RESEARCH PAPER

Mucilage production during the incompatible interaction between *Orobanche crenata* and *Vicia sativa*

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Abstract

*Orobanche* spp. (broomrapes) are holoparasites lacking in chlorophyll and totally dependent on their host for their supply of nutrients. *O. crenata* is a severe constraint to legumes cultivation and breeding for resistance remains as one of the best available methods of control. However, little is known about the basis of host resistance to broomrapes. It is a multicomponent event, and resistance based on hampering development and necrosis of broomrape tubercles has been reported. In the present work, the formation of mucilage and occlusion of host xylem vessels associated with the death of *O. crenata* tubercles were studied histologically. Samples of necrotic *O. crenata* tubercles established on resistant and susceptible vetch genotypes were collected. The samples were fixed, sectioned and stained using different procedures. The sections were observed at the light microscopy level, either under bright field, epi-fluorescence or confocal laser scanning microscopy. A higher proportion of necrotic tubercles was found on the resistant genotype and this was associated with a higher percentage of occluded vessels. Mucilage is composed mainly by carbohydrates (non-esterified pectins) and the presence of polyphenols was also detected. The mucilage and other substances composed by parasite secretions and host-degraded products was found to block host vessels and obstruct the parasite supply channel, being a quantitative defensive response against *Orobanche* tubercles.

**Key words:** Histology, legumes, necrosis, *Orobanche crenata*, parasitic plants, resistance, vascular gel, *Vicia sativa*, wilt diseases, xylem mucilage.

Introduction

Broomrapes (*Orobanche* spp.) are obligate root holoparasites which connect to the vascular system of their host plants through a specialized structure known as a haustorium. These parasites are devoid of chlorophyll and totally depend on their hosts for their supply of carbon, nitrogen, and inorganic solutes. Some of these species have become a severe constraint to major crops including legumes. This is the case of *O. crenata* (crenate broomrape) which has been known to threaten legume crops since ancient times (Cubero, 1994). It is an important pest in faba bean (*Vicia faba*), pea (*Pisum sativum*), lentil (*Lens culinaris*), vetches (*Vicia* spp.), grass and chickling pea (*Lathyrus sativus* and *L. cicera*), and other grain and forage legumes in the Mediterranean basin and the Middle East (Rubiales et al., 2006).

Breeding for resistance is the most economic, feasible and environmentally friendly method of control against this parasite. However, despite the fact that resistance to *Orobanche* spp. has been reported (Labrousse et al., 2001; Rubiales et al., 2003a, b, 2004; Pérez-de-Luque *et al.*, 2005).
et al., 2005a), little is known about the basis of host resistance to these parasites (Joel et al., 1996; Pérez-de-Luque et al., 2005b). One of the most common incompatible interactions described in the literature is the darkening and/or necrosis of developing tubercles (Dörr et al., 1994; Labrousse et al., 2001; Pérez-de-Luque et al., 2005a, b). Histological studies have revealed that initial vascular connections are established and tubercles develop, but they then become dark and the parasite dies at an early developmental stage (Dörr et al., 1994; Labrousse et al., 2001; Pérez-de-Luque et al., 2005b). The presence of substances inside host vessels has been associated with the darkening of broomrape tubercles (Labrousse et al., 2001; Zehhar et al., 2003; Pérez-de-Luque et al., 2005b) and it is possible that these substances block the vessels and interfere with the nutrient flux between host and parasite (Pérez-de-Luque et al., 2005b).

Xylem occlusion is a common response to vascular invading pathogens such as bacteria, fungi, and viruses, and formation of mucilages and tylosis is typically associated with resistance against wilt diseases (Beckman and Zaroogian, 1967; VanderMolen et al., 1983; Rioux et al., 1998; Beckman, 2000). Furthermore, it has also been reported as a response to wounded tissue (Crews et al., 2003). However, it has not been demonstrated as a common response against parasitic plants. In the present work, the composition and the role of these substances appearing inside host vessels of Vicia sativa resistant to O. crenata were studied by the use of different cytochemical and immunofluorescence assays.

**Materials and methods**

**Plant material and growth conditions**

Orobanche crenata seeds were collected on Pisum sativum infected plants in Cordoba (Spain) during year 2003. O. crenata was grown on resistant and susceptible genotypes of common vetch (Vicia sativa L., A01 and V27, respectively). The polyethylene bag system described by Linke et al. (2001) was used. A strip (11×28 cm) of glass fibre paper (Whatmann GF/A) with disinfectant O. crenata seeds (40 mg) spread on it was inserted in a polyethylene bag (25×35 cm). In order to ensure homogeneous seed germination, the synthetic germination stimulant GR-24 (10 mg l⁻¹, 5 ml per bag) was added. Fifteen days later germinated vetch seedlings having a radicle length of about 4–5 cm were transferred to the bag, placing them on the upper side of the system. Twenty ml of Hoagland nutrient solution (Hoagland and Arnon, 1950) was added to each bag, and refilled later when necessary. The bags were suspended vertically in boxes and the plants were grown in a controlled environment chamber with a day/night temperature of 20±0.5 °C, 14 h photoperiod and an irradiance of 200 µmol m⁻² s⁻¹.

At days 35, 40, and 45, the number of O. crenata tubercles was counted, referring those that became necrotic and died as a percentage of the total.

**Collection and fixation of samples**

Observations were taken using a binocular microscope (Nikon SMZ1000; Nikon Europe BV, Badhoevedorp, The Netherlands).

At 35, 40, and 45 d after inoculation, tubercles of O. crenata were sampled at random with the corresponding attached parts of host roots.

For bright field and epi-fluorescence observations at the light microscope, the sampled material was fixed in FAA (50% ethanol+5% formaldehyde+10% glacial acetic acid, in water) for 48 h. Fixed samples were then dehydrated in ethanol series (50, 80, 95, 100, 100% for 12 h each) and transferred to an embedding solvent (Xylene; Panreac Quimica SA, Montcada i Reixac, Spain) through a xylene-ethanol series (30, 50, 80, 100, 100% for 12 h each) and finally saturated with paraaffin (Paraplast Xtra; Sigma, St Louis, USA). 7 µm-thick sections were cut with a rotary microtome (Nahita 534; Auxilab SA, Beriaín, Spain) and attached to adhesive-treated microscope slides (polysine slides; Menzel GmbH & Co KG, Braunschweig, Germany).

For immunofluorescence experiments analysed through confocal laser scanning microscopy, the samples were fixed in 4% formaldehyde in phosphate buffered saline (PBS), pH 7.3 at 4 °C overnight. After washing in PBS (3×15 min), they were stored in 0.1% formaldehyde in PBS at 4 °C. 40 µm vibratome sections (Vibratome series 1000, Intracel Electrophysiology and Biochemistry Equipment, Herts, UK) were cut under water and dried down on 3-aminopropyl triethoxy silane (APTES, Sigma)-coated multi-well slides. The sections were dehydrated in a series of 30, 50, 70, and 100% methanol/water, then rehydrated in a series of 70, 50, 30% methanol/water, and finally in PBS, for 5 min at each step. To facilitate penetration of the labelling reagents, the sections were treated with 0.1% Tween 20 in PBS for 15 min at room temperature, then washed in PBS for 5 min and allowed to dry.

**Cytochemical methods for light microscopy**

After removal of paraaffin, the sections were stained with different dyes: (i) Alcian green: safranin (AGS) (Joel, 1983). The slides were dried and mounted with DePeX (BDH). With this staining method, carbohydrates (including cell walls and mucilage) appeared green, yellow or blue, while lignified, cutinized and suberized walls, as well as tannin and lipid material inside cells appeared red (Joel, 1983). Non-stained sections were kept as control, and for examination under the fluorescence microscope, (ii) Staining with 0.05% toluidine blue O (TBO) in PO₄ buffer (pH 5.5) during 5–10 min was used. In this case the dye was applied before removal of paraaffin (Ruzin, 1999). This method allows the detection of phenolics as well as tannins, lignin, and suberin (Baayen et al., 1996; Bordallo et al., 2002; Mellersh et al., 2002; Crews et al., 2003). (iii) Phloroglucinol (2% in ethanol)–HCl (35%) (Ruzin, 1999) stains the aldehyde groups of lignin and suberin, but quenches lignin autofluorescence and retains suberin fluorescence (Baayen et al., 1996; Rioux et al., 1998). (iv) Aniline blue fluorochrome was used for callose detection under UV fluorescence. The samples were stained during 15–30 min in a solution 0.1% aniline blue fluorochrome in water (Bordallo et al., 2002). (v) Pectins were detected using ruthenium red. The samples were immersed during 5 min in a solution 0.05% ruthenium red in water. Non-methyl-esterified pectins take a red/pink coloration with this dye (Vallet et al., 1996).

The sections were observed using a light microscope (Leica DM- LB, magnification ×100 to ×400; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) and photographed using a digital camera (Nikon DXM1200F; Nikon Europe BV, Badhoevedorp, The Netherlands). The samples were also observed by epi-fluorescence under excitation at 450–490 nm (blue-violet).

**Immunofluorescence for confocal laser scanning microscopy**

After blocking with 5% bovine serum albumin (BSA) in PBS for 5 min, the slides carrying the vibratome sections were incubated with the first antibody undiluted for 1 h: JIM 5 for non-methyl-esterified...
pectins and JIM 7 for methyl-esterified pectins (Professor Keith Roberts, John Innes Centre, Norwich, UK). After washing in PBS, they were incubated with a fluorescent anti-rat ALEXA 546 (Molecular Probes Inc., Eugene, OR, USA) antibody applied 1/25 in 3% BSA in PBS for 45 min at room temperature in the dark. Confocal optical section stacks were collected using a Leica TCS-SP2-AOBS-UV confocal laser scanning microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

**Dye tracer**

Naphthol blue black was used as a dye tracer to test the continuity of water and nutrient fluxes between the host root and the parasite tubercles (Jacobsen *et al.*, 1992). The distal parts of infected vetch roots were immersed into a dye solution 0.1% naphthol blue black and the root tips cut under the solution. Transpiration was then allowed to proceed for 4–5 h. Attached tubercles with the corresponding host root part as well as root sections cut at various points (before and after attached tubercles) were sampled. Transverse fresh hand-cut sections of the samples were observed under a light microscope.

**Determination of sealed vessels**

Samples stained with AGS were used to determine the proportion of vessels filled with different substances. Transverse sections of the

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Necrotic tubercles (%)</th>
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<tbody>
<tr>
<td><em>V. sativa</em> V27 (susceptible)</td>
<td>8.0 (0.21±0.06) 15.9 (0.33±0.08) 27.6 (0.48±0.09)</td>
</tr>
<tr>
<td><em>V. sativa</em> A01 (resistant)</td>
<td>13.8 (0.31±0.09) 51.4 (0.80±0.14) 61.5 (0.95±0.17)</td>
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Table 1. Proportion of *O. crenata* necrotic tubercles (incompatible interactions) after their establishment on susceptible and resistant vetches

Data are expressed as a percentage of the total number of established tubercles. dai, days after inoculation. Log-transformed data with SE shown in parentheses alongside back-transformed means.

Fig. 1. Proportion of vessels filled with mucilage and other substances depending on the interaction between host and parasite: IIR, incompatible interaction on resistant host; IIS, incompatible interaction on susceptible host; CIS, compatible interaction on susceptible host. Samples collected 45 d after inoculation were used.

| Cytochemical assays | Specificity | Colour | ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l ml w l |
|---------------------|-------------|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------
Fig. 2. Cross-sections of incompatible interactions of *Orobanche crenata* on resistant vetch (*Vicia sativa* line A01) stained following different procedures. (A) AGS staining showing accumulation of carbohydrates (arrows, green colour) in the apoplast of areas opposite the haustorium. Neighbouring xylem vessels filled with mucilage can also be observed (asterisks). Arrowheads indicate the direction where the haustorium was located. (B) The same as (A) with TBO staining showing the accumulation of carboxylated polysaccharides (arrows, pink colour) in parenchymatic cells. (C) The same as (A) with ruthenium red staining indicating the presence of non-esterified pectins (arrows, pink colour). (D) AGS staining showing areas and vessels dyed with safranin next to the haustorium (arrows, red colour). (E) TBO staining indicating the presence of polyphenols and lignins (arrows, blue-turquoise colour). (F) The same as (E) with phloroglucinol-HCl staining showing lignins and polyphenols (arrows, red colour). Yellow lines delimit parasite from host tissues. CC, central cylinder; Ctx, cortex; Pic, parasite intrusive cells (haustorium); Xyl, xylem vessel.
haustoria were observed under the light microscope, with the host central cylinder centred within the field of view. The central cylinder was divided in four equitable quadrants and the relative position of the haustorium with respect to these quadrants was recorded. Vessels within quadrants containing the haustorium were considered close to the haustorium, and vessels within quadrants not containing the haustorium were considered away from the haustorium. The number of vessels filled with safranin and alcian green-stained substances were recorded, and expressed as a percentage with respect to the total number of vessels in each quadrant.

Statistical analysis
Assays were performed with ten replicates per treatment with a completely randomized design. Statistical analysis (ANOVA) was performed with SPSS 10.0 and Statistix 8.0 for Windows. Percentages were transformed according to the formula

\[ Y = \arcsin\left(\sqrt{\frac{X\%}{100}}\right). \]

A minimum of ten samples from a pull collected from ten replicates were used for each cytochemical assay.

Results
The highest proportion of necrotic tubercles was found on the resistant genotype (Table 1). The percentage of necrotic tubercles increased with time in both resistant and susceptible genotypes, but at a much higher rate in the resistant one.

Vessels of susceptible roots in a compatible interaction contained no mucilage, but in some cases the presence of safranin-staining substance could be detected at low proportions (Fig. 1). During an incompatible interaction there was a higher percentage of vessels filled with substances in resistant vetch than in susceptible vetch. Within an incompatible interaction more than half of the vessels near the haustorium were filled with the safranin-staining substance in the resistant genotype. There was a higher proportion of vessels filled with mucilage opposite to rather than next to the haustorium in resistant vetch.

Table 2 shows the reactions of different tissues to the histochemical tests in incompatible interactions in resistant vetch. Carbohydrates, including carboxylated polysaccharides and pectins, were detected using alcian green, TBO, and ruthenium red dyes mainly in the apoplast (Fig. 2A–C). A more intense staining for carbohydrates was detected in zones opposite the haustorium of necrotic tubercles than that of healthy tubercles. An intense staining for polyphenols and lignins with AGS, TBO and phloroglucinol was seen in zones next to the haustorium of necrotic tubercles (Fig. 2D–F). The staining also appeared in the apoplast and in the interface between host and parasite. Lignification of host cell walls in contact with parasite tissues was observed at different points and on consecutive sections. These lignified walls formed a ring surrounding the parasite intruding tissues and corresponded to cells from the host endodermis and/or pericycle. Figure 3 shows a 3D diagram of this phenomenon. The presence of suberin was restricted only to the endodermal cells (Fig. 4A, B), and callose accumulated in some parenchyma cells, in the host–parasite interface and in the middle lamellae and cell wall of some xylem vessels (Fig. 4C–F).

Staining consecutive sections with different dyes allowed the composition of mucilage and other substances inside the xylem vessels to be characterized (Fig. 5). Mucilage stained with alcian green from AGS staining, indicates a carbohydrate composition (Fig. 5A, E). This was confirmed by the pink colour after TBO staining, that corresponds to carboxylated polysaccharides (Fig. 5B). The pink/red colour obtained with ruthenium red indicated the presence of non-methyl-esterified pectins (Fig. 5B). The presence of polyphenols was also confirmed by blue–turquoise staining with TBO (Fig. 5B) and red staining with phloroglucinol (Fig. 5F) and the absence of lipids, tannins, suberin, and lignin because of the negative staining with safranin from AGS (Fig. 5A, E). The mucilage was colourless in non-stained sections, whereas the other
Fig. 4. Cross-sections of incompatible interactions of *Orobanche crenata* on resistant vetch (*Vicia sativa* line A01) stained for suberin and callose detection. (A) Section stained with phloroglucinol-HCl in order to quench lignin autofluorescence. (B) The same as (A) observed by epi-fluorescence under blue-violet excitation. Suberized cell walls can be observed (blue fluorescence), mainly corresponding to the endodermis. The absence of lignin fluorescence can be observed (for example, xylem vessels). The fluorescence of endodermal cells is disrupted at the point of penetration of the parasite (haustorium). (C) Light micrograph of a section stained for callose detection. (D) The same as (C) observed by epi-fluorescence under blue-violet excitation. Callose depositions show a blue-white fluorescence (arrows). (E) Detail of (D) showing callose deposition in cortical cell walls in contact with parasite tissues (arrows). (F) Detail of a central cylinder showing callose depositions in xylem walls and parenchyma cells (arrows). Arrowheads indicate the direction where the haustorium was located. CC, central cylinder; Ctx, cortex; Pic, parasite intrusive cells (haustorium); Xyl, xylem vessel.
Fig. 5. Cross-sections of incompatible interactions of Orobanche crenata on resistant vetch (Vicia sativa line A01) stained for characterization of mucilage composition. (A–C) Sections from the same series stained with AGS, TBO, and ruthenium red, respectively. Arrows indicate mucilage inside xylem vessels and arrowheads other filling substances. Asterisk indicates sections of the same xylem vessel as a reference. Green colour in (A) corresponds to carbohydrates and red colour with lignin, and/or lipids. Pink colour in (B) corresponds to carboxylated polysaccharides and blue–turquoise with polyphenols. Pink colour in (C) corresponds to non-methyl-esterified pectins. (D) Detail of the other substance(s) found inside xylem vessels, in this case located within the interface host–parasite, and stained with TBO. A granular structure and heterogeneous aspect can be observed. (E, F) Sections from the same series stained with AGS and phloroglucinol-HCl, respectively. Arrows indicate mucilage inside xylem vessels and arrowheads other filling substances. A yellow circle indicates the same xylem vessel in both sections. Asterisk indicates sections of the same xylem vessel as a reference. Green colour in (E) corresponds to carbohydrates and red colour with lignin and/or lipids. Red colour in (F) corresponds to lignin and polyphenols. Xyl, xylem vessel.
substances found inside host vessels showed a natural brownish yellow coloration. This substance stained strongly red with safranin from AGS (Fig. 5A, E), was positive also for ruthenium red (Fig. 5C), phloroglucinol (Fig. 5F), and TBO originating a green staining (Fig. 5B, D). All this indicated the presence of polyphenols, pectins, lignins, and probably lipids and/or tannins, but not suberin due to the lack of fluorescence using phloroglucinol staining (Fig. 4B). Positive staining with ruthenium red showed non-esterified pectins also being part of these components (Fig. 5C). Contrary to the homogeneous aspect showed by the mucilage, this substance presented a heterogeneous aspect and a granular structure (Fig. 5D).

In order to confirm that the mucilage was mainly composed of non-methyl-esterified pectins, immunostaining was performed with antibodies JIM 5 and JIM 7. An intense staining was observed in the core of the haustorium and adjacent areas for non-esterified pectins (Fig. 6A), whereas little presence of esterified pectins was detected (Fig. 6B) and was mainly restricted to host cortical cell walls opposite the haustorium. In no case were esterified pectins located inside host vessels (Fig. 6D). Non-esterified pectins were located in the apoplast, between intercellular spaces, and inside host xylem vessels (Fig. 6C, E, F), and in vascular parenchyma cells opposite the haustorium (Fig. 6E, F), this last being a confirmation of the important role of these cells in the synthesis and secretion of the mucilage.

When naphthol blue black was applied into the transpiration stream of roots, it was confined to the vascular bundles and stained the areas it was moving through. In compatible interactions, the vessels of the healthy tubercles and the haustorium were stained (Fig. 7A, B). By contrast, the dye did not reach the necrotic tubercles of incompatible interactions either in the haustorium or nearby host vascular tissues, but reached host vessels opposite to and away from the haustorium (Fig. 7C, D).

Discussion

Xylem occlusion as a putative defensive response against parasitic plants has been reported previously (Labrousse et al., 2001; Zehhar et al., 2003; Pérez-de-Luque et al., 2005b). However, no detailed studies about this phenomenon had been undertaken. The results presented here suggest that it is a quantitative trait, expressed against O. crenata at a higher intensity within resistant compared with susceptible vetch genotypes. The response is quantitative not only in the amount of incompatible infection units (Table 1), but also in the amount of mucilage accumulating inside vessels within incompatible interactions (Fig. 1).

The mucilage is presumably produced by vascular parenchyma cells near xylem vessels, as indicated by the more intense staining for carbohydrates observed in resistant plants (Fig. 2A), the immunostaining for non-esterified pectins with JIM 5 (Fig. 6E, F) and by the presence of a higher proportion of vessels away from the haustorium filled with mucilage (Fig. 1). This is in agreement with previous reports about mucilage production and secretion (Shi et al., 1992; Baayen et al., 1996; Dong et al., 1997; Rioux et al., 1998; Crews et al., 2003). Despite VanderMolen et al. (1983) suggesting that occlusion gels congeal in host vessels after dissolution of carbohydrates from host cell walls by pathogen enzymes, no evidence for this has been found. However, the safranin-staining substance accumulated in vessels near the haustorium is probably composed, partially at least, by host cell components released by O. crenata enzymatic activity.

The main components of the mucilage seem to be carbohydrates, specifically non-methyl-esterified pectins, and polyphenols to a lesser extent. This composition confers good properties to the gel in order to act as a permanent seal: the mixture can be polymerized by peroxidases to form a stable adhesive (Crews et al., 2003). This idea is supported by the accumulation of peroxidase in vessels of rice challenged by a vascular pathogen reported by Young et al. (1995) and the increased levels of peroxidase activity found by Pérez-de-Luque et al. (2005a) in resistant peas to O. crenata showing this kind of incompatible reaction (i.e. necrotic tubercles and mucilage production). Being of a composition similar to that described for mucilage produced against other vascular pathogens (Shi et al., 1992; Baayen et al., 1996; Kpémoua et al., 1996; Dong et al., 1997; Rioux et al., 1998; Tagne et al., 2002) it was assumed that this is the same response as found in those other pathogenic systems.

The question arising is why this defensive mechanism is activated if the parasite does not invade host vessels in the
same way as a vascular pathogen (i.e. fungi or bacteria spread along the xylem lumen). A first hypothesis could be that the host recognizes a wound in the tissues when the parasite penetrates, and mucilage production is activated as reported by Crews et al. (2003). However, the mucilage production would also be observed in susceptible plants during compatible interactions, and that is not the case (Fig. 1). Thus, in order to find out a more satisfactory answer, attention was paid to the safranin-staining substance accumulating in vessels near the haustorium. As previously stated (Pérez-de-Luque et al., 2005b) and confirmed by the present results (Table 1), this substance always accumulates in areas near the core of the haustorium and a similar substance was found at the attachment area of O. crenata seedlings and surrounding parasite intrusive tissues (Pérez-de-Luque et al., 2005b). This substance seems to be, at least partially, parasite originated, because it only appears on the external parasite interface near the attachment and penetration point (Pérez-de-Luque et al., 2005b). The role of this substance seems to be an anchoring device for the parasite in order to penetrate host tissues by mechanical pressure (Joel et al., 1996). Lipids probably take part in the composition of this secretion as well as non-methyl-esterified pectins (Figs 5A, C, 6A, C), conferring the adhesive properties. But the parasite also releases enzymes to allow penetration between host cells (Singh and Singh, 1993; Antonova and Ter Borg, 1996; Losner-Goshen et al., 1998). These enzymes dissolve components from the host cell walls and middle lamellae, such as pectins, polyphenols, and lignins, which will became part of the secretion, as suggested by the staining with AGS, ruthenium red, phloroglucinol, and TBO (Fig. 5). In fact, the secretion can be considered as a pull of different substances and components, including enzymes, originated mainly from the parasite, but also from the host. This could explain the heterogeneous aspect and granular structure it presents (Fig. 5D). When the parasite is hampered during the penetration attempts, it releases a higher amount of secretions (i.e. enzymes and adhesives) to overcome the host resistance (Joel et al., 1996; Pérez-de-Luque et al., 2005b). The presence of a ring of lignified host cells surrounding the parasite intruding tissues probably
corresponds to a previous barrier to penetration broken by the parasite (Fig. 3). If the parasite is successful and establishes vascular connections with the host, the excess of secretions and dissolved cell wall components leak through the apoplast and reach the neighbouring host vessels (Pérez-de-Luque et al., 2005b). At this time, the presence of foreign substances inside host vessels may activate the production and accumulation of mucilage. The degraded products from host cell walls, as carbohydrates, can act as the endogenous elicitors of defence responses (Aldington and Fry, 1993; Moerschbacher et al., 1999). Finally, the accumulation of mucilage, secretions, and degraded products block host vessels and does not allow nutrient flux between host and parasite (Fig. 7), causing further parasite death.

In conclusion, mucilage production can be considered as a quantitative defensive reaction taking place against O. crenata in vetch, and probably also in other legumes (Pérez-de-Luque et al., 2005b) and plants (Labrousse et al., 2001; Zehhar et al., 2003). It seems to be activated by the presence of foreign substances (i.e. parasite secretions) and host-degraded products (i.e. carbohydrates from cell walls) inside host vessels, and leads to the obstruction of the parasite supply channel and to the death of established Orobanche tubercles.

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