a) and (b). The parasite cell wall is coated with a looser endodermal cell wall to which it has attached. C. Remains of a host cell wall (a) captured between an intrusive cell (bottom) and two host cells (b and c). Intercellular wall space evident within the compound wall.

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were seen between host walls.

Intact Casparian strips were not found adjacent to intrusive cells. Cell walls of crushed host cells were often present between intact Casparian strips and parasite cells (Fig. 5B). Crushed host cells were found around and within *Orobanche* intrusive body (Fig. 5A). The walls of these crushed cells were folded, some of them forming a compact barrier between the endophyte and the living host cells (Fig 5C). Fig. 5A shows an intrusive cell pushing a wall of one elongated parenchyma cell (cell *b*). The narrow space between these cells captures the crushed cell walls of another cell (cell *a*), which was pushed by the intrusive cell before. The remains of cell *a* can be followed on the other side of the intrusive cell, where they were captured between two intrusive cells. This figure illustrates how cell walls can be followed and how the boundary between host and parasite cells was analyzed.

In a few cases host cell walls were considerably changed showing typical swellings, which are symptoms of *enzymatic* penetration (Fig. 6). This could be found only on the boundary with cells of the central vascular cylinder. In such areas partial dissolution of walls was also found (see next chapter).

DISCUSSION

There has been a long controversy as to the mechanisms involved in haustorial penetration. The results presented in our study give evidence to a multi step process, that corresponds to the wall features of the various host root tissues.

Anchoring of the parasite to host surface is a prerequisite to a successful penetration of intrusive cells. In a previous paper (Losner et al. 1991, Joel and Losner 1993) we described the attachment of the young haustorium to host root, which is facilitated by a secreted substance. Our recent results reveal that the first stage of penetration is associated with secretion of a similar adhesive substance that allows internal anchoring of the parasite to the host. Cell walls of the intrusive cells are bound to anticlinal cell walls of the root epidermis, together forming the 'penetration neck' of the haustorium. The

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Fig. 6. Wall swellings in boundary between host and parasite cells in central cylinder of tomato root infected with *Orobanchae*. A. General view of the infection site. x3400. B. Magnified portion of A showing the swollen portion of host cell wall adjacent to intact parasite cell wall. x 22,800.

contact between host and parasite cells in other root tissues is not always tight, and rarely includes any secreted substance. A similar substance was described by Baird and Riopel (1986) for *Conopholis* (Orobanchaceae) and by Krause (1990) for *Striga* (Scrophulariaceae). Our observations that the interface between the parasite and resistant hosts contains a secreted substance resemble, in part, those described by other researchers for resistant sunflower (cf Dörr et al. 1994). They describe the walls of the xylem elements at the point of penetration as "more darkly stained and up to twice as thick" compared to non-infected vessels. It was thought that this phenomenon is a kind of a host xylem reaction. Dörr (personal communication) was in the opinion that the "additional wall layers" are deposited by differentiating young xylem cells at the time of broomrape invasion.

Examination of living and unstained rather than fixed plant material has proven highly informative. Based on these observations we believe that the parasite, not the host, is the source of the brown-yellow substance in the apoplast of the vascular cylinder at the incompatible infection site, for several reasons:

- (a) there is a continuity between this substance, in all its locations, along the parasite haustorium.
- (b) the substance occurs also in compatible interactions, though only in limited areas around the penetration neck, away from tissues that are loaded with it in incompatible interactions.
- (c) the substance seems to occupy only apoplastic areas in host tissues.
- (d) in the host it occurs near a variety of different cell types, including non-living cells.
- (e) Secretion was observed in all cases where penetration was blocked, no matter ~~at~~ which host did it meet.

The nature of this substance is obscure, still it is clear that contrary to previous opinions, it is not lignin. Its natural color might have been the reason for its misinterpretation as being lignin.

It is hard to explain the biological meaning of a substance that is secreted in excess at

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the parasite's surface area inside a incompatible host roots. We are in the opinion that this secretion is the parasite's reaction to difficulties in penetrating host root tissues. We suggest that broomrape behavior in resistant host roots is analogous to that of the Santalaceae, where haustorial folds are repeatedly developed in the outer surface of the haustorium, again and again building a new external anchoring device that eventually enables the intrusive body to penetrate host tissues (Kuijt 1969). *Orobanche* secretes additional adhesive substance that may provide an effective internal anchoring to allow complete penetration and connection to host vascular systems. This hypothesis may explain the few cases where we observed an osmiophilic substance along the well developed host-parasite boundary in internal host tissues. If this hypothesis proves true, the explanations hitherto given for resistance mechanisms of broomrape hosts, based on a description of an extra lignification of host vessel elements, need a substantial revision. Our three dimensional reconstruction of the infected zone (Joel et al. 1993) revealed that penetration through host tissue by the parasitic intrusive cells does not involve any dissolution of host cells, in contrast to Krenner (1958) that described a complete disappearance of parts of the cell walls of the cortical parenchyma in infected sunflower roots. At the same time, however, host cells at the infection zone are not only pushed aside; their shape is changed by intrusive cells occupying the space formerly held by them. Thus, while certain parts of elongated parenchyma host cells are untouched and show normal shape and wall structure, other parts are completely distorted, their cell wall greatly extended and pushed aside (Joel et al. 1993). The identification of totally crushed cells was facilitated using the method of Joel and Fahn (1980) who distinguished between tightly bound cell walls according to the appearance of cell corners within walls. According to these authors, a lysigenous corner-space is formed when a cell is destructed, and the space it releases is partly captured by neighbouring cells that eventually form a new region of contact, narrowing the space between them whereby the cell walls of the destructed cell are bound between them. The triangular intercellular spaces that remain within the boundary walls are perhaps the most significant evidence of the multi-layer origin of these compound walls.

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Previous studies suggested a schizogenous penetration of the intrusive cells into host tissues, i.e. penetration between host cells by enzymatic dissolution of the middle lamella with no damage to the cells themselves. Dörr and Kollmann (1974) showed degradation of middle lamella and primary cell wall regions around intrusive cells of *Orobanche* which is consistent with hydrolytic activity. Our results point to a more complex process, involving both enzymatic activities that dissolve certain wall components (see next chapter), and the mechanical pressure that pushes portions of cell walls aside, or extends host cell walls in these regions. This is clear ~~for the~~ in particular in cortex parenchyma. Penetration through the endodermis (Joel et al 1993) and through the vascular cylinder was shown to be the result of enzymatic separation between neighboring endodermal cells (see next chapter), while endodermal cells near the penetration sites are pushed aside and their Casparian strips remain intact, though between crushed cell walls.

Okonkwo and Nwoke (1978) and Smith and Stewart (1987) suggested that hyperplasia of underlying host pericycle cells of mature *Striga gesnerioides* tissues inside cowpea root cause outward rupture of the endodermis at the site of penetration. Krause (1990) confirmed these results in his light microscopical developmental study of the same species, but not in other *Striga species*. Nevertheless, no such events could be seen in any of the examined *Orobanche* infections. We also did not find any proliferation of host cells in any root tissues in the infection area, though Dörr and Kollmann (1974) described proliferation of cortex cells resulting in thickening of the host root at the site of parasitic invasion. The only host root thickening that we have observed resulted from cell growth and proliferation of the parasite inside host root.

Wall swelling and dissolution of cell walls could only be traced in the host-parasite interface in the vascular cylinder of infected host roots, as also seen by Olivier et al (1991) who described partial wall dissolution as a typical feature occurring in sorghum root tissues penetrated by *Striga*. This wall dissolution may be leading to future vascular connections between host and parasite.

The involvement of enzymes in penetration is dealt with in the next chapter.

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Penetration Mechanism of *Orobanch* Haustorium.

2. Pectolytic Activity by the Haustorium *in situ*

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INTRODUCTION

The haustorium is the key organ in all parasitic plants, physically and physiologically bridging between the parasite and the host (Kuijt 1969). Nutrients and water are transferred through the haustorium from the conductive system of the host into that of the parasite, and all hormonal interactions between the two organisms are facilitated by this organ (Press and Graves, 1995). Holoparasites, like the root parasite *Orobanch* (broomrape) are fully dependent on their hosts for nutrients because they are incapable of any photosynthetic activity (Kuijt 1969, Timko *et al.* 1989, Stewart and Press 1990).

The haustorium develops when intrusive cells of the parasite penetrate host tissues, eventually reaching the conductive system of the host. Different opinions have been expressed as to the possible mechanism of intrusion, namely whether the intrusive cells of the parasite employ mechanical pressure to force their way through host tissues, or whether they release enzymes, to allow a smoother penetration. These opinions were based on microscopical observations using mainly staining procedures that highlight cell-wall morphology (examples for *Orobanch*: Krenner, 1958; Dörr and Kollmann, 1974, Attawi and Weber, 1980, Aber, 1984; for *Alectra*: Visser *et al.* 1990 and for *Striga*: Olivier *et al.* 1991) and on indirect biochemical evidence (Singh and Singh 1993 for *Orobanch*, Renaudin 1977 for *Lathraea*, Reddy *et al.* 1981 for *Cassytha*, Nagar *et al.* 1984 for *Cuscuta*). In a previous paper we showed that seedlings and infective calli of *Orobanch* are able to secrete pectin methylesterase

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(PME) and polygalacturonase *in-vitro* (Ben-Hod *et al.* 1993). Nevertheless all these studies provide us with hints rather than proofs as to the possible involvement of pectin degrading enzymes in the penetration process. There has been no direct *in situ* evidence of the precise location of wall degrading enzymes in the infection area, and proof of enzymatic activity in the relevant wall regions.

The following immunocytochemical study describes the presence of pectin methyl esterase and changes in host cell wall composition at the site of *Orobanch*e haustorium penetration, thus providing the first direct evidence for the involvement of pectolytic enzymes in the process of parasite intrusion into host tissues.

MATERIAL AND METHODS

Plant material

*Orobanch*e *cumana* Wallr. was grown on sunflower (*Helianthus annuus* L.); *O. aegyptiaca* Pers. was grown on tomato (*Lycopersicon esculentum* Mill.). The plants were grown in three different systems:

- (a) In pots, using the "sandwich method": surface-sterilized *Orobanch*e seeds were spread between two layers of glass-fiber paper (Whatman GFA) placed in sterile sand as shown in Fig. 1. Young host plants were then planted in the pots. Some of their roots grew into the space between the two GFA layers, reached the *Orobanch*e seeds, stimulated their germination, and were eventually infected by the young seedlings of the parasite.
- (b) *In vitro* in Peter dishes after Losner *et al.* (1996).
- (c) *In vitro* in aseptic transparent bags, modified from Parker and Dixon (1983) as follows: surface-sterilized and antibiotics-treated *Orobanch*e seeds (Losner-Goshen and Joel, 1996) were preconditioned for germination for 10 d in Petri dishes on sterile agar medium (0.8% agar in water), and transferred inside a sterile "Biohazard" bag containing glass-fiber paper (Whatman GF/A) on which a host plant has grown aseptically and developed a root system. Sterile Haugland solution was added to the bottom of the bag. The *Orobanch*e seeds were placed adjacent to young developing host roots and allowed to germinate and infect the roots.

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The whole system was strictly kept sealed under sterile conditions, in a growth chamber.

Fig. 1. The "sandwich method" for raising *Orobanche* infections on host roots: Surface-sterilized *Orobanche* seeds are spread between two layers of glass-fiber paper. The upper layer is smaller, and is perforated to allow penetration of host roots between them. This "sandwich" is buried in autoclaved sand in a small pot. A young host plant is planted in sand, above the "sandwich". Some of its roots grow into the space between the two GFA layers, reaching the *Orobanche* seeds, stimulating their germination, and eventually infected by them.

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Preparation of plant material for microscopy

Infected roots were fixed either in glutaraldehyde followed by OsO₄ or only in glutaraldehyde, dehydrated and embedded in either AGAR 100 resin or LR. White. 2-4 µm sections were prepared using Ralph glass knives on a rotary microtome. The sections were stained with toluidine blue and basic fuchsin and examined under the light microscope. For electron microscopy, ultrathin sections were prepared with a diamond knife and mounted on nickel or gold grids. Some grids were stained with uranyl acetate and lead citrate, other grids were either stained for carbohydrates using PATAg (periodic acid-thiocarbohydrazide-Ag proteinate, Roland and Vain 1993) or immuno-stained with the following antibodies: polyclonal antibodies against pectin methylesterase (anti-PME) from tomato fruit, courtesy of Dr. G.A. Tucker, University of Nottingham, UK; JIM 5, a monoclonal antibody that recognizes pectin with up to 50% esterification, and JIM 7, a monoclonal antibody that recognizes pectins with 35-90% esterification, both kindly donated by Dr. Knox, Norwich, UK (Vanderbosch *et al.*, 1989; Knox *et al.*, 1990).

RESULTS

Immunolocalization of pectin methyl esterase (PME)

Abundant PME was gold labelled in the external cells of *in vitro* grown *Orobanche* calli with PME antibodies. Gold labelling was found in external cell walls of peripheral cells of *Orobanche* callus protrusions (Fig. 2A). In the intrusive body inside host tissues PME labelling was rather difficult to find, and was seen only in a few fixed samples. In intrusive cells the enzyme was located in cell walls attached to host cells, between the plasma membrane and the wall, and also in the cytoplasm (Fig. 2B). In some cases Golgi vesicles of intrusive cells were clearly labelled (Fig. 2C). PME was also found in the adhesive substance that binds the external walls of intrusive cells with host root cell walls (Fig. 2D). Immunogold labelling of PME in infected roots showed labelling in host cells only in walls attached to or in the vicinity of parasite cells (Fig. 2E). No such labelling was found in equivalent non-infected host tissues.

Fig. 2. Immunogold localization of pectin methyl esterase (PME). A. PME labelling in outer cell of an *in vitro* grown callus of *O. aegyptiaca*. x42 800. B-C. PME in intrusive cells of *Orobancha* inside host root. B. PME labelling in cell wall of *O. cumana* that is attached to a sunflower root cell. Labelling also in the outer cytoplasm of the intrusive cell. x20 000. C. PME labelling associated with Golgi body and vesicles in intrusive cells of *O. aegyptiaca*. x60 000. D. PME labelling in the adhesive substance between tomato root cells and intrusive cells of *O. aegyptiaca*. x40 700. E. PME labelling in a sunflower root cell junction adjacent to a parasite cell. x25 000. G=Golgi body. H=parasite. H=host. arrow=PME labelling.

Fig. 3. Immuno electron micrograph of outer callus cells of *Orobancha*, illustrating spatial differences in pectin methylation that are consistent with PME release along the outer cell walls. x3600.

A. Gold labelling with JIM5 illustrating the occurrence of low methylated pectins mainly in the outer cell walls.

B. Gold labelling with JIM7 illustrating the occurrence of high methylated pectins also in inner anticlinal cell walls.

Changes in outer cell walls of infecting Orobanche seedling

Activity of PME could be followed by differential labelling of wall pectins with the monoclonal

pectin antibody JIM 5 that binds to low-methylated pectins, and with the monoclonal antibody JIM 7 that binds high-methylated pectins. We hypothesized that cell walls will stain differently with these two antibodies respective to PME activity that demethylates pectins. Analysis of the outer cells of the attachment organ of *Orobanche* that secrete the enzyme PME, using the pectin antibodies Jim 5 and Jim 7 revealed that low methylated pectins occur mainly in the outer cell walls (Fig. 3A), while the inner cell walls stain only with Jim 7 indicating high methylated pectins (Fig. 3B). These results are consistent with pectin demethylation by PME in the outer cell walls through which PME is secreted.

Changes in wall composition at the infection site

Fig. 4A shows a wall portion of host root cortex that lies perpendicular to the cell wall of a young *Orobanche* intrusive cell (P). Immunocytochemical staining of the section using JIM 5, after post-fixation with OsO₄, revealed that abundant low esterified pectins occur in the middle lamella only adjacent to the parasite cell, not in the distal portion of that wall. Near mature parasite cells, host cell walls often do not stain at all with both pectin antibodies, unlike host cell walls that are not in direct contact with the parasite (Fig. 4B), indicating a complete absence of pectins. In a similar manner, while intercellular regions of cortex cell walls in tomato roots normally stain with PATAg (Fig. 5A), in the infection site they stain only weakly, and often do not stain at all (Fig. 5B). Figs. 5C and 5D, of consecutive ultrathin sections, show the absence of wall pectins in the boundary between tomato vessel elements adjacent to an intrusive cell of *O. aegyptiaca*. The compound middle lamella that normally binds pectin antibodies and stains with PATAg, as seen in intercellular areas and in pit membranes that are not in contact with the parasite, does not stain in the vicinity of a

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Fig. 4. Changes in pectin in cell walls of sunflower root, consistent with PME release by neighbouring intrusive cells of *O. cumana*. A. Appearance of low-esterified pectins in a root cortex cell wall perpendicular to the parasite. Only the proximal portion of the wall is labelled with JIM 5 (large arrows). Distal wall not labelled (small arrows). x21 800. B. Disappearance of pectins in outer cortex cell wall adjacent to the parasite, seen after double labelling with JIM 5 (5 nm gold particles) and JIM 7 (10 nm gold particles). Host cell walls distant from the parasite are labelled (large arrows), whereas those touched by a parasite cell are not (small arrows). x20 000. (P) parasite cell. (H) host cell.

Fig. 5. A. Intercellular space in uninfected tomato root cortex, densely stained with PATAg. x16 000. B. Intercellular space adjacent to *Orobanch* cell (P), no PATAg staining of the intercellular material between host cells (H). x10 300. C-D. Consecutive sections. Disappearance of pectins in the boundary between tomato vessel element (H) and *Orobanch* cell (P). C. JIM 7, x12 300. D. PATAg, x10 000.

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parasite cell. In these two electron micrographs (Fig. 5C,D) the distant intercellular space stains densely for pectins in both methods, the space adjacent to *Orobanch*e cell does not stain at all, and the one in between stains only partly. The pit membrane that lies distant from the parasite stains very clearly with PATAg, while the one near the parasite does not stain, and shows a swollen texture.

Fate of the Casparian strips of host root endodermis

*Orobanch*e haustorium penetrates host root endodermis by separation of endodermal cells and pushing them aside. Whereas non infected Casparian strips do not stain with pectin antibodies (Fig. 6A), at the infection site itself they do, indicating cutin dissolution (Fig. 6C). Once a Casparian strip has been opened, the haustorium pushes the neighbouring endodermal cells sideward, and they collapse (Fig. 6B).

Cell walls containing *Casparian Strips* that lie in the vicinity of the penetration site (not at the site itself) showed differential labelling with pectin antibodies. The wall facing the parasite at the cortex side of the endodermis stained both with Jim 5 and Jim 7 while the wall on the other side of the *Casparian Strip*, facing the central cylinder, stained only with Jim 7 (Fig. 6A).

Wall dissolution

In a few cases host cell walls were considerably changed showing perforations that extended from the parasite side of the wall to the host side. This was found only on the boundary with cells of the central vascular cylinder. With conventional staining for the EM the perforations look as wall regions that are less dense than normal (Fig. 7A). After PATAg staining these regions were much more pronounced, because they were not stained, in contrast to neighbouring wall regions (Fig. 7B). Similarly, these wall perforations did not stain with any of the pectin antibodies (not shown). Immunogold staining using anti PG antibodies revealed that the enzyme was mainly located in these dissolved wall regions (Fig. 7C).

Fig. 6 *Orobanchae* haustorium penetrating host root endodermis. Double immunostaining with JIM 7 (10 nm gold particles) and JIM 5 (5 nm). A. Enlargement of site A in Fig. 6B. A few micrometers away from the parasite, differential pectin labelling on two sides of Casparian strip: JIM 5 labelling only where passage of PME in apoplast was not blocked. B. General view showing site of *Orobanchae* intrusion in host endodermis. C. Enlargement of site C in Fig. 6B. Site of haustorium penetration between endodermis cells. Both high and low methylated pectins are labelled inside a Casparian strip, indicating possible enzymatic cutin dissolution.

DISCUSSION

Unlike the intrusive cells of *Cuscuta*, which often penetrate host cells (Dawson *et al.* 1994), the intrusive cells of *Orobanch*e push their way between host cells rather than through them. This schizogenous penetration seems to be possible due to the activity of enzymes that change the composition and physical properties of the host cell walls and middle lamella in the path of the haustorium. The present paper presents, for the first time, evidence of the presence and activity of the enzyme pectin methyl esterase (PME) in the host/parasite interface.

The evidence required to prove the involvement of a particular enzyme in penetration include: (a) proof that the enzyme is present *in situ* during penetration; (b) proof of depletion of the substrate of enzymatic activity *in situ*; (c) proof of the appearance of products of its activity *in situ*; (d) evidence that the enzyme is produced by the parasite in culture. We have addressed all these questions.

By immunogold staining with specific antibodies for PME we have shown that the enzyme is present *in situ* in the intrusion site. Gold labelling indicating the presence of PME was found only in host cell walls attached to or located close to parasite cells. In addition it was also found in the secretion that fills the interface between parasite and host cells in the epidermal and outer cortex regions of host root. In intrusive cells the enzyme was found in Golgi vesicles and in cell walls attached to host cells. These results indicate that the enzyme is of parasite origin.

Detection of the enzyme in the infection zone was difficult probably because penetration is a very rapid process and the enzyme is only transiently localized in the cell apoplast. This is not the case with *Orobanch*e grown in culture, that continuously secretes the enzyme to the medium (Ben-Hod *et al.* 1993). Detecting the enzymes' substrates and its products, which are more abundant in cell walls, clearly allows the detection of enzymatic activity during parasite penetration. The changes in cell wall composition persist in the infection zone long after the haustorium has penetrated, and are therefore easier to detect. Localization of the enzymes and visualization of the minute changes in the distribution of their substrates are complementary methods.

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Fig. 7. Wall perforations between parasite and host in host vascular cylinder. A. conventional EM staining. x12,200. B. PATAg staining. x12,200. C. Immunogold staining using anti PG antibodies. PG located in dissolved wall regions. x21,500.

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We anticipated a drop in the degree of esterification of wall pectins in host cells adjacent to young intrusive cells of the parasite as the result of PME activity. And indeed, JIM 5 staining was considerably more pronounced in sunflower root cell walls adjacent to young intrusive cells, indicating the appearance of galacturonic sequences with less than 50% esterification in these wall regions, which is consistent with PME activity that de-methylates pectins. This change in wall pectins was especially pronounced when we examined osmium treated tissues. Under these conditions only the most abundant of the JIM 5 epitope is detectable (JP Knox, pers. comm.), allowing the visualization of differences in wall pectin esterification along a single cell wall. The two monoclonal antibodies JIM 5 and JIM 7 have been well characterized (Vanderbosch *et al.*, 1989; Knox *et al.*, 1990) regarding their respective range of recognition of methylated homogalacturonan. For an ultrastructural immunocytochemical approach these antibodies are very useful because they allowed the direct visualization of spatial changes in the presence of pectin and in the degree of esterification of polygalacturonates in infected tissues.

Host cell walls neighbouring mature parasite cells often did not stain with both pectin antibodies, in contrast to similar cell walls of non-infected tissues. Degradation of wall pectins was also demonstrated with PATAg staining, which detects carbohydrates that are sensitive to periodic acid, especially pectins. PATAg staining often disappeared at the host/parasite interface. It is not clear, however, whether the changes in PATAg staining correspond only to changes in pectin, since this staining protocol is not specific only for pectins. Similar results were reported for cellulose in the sorghum-*Striga* interaction, using gold-labelled exo-glucanase (Olivier *et al.*, 1991), though so far no direct evidence was brought for cellulase activity in such systems. In most cases only host cell walls were found to be affected by enzymatic activities of the parasite. In some cases, both host and parasite cells were affected. Real holes were observed in the host and parasite cell walls in host-parasite interface at the vascular cylinder of the host. These holes could be seen when the tissue was stained with PATAg or with pectin antibodies, but also with conventional staining. They may represent the sites where direct connections develop between host and parasite vessel elements.

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The fate of endodermal cells during penetration was hitherto obscure. Here we show, for the first time, that *Orobanche* has means to open the gap between endodermal cells, perhaps by cutinase activity on Casparian strips, and that endodermal cells are pushed sideward by the haustorium and crush by neighbouring cells.

There are two stages in the pectolytic activity of the intrusive cells of *Orobanche*. The first is performed by PME, de-esterifying pectins in host wall and middle lamella near the intrusive cells, and the second is performed by another enzyme, presumably polygalacturonase, the result of which being a complete degradation of wall pectins that allows separation of cells and a smooth penetration of the intrusive cells between host cells without rupturing them.

Although bacteria were shown to be common on the surface of the attachment organ of *Orobanche* (Joel and Losner-Goshen 1994), the possible involvement of microorganisms as a source of PME during haustorium penetration can be excluded since our results were confirmed with plant material which was grown *in vitro* under aseptic conditions. In a previous paper we have already shown that PME is produced by *Orobanche* in culture (Ben-Hod *et al.* 1993).

Changes in host cell wall structure at the infection site were often related to enzymatic activities of the parasite (Dörr and Kollmann, 1974; Visser *et al.*, 1990; Olivier *et al.*, 1991). Renaudin (1977) found proteolytic and cellulolytic activities in mature haustoria of *Lathraea*, by examining thin films of protein and of cellulose, respectively, that were attached to the surface of sectioned host-parasite interface. Reddy *et al.* (1981) reported cellulase activity in haustoria of *Cassytha*. Nagar *et al.* (1984) were able to show elevated activity of some wall degrading enzymes in homogenates prepared of the infecting organs of *Cuscuta*, and Singh and Singh (1993), using similar methods, showed that *Orobanche* plants had higher polygalacturonase (PG) and cellulase activity than *Brassica* roots that served as hosts, and suggested that these enzymes may be active in the penetration of haustoria. In a previous paper we showed that seedlings and infective calli of *Orobanche* are able to secrete pectin methylesterase (PME) and polygalacturonase *in-vitro* (Ben-Hod *et al.* 1993). But until recently there has been no direct *in situ* evidence of any involvement of wall degrading enzymes in the penetration process itself. The results described above are therefore the first to directly

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demonstrate the *in situ* presence of pectolytic enzymes of parasite origin at the host-parasite interface, together with direct evidence of chemical changes in host cell walls that correspond to the activity of the respective enzymes, which is a clear evidence of the activity of pectinases in the *Orobanche* intrusive system.

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Evaluation of the research achievements

Some of the most serious pests in middle east agriculture are the broomrapes, parasitic weeds belonging to the genus *Orobanche*. These parasites attach to roots of many different vegetables and field crops, sucking water and nutrients from their hosts thus causing tremendous damage. In heavily infested fields both the quality and quantity of harvest is seriously reduced, often leading to total yield loss. Although some methods which are currently used for the control of these parasitic weeds have encountered various degrees of success, they are generally ineffective in many important crops and often prohibitively costly.

The purpose of this BARD project was to contribute understanding of the mechanisms of parasitism by *Orobanche* as a basis to search for novel methods for its control. It was also aimed to throw light on recognition processes in parasitic weeds and on basic mechanisms of parasitism in plants.

(a) The first objective of this proposal was to characterize *Orobanche* germination stimulants. So far no specific germination stimulant was identified from *Orobanche* hosts. Though *Striga* stimulants of the strigol group do, in most cases, stimulate germination of *Orobanche* species, it was not clear whether natural *Orobanche* and *Striga* stimulants have similar structures.

The characterization of costuslactone as the germination stimulant for *Orobanche cumana* represents one of the first examples of a host-derived germination stimulant for this agriculturally important genus.

A major finding was that costuslactone possesses a lactone moiety which is required for activity. Costoslacto contains a lactone moiety as does strigol, which is a highly functionalized sesquiterpene lactone derived from cotton which exhibits germination stimulant activity against a fairly wide range of parasitic *Orobanche* and *Striga* species. However, costuslactone appears to be fairly species-specific: while it is a potent stimulant for *Orobanche cumana*, it has little activity in promoting germination of *O. aegyptiaca*.

The germination stimulant from sunflower roots proved to be extremely hydrophobic. Nevertheless it could be purified from root exudates and quantified in root exudates at levels sufficient to account for all the germination stimulant activity of sunflower root exudate.

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Thus the stimulant is probably released along with a number of other compounds in root exudate that promote its aqueous solubility. In terms of developing reproducible activity assays for compounds as hydrophobic as costuslactone, we found it was necessary during purification to apply the stimulant to glass fiber discs in solvents sufficiently nonpolar to ensure that the material was adsorbed to the disc in a solution, rather than as a suspension.

In retrospect the finding of a sesquiterpene lactone germination stimulant in sunflower is not surprising. The aerial portions of sunflower are known to produce a wide range of sesquiterpene lactones although roots have not been investigated heretofore. Costuslactone was unusual in being both the primary, and a highly abundant, extra cellular terpenoid in roots.

A number of studies in other laboratories have suggested that strigol-like sesquiterpene lactones are present in all plants species and that these compounds are responsible for all germination activity for parasitic plants. We believe the chemistry underlying some of these studies is of questionable quality, and our own findings do not support these ideas. We found no indication of strigol-like compounds in sunflower which were active on *O. cumana* seeds. Although the lactone moiety was found, similar to the case with strigol, to be necessary for activity, the rest of the molecule does not share significant homology to strigol. New classes of synthetic germination stimulants, at least for *Orobancha cumana*, will still have to rely upon the lactone model as a starting point for design of analogs which could be applied to soils to promote suicidal germination of the parasite.

While the biochemistry and molecular biology of sesquiterpene biosynthesis is now well enough developed to justify an effort to interfere with cyclization steps in transgenic plants, this solution would be specific to sunflower and *O. cumana*. Other important crop species attacked by *Orobancha* spp., such as tomato, do not possess molecules similar to costuslactone. Conversely, costuslactone is significantly less active against *O. egyptiaca*, which decreases the utility of a trap crop approach to engineering the ability to produce this compound in tomato.

However, the tractability of tomato as a genetic system and its importance to Israeli and U.S. agriculture suggest that transgenic approaches would be warranted if the biochemical nature of the *O. egyptiaca* stimulant were known. Our preliminary efforts, having developed

methods for assaying extremely hydrophobic molecules such as costuslactone, to search for the *O. aegyptiaca* germination stimulant from tomato have proven negative in terms of identifying a single compound or group of compounds that account for the bulk of the germination stimulant activity of this host.

(b) Histological and immunocytochemical analysis of mechanisms involved in penetration of host root tissues by intrusive cells of *Orobanch*e haustoria.

With this 2nd objective in this BARD project we have extended our understanding of haustorial invasion, developed a new *in vitro* system for infection of host root cultures with seedlings of the parasite, and followed the expression of PR proteins in an infected transgenic host. In addition we present new findings regarding host/parasite interaction in incompatible systems, illustrating for the first time the reaction of the parasite to host resistance.

Wall remains that result from host cell crushing along the penetration route of the intrusive body of parasitic plants were already recorded in previous studies. However, the fate of cell wall remains was not always established, and there was no clear understanding of the role enzymes play in the penetration process. A major finding was therefore that penetration combines enzymatic and mechanical processes. Whether the inhibition of one of them could help in preventing penetration is yet to be experimentally examined.

Two main challenges stood on our way to understand whether biochemical mechanisms are involved in parasitism. The first was the necessity to get rid of microorganisms in the experimental systems, the second was the need to detect enzymes *in situ*. We therefore developed a series of aseptic *in-vitro* systems, the last of which includes normal broomrape seedlings that infect host roots grown in culture. In order to succeed in this system we had to overcome a few technical obstacles, such as the inability of the parasite to develop haustoria when it is fully nourished by the medium. This novel system provided to our knowledge the first reported infection of isolated roots by an angiospermous parasite. In addition to its advantages in the present research project, this new system should also provide a convenient model for the study of some other biochemical and physiological aspects of parasitism in *Orobanch*e and similar important root parasites, like *Striga*. The availability of

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immunocytochemical methods allowed us to locate enzymes and various pectins *in situ* using the relevant antibodies.

Our developmental study proved to be difficult because haustorial penetration is a rapid process that is difficult to follow in chemically fixed material. In most examined specimens we found either young seedlings that were attached to host roots but did not develop a haustorium, or *Orobanch*e seedlings with a haustorium that already reached the central cylinder of host root. Only very few samples, of the many hundreds that we have examined, included a haustorium that penetrated the root but did not reach the vascular system. We therefore assume that detection of the relevant enzymes in the infection zone was difficult because penetration is a very rapid process and the enzymes are only transiently localized in the cell apoplast. This is not the case with *Orobanch*e grown in culture, that continuously secretes the enzymes to the medium, and where enzymes could be located immunocytochemically with no difficulty. Detecting the enzymes' substrates and their products, which were more abundant in cell walls, clearly allowed the detection of enzymatic activity *in situ*. The changes in cell wall composition persisted in the infection zone long after the haustorium has penetrated, and were therefore easier to detect.

Using these methods we found for the first time clear evidence of the presence and activity of the enzyme pectin methyl esterase (PME) in the host/parasite interface. Based on (a) evidence that the enzyme is present *in situ* during penetration; (b) evidence of depletion of the substrate of enzymatic activity *in situ*; (c) evidence of the appearance of products of its activity *in situ*; (d) evidence that the enzyme is produced by the parasite in culture, we obtained clear and comprehensive proof of the involvement of pectinases in the process of *Orobanch*e haustorium penetration inside host roots.

The promoter of the *PRb-1b* gene (one of the genes for the formation of pathogenesis related proteins) that was shown to be activated by *Orobanch*e attack may potentially serve as a powerful tool in engineering *Orobanch*e resistant crops, if used with appropriate genes for resistance against this parasite, such as genes encoding the synthesis of anti-*Orobanch*e toxins or genes that can create a hypersensitive reaction in the infection area.

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(c) Characterization and isolation of penetration enzymes.

The purification of pectin methyl esterase proved to be far more difficult than we have envisaged. The difficulties encountered were:

1. Quite low levels of the enzyme in the tissue.
2. The PME from *Orobanch*e did not behave like other PMEs that were previously reported to be present in other plants.
3. The PME from *Orobanch*e could not be purified by standard procedures on ion exchange columns.
4. PME from *Orobanch*e was activated by K^+ and inhibited by Na^+ , which made it extremely difficult to recover the enzyme and estimate it after ion exchange column chromatography.
5. The enzyme could be extracted by low salt concentrations as opposed to the cell wall bound PMEs usually reported which require extraction with high salt concentrations.

Eventually a procedure of biogel separation and electrophoresis allowed purification of the enzyme.

A further difficulty was met with regard to the preparation of antibodies. When the normal serum of candidate-rabbits was checked, it contained a considerable number of non-specific epitopes that reacted with extracts of *Orobanch*e, probably because these rabbits were fed with vegetable material that contains epitopes that are also present in *Orobanch*e. The solution to this problem was to prepare the antibodies in guineapigs, which was more tedious and time consuming and afforded smaller amounts of antibodies.

PME from *Orobanch*e is clearly a glycoprotein and therefore a number of non-specific bands were observed as reacting in crude *Orobanch*e extracts. These bands could essentially be removed by the exposure of blots to sodium periodate prior to the blocking step. Non-specific carbohydrate groups were eliminated by this procedure.

As far as PME is concerned, we can conclude that the enzyme has unusual properties but its purification and characterization was achieved and antibodies against it prepared.

Purification of Polygalacturonase was complicated by three main factors:

1. The presence of a number of PGases, some endo, some exo-

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2. All the PGases were very unstable. They could be readily extracted but not concentrated.
3. *Orobanch*e tissues contain two types of inhibitors of PGases: a low molecular, non-phenolic inhibitor that is stable to boiling, and small amounts of a proteinaceous inhibitor that is not removable by dialysis.

These three factors combined greatly complicated purification of the enzyme. Only partial purification was achieved and antibodies could not be prepared. However, the partial characterization of the inhibitors presents considerable step forward. The inhibitors may be of significance in the infection step, by preventing self damage of the parasite by its secreted enzyme PGase. Although not all the objectives were achieved, the major part of the enzymatic study was realized, and has led to two publications on the enzymes.

Description of the cooperation

Collaboration in this project results from the common interests of all three PIs in developmental mechanisms of parasitism in *Orobanch*e, and the desire of all three PIs to see knowledge derived from basic studies of *Orobanch*e applied to development of novel methods for control of this agricultural important parasite. During the last years this binational team conducted the BARD project with continuous mutual exchange of information, results, views, and queries. This scientific exchange was supplemented by a series of meetings and visits to each other's laboratories and resulted in shared authorship on several papers.

The Israeli effort in the current project was primarily directed toward understanding post-germination events in parasitism by *Orobanch*e, and the U.S. effort focused on understanding the stimulation of *Orobanch*e seed germination by host root exudates. DMJ has spent an academic year at Cornell prior to this project to carry out joint experiments with JCS and to acquire further experience in protein biochemistry and the immunocytochemical methods essential to successful completion of the project, and later visited the US team in 1994. JCS visited the Israeli teams in Jerusalem and in Newe-Ya'ar in spring 1994, and AMM visited JCS at Cornell in 1993 and in 1995. Seeds of the same lots of *Orobanch*e species were used in all three labs, and assays were inter calibrated. The fact that the Israeli teams were active in

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an equivalent project on germination physiology of broomrape seeds helped in deepening the discussions within the group.

The basic framework of collaboration :

DMJ Principal investigator, coordinating the research in the three different institutions. Leading the Newe-Ya'ar research group which concentrated on the anatomical, ultrastructural, histochemical and immunocytochemical studies of host penetration by *Orobanch*e.

AMM Leading the Jerusalem group which worked on the biochemical aspects of haustorial enzymes.

JCS Leading the Cornell group which concentrated on the chemistry of germination stimulants.

The two Israeli teams, one in Jerusalem and one in Newe-Ya'ar, worked in close collaboration, using the same plant material and sharing facilities. D. Losner-Goshen, a PhD student under joint supervision of DMJ and AMM (awarded the degree in December 1995), bridged between the two labs on a weekly basis. Plant material which was grown in culture in Jerusalem was frequently used for experimentation in Newe-Ya'ar, while young *Orobanch*e seedlings and plants grown in Newe-Ya'ar served in experiments in Jerusalem. Transmission electron microscopy by the Newe-Ya'ar team was carried out also in Jerusalem.

Communication was also open between the Israeli PIs and JCS at Cornell, using electronic mail and telephone when necessary. All current results in Israel and the US were dealt with on the spot, and overall discussions were held during the visits of the Israeli PIs at Cornell and the American PI in Israel.

List of Publications:

Bar-Nun, N., Ben-Hod, G., Lavi, E. and A.M. Mayer (1996) Purification of pectin methyl esterase from *Orobanch*e *aegyptiaca*. *Phytochem.* **41**: 403-6.

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Swapan K. Chaudhuri, and John C. Steffens (1997) Host recognition by the root parasite, *Orobanche cumana*: Isolation and structural characterization of the germination stimulant from sunflower root exudate (in preparation).

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