Medicago truncatula as a Model for Nonhost Resistance in Legume-Parasitic Plant Interactions\[C\]

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Crenate broomrape (Orobanche crenata) is a root parasitic weed that represents a major constraint for grain legume production in Mediterranean and West Asian countries. Medicago truncatula has emerged as an important model plant species for structural and functional genomics. The close phylogenetic relationship of M. truncatula with crop legumes increases its value as a resource for understanding resistance against Orobanche spp. Different cytological methods were used to study the mechanisms of resistance against crenate broomrape of two accessions of M. truncatula, showing early and late acting resistance. In the early resistance accession (SA27774) we found that the parasite died before a tubercle had formed. In the late resistance accession (SA4327) the parasite became attached without apparent problems to the host roots but most of the established tubercles turned dark and died before emergence. The results suggest that there are defensive mechanisms acting in both accessions but with a time gap that is crucial for a higher success avoiding parasite infection.

Medicago truncatula as a crenate broomrape host model plant due to its characteristics.

M. truncatula is an annual forage legume in the Mediterranean area. Contrary to other legume crops, M. truncatula is an autogamous self-fertile plant with a small and diploid genome, a short life cycle, and a prolific seed production (Blondon et al., 1994). Its simple genetics, the development of new tools and methods for molecular and genetic analysis, and the complete genome sequence (http://www.medicago.org) provide researchers with a valuable data set and making it interesting as a legume model species for laboratory studies (Cook et al., 1997; Cook, 1999) and also in pathogenic interactions (Ellwood et al., 2007; Pérez-de-Luque et al., 2007a).

Nowadays, the most numerous and important works about parasitic plants were focused on the development in susceptible host, as Orobanche spp. (Joel and Losner-Goshen, 1994; Neumann et al., 1999), Striga spp. (Dörr, 1997; Reiss and Bailey, 1998), Cuscuta spp. (Vaughn, 2002, 2003), Viscum spp. (Heide-Jørgensen, 1987), and others (Heide-Jørgensen and Kuijt, 1993, 1995). But little is known about the basis of host resistance to these parasites, just finding the work of Joel et al. (1996) introducing this subject. In the last years only some histological studies of the resistant interactions have been undertaken (Dörr et al., 1994; Antonova and Ter Borg, 1996; Gowda et al., 1999; Goldwasser et al., 2000; Labrousse et al., 2001; Rubiales et al., 2003; Zehhar et al., 2003; Pérez-de-Luque et al., 2005a, 2006b).

This lack of knowledge is due to the complexity of this interaction. The study of this host-parasite interaction presents important limitations because they both are plants, which implies sharing similar morphological, physiological, and biochemical traits. To solve it, we have chosen cytological and cytochemical techniques as powerful tools to reveal the mechanisms underlying the host-parasitic plant interaction. These studies, complemented with omic studies using M. truncatula as a host model, will be a valuable addition to our knowledge about the plant-parasitic plant interactions.

In this work we have used two M. truncatula accessions previously evaluated against crenate broomrape infection (Rodríguez-Conde et al., 2004). We have identified one of them as early resistant to broomrape...
infection (SA27774) because no establishment or development of parasites can be observed. The other one was characterized as late resistant (SA4327) because despite parasites established and developed, most of them were unable to evolve into mature plants. Cytochemical studies revealed that the defensive mechanisms are activated at different time points in each accession, which implies a different observation of the phenotype of the resistance and a higher success avoiding parasite infection for the early resistant accession.

RESULTS

Minirhizotron Studies

This system allowed the study of the infection process in both accessions of M. truncatula and the collection of samples at the right time for cytochemical analysis. It also permitted a quantitative study of the expression of the resistance, allowing characterization of both accessions as early and late resistant plants.

Addition of GR24 to the minirhizotrons assured a high and homogeneous germination of crenate broomrape seeds (40%). A similar number of attachments was formed on both accessions during the first 3 weeks (Table I). However, both accessions differed already by this time on percentage of crenate broomrape germinated seeds in contact with M. truncatula roots that successfully formed a tubercle, being the success on tubercle formation much lower in SA27774 (7.7%). This reaction was characterized by stoppage of parasite seedlings penetration into the host root, usually accompanied by darkening of host and/or parasite tissues around the point of attachment. On the contrary, at the same observation date, most of the attachments in SA4327 had evolved to small tubercles. As a consequence, 30 d after GR24 application there were almost no tubercles (4.3) in accession SA27774 contrasting with the number of them established on SA4327 (23.6). But a new incompatible reaction was found at this point: More than half of the tubercles (67.9%) on SA4327 had become dark and stopped their development.

### Table I. Phenotypic components of the resistance to crenate broomrape observed in M. truncatula per plant

Data shown as mean ± st. Data with the same letter within the same column are not significantly different (HSD, P < 0.05). * From a total of 200 seedlings per plant.

<table>
<thead>
<tr>
<th>Accession</th>
<th>15 d after GR24</th>
<th>22 d after GR24</th>
<th>30 d after GR24</th>
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<tr>
<td></td>
<td>Total No. of</td>
<td>% Oc Seedlings</td>
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<td></td>
<td>Oc Seedlings</td>
<td>Attached to Mt Roots*</td>
<td>Oc Seedlings</td>
</tr>
<tr>
<td>SA4327</td>
<td>23.0 ± 1.58</td>
<td>11.5 ± 0.79</td>
<td>27.4 ± 2.87</td>
</tr>
<tr>
<td>SA27774</td>
<td>23.4 ± 1.82</td>
<td>11.7 ± 0.91</td>
<td>31.6 ± 1.51</td>
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Figure 1. Sections stained with TBO. A, Cross section of a successful crenate broomrape seedling penetration on SA4087 accession of M. truncatula. B, Detail of A showing the central cylinder and host xylem vessels in contact with parasite cells. Some parasite vessels begin to develop connecting with the host xylem vessels. C, Cross section of a successful crenate broomrape seedling penetration on SA4327 accession of M. truncatula. D, Detail of C showing the central cylinder and host xylem vessels in contact with parasite cells. E, Cross section of an unsuccessful crenate broomrape seedling penetration on SA27774 accession of M. truncatula. F, Detail of E showing the thickening of host xylem walls (arrows) in contact with parasite cells and accumulation of a dark stained substance (arrowhead). ps, Parasite seedling; pic, parasite intrusive cells; hc, host cortex; hcc, host central cylinder; hx, host xylem vessels; px, parasite xylem vessels. [See online article for color version of this figure.]
Figure 2. Sections stained with AGS. A, Cross section of an unsuccessful crenate broomrape seedling penetration on SA27774 accession of *M. truncatula*. B, The same section observed under UV excitation (340–380 nm) showing an intense fluorescence in host cells in contact with parasite tissues (arrows). C, Overlay of A and B showing the localization of the fluorescent cells. D, Detail of A showing accumulation of substances (noncarbohydrates) inside host xylem vessels (arrows) and thickening of xylem vessels in contact with parasite cells (arrowhead). E, The same section as D observed under UV excitation (340–380 nm) and showing blue fluorescence from the thickened xylem cell walls (arrowhead). F, The same section as D observed under polarized light showing no changes in the cell walls birefringence of thickened xylem vessels. G, Cross section of a successful established crenate broomrape tubercle on SA4087 accession. H, Cross section of a darkened established crenate broomrape tubercle on SA4327 accession. I, The same section as H observed under polarized light. J, Detail of a cross section of a darkened established crenate broomrape tubercle on SA4327 accession showing accumulation of a dark brown substance inside parasite xylem vessels and the apoplast of the haustorium (arrow). K, The same section as J observed under UV excitation (340–380 nm) and showing quenched autofluorescence from the vessels covered by the dark brown substance (arrow). ps, Parasite seedling; pic, parasite intrusive cells; hc, host cortex; hcc, host central cylinder; hx, host xylem vessels; pba, parasite haustorium; pt, parasite tubercle; px, parasite xylem vessels.
Samples of both kinds of incompatible interactions and compatible interactions were collected and used for cytochemical studies.

**Light and Fluorescence Microscopy**

To characterize the mechanisms of resistance setting in play by the host, different histochemical procedures were employed. For comparison, tissues of the *M. truncatula* accession SA4087 were included as controls. This accession is the most susceptible one to crenate broomrape attack described to date (Rodrı́guez-Conde et al., 2004).

Sections of both successful and unsuccessful penetration attempts are presented in Figure 1, corresponding to accessions SA4087 (Fig. 1, A and B), SA4327 (Fig. 1, C and D), and SA27774 (Fig. 1, E and F), respectively. The toluidine blue O (TBO) staining was used as a general dye to get preliminary information about the incompatible interaction. In accessions SA4087 and SA4327 the parasite was able to pierce through the cortex and penetrated into the host central cylinder, beginning the development of the haustorium. Parasite intrusive cells in SA27774 also reached the host central cylinder but some abnormalities were observed. A dark stained deposit accumulated at the interface between host and parasite and a wall thickening inside host xylem vessels next to parasite tissues was developed (Fig. 1F).

Staining of sections from the early resistance accession with alcian green safranin (AGS) confirmed that parasite intrusive cells reached the host central cylinder, but an intense red coloration of the parasite tissues (corresponding to noncarbohydrate substances) was observed (Fig. 2, A and D), contrary to the common green staining (carbohydrates) expected for normal and healthy tissues in SA4087 (Fig. 2G). The parasite tissues also presented a disrupted and disorganized aspect. Accumulation of a red-stained material in the apoplast within and around the penetration pathway of the parasite was observed (Fig. 2, A and D) corresponding with the dark-stained material (unspecific substances) found using the TBO procedure. Host cells in contact and near the parasite tissues were impregnated with this red substance and it was also present inside some host xylem vessels (Fig. 2D). Observation of samples under UV excitation (340–380 nm) revealed a strong blue-white fluorescence from the walls and middle lamellae of cells surrounding the parasite intrusive tissues, including some xylem vessels (Fig. 2, E and F).
This fluorescence was shown only in host vessels near or in contact with the parasite cells (Fig. 2, C, and E), but not in those located away from the infection point. The thickening of the cell walls found inside the xylem vessels also presented this fluorescence (Fig. 2E). Observation of samples under polarized light proved that the secondary thickening of xylem walls was not due to birefringence (O’Brien and McCully, 1981), as it is common in the case of lignified and suberized walls (Fig. 2F). Sections of tubercles becoming dark on the late resistant accession and stained with the same technique (AGS) showed a normal aspect at the first sight (Fig. 2H) compared with those corresponding to healthy tubercles (Fig. 2G). However, a few details were observed: First, the presence of a dark brown deposit in the apoplast and some vessels from the haustorium (Fig. 2J), which did not stain; and second, the presence of a slight blue-white fluorescence in host xylem walls in contact with the haustorium (Fig. 2K). The dark deposit did not present fluorescence and quenched that of the impregnated vessels (Fig. 2K). It also eliminated the birefringence of the affected vessels (Fig. 2I) under polarized light. The blue-white fluorescence was very similar to that found in the case of the early resistant accession (Fig. 2, B, C, and E).

To check the possible role of lignins and suberins in this resistance, phloroglucinol-HCl staining was used (Fig. 3). In compatible interactions on SA4087, xylem walls appeared with a light pink stain (Fig. 3, E and F), indicating their normal lignification. However, an intense red coloration was observed in walls of xylem vessels near the parasite intrusive cells in sections of incompatible interactions on SA27774 (Fig. 3, A and C). These vessels presented also the thickened walls observed with TBO and AGS staining. Some xylem vessels were also filled with a material that was strongly stained with this method. When observed under UV excitation (340–380 nm; Fig. 3, B and D), lignin auto-fluorescence was quenched by the staining and only suberin fluorescence remained. As can be seen in Figure 3, B and D, only the suberized walls corresponding to the endodermal cells showed fluorescence; lignified cell walls did not fluoresce.

To identify other mechanisms implicated in reinforcement of host cell walls against Orobanche penetration, aniline blue fluorochrome was used for identification of callose under UV excitation (340–380 nm; Fig. 4). Our results showed that host cell walls from the cortex (Fig. 4C) in contact with parasite intrusive tissues and some cells in the central cylinder

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Sections stained with aniline blue fluorochrome. A, General view of a cross section of an incompatible interaction on SA27774 accession of *M. truncatula* observed under bright field. B, The same section as A observed under UV excitation (340–380 nm). C, Detail of B showing callose depositions (arrow) in cell walls from the host cortex in contact with parasite cells. D, The same section as C showing callose depositions (arrow) in cell walls from the host central cylinder. E, Cross section of a compatible interaction on SA4087 accession of *M. truncatula* observed under bright field. F, The same section as E observed under UV excitation (340–380 nm). No presence of callose is detected. pt, Parasite tubercle; pic, parasite intrusive cells; hc, host cortex; hcc, host central cylinder.
Fig. 4D presented a slight accumulation of callose. No presence of callose was detected in compatible interactions (Fig. 4F).

Observations of fresh hand cut sections were taken under UV excitation (340–380 nm; Fig. 5) to detect the presence of phenolic compounds. No fluorescence in tissues was detected neither in uninfected roots (Fig. 5, A and B) or compatible interactions (Fig. 5, C and D). On the contrary, a strong fluorescence was found in host tissues adjacent to parasite intrusive cells (Fig. 5, E and F) and haustoria (Fig. 5, G and H) in sections of incompatible interactions.

Confocal Laser Scanning Microscopy

Confocal microscopy studies were developed to get a more secure localization of phenolic compounds in tissues (Fig. 6). The emission spectra were collected using two channels (green and red) for the same excitation, to check differences in the fluorescence of the accumulated compounds. The fluorescence was detected within the host central cylinder (Fig. 6, A–D) and cortical cells (Fig. 6, E–H) in incompatible interactions on the early resistant accession (SA27774). Also, accumulation of phenolics was observed in the

**Figure 5.** Hand cut fresh sections for fluorescence observation. A, Longitudinal section of an uninfected *M. truncatula* root observed under bright field. B, The same section as A observed under UV excitation (340–380 nm). C, Longitudinal section of a successful established crenate broomrape tubercle on SA4327 accession of *M. truncatula* observed under bright field. D, The same section as C observed under UV excitation (340–380 nm). E, Longitudinal section of an unsuccessful crenate broomrape seedling penetration on SA27774 accession of *M. truncatula* observed under bright field. F, The same section as E observed under UV excitation (340–380 nm) and showing a strong fluorescence from host cells in contact with parasite tissues (arrows). G, Cross section of a darkened crenate broomrape tubercle on SA4327 accession observed under bright field. H, The same section as G observed under UV excitation (340–380 nm) and showing a strong fluorescence from host cells in contact with parasite tissues (arrows). hc, Host cortex; hcc, host central cylinder; pha, parasite haustorium; pt, parasite tubercle; pao, parasite attachment organ; pic, parasite intrusive cells.
Figure 6. Localization of phenolic compounds using confocal laser microscopy. The images are single optical sections in B, C, F, G, J, K, M, N, and P. Figures B, F, J, and M correspond to emission spectra collected within the red channel (590–670 nm). Figures C, G, K, N, and P correspond to emission spectra collected within the green channel (515–590 nm). A, Incompatible interaction of crenate broomrape on SA27774 accession of *M. truncatula* (transmission). A darkening of the attachment organ and root tissues can be observed. B, Optical section of A showing intense fluorescence (red channel) in the host central cylinder and in the youngest attachment organ. C, The same section as B through the green channel. D, Overlay of A, B, and C showing the localization of the fluorescence in tissues. E, Detail of an unsuccessful crenate broomrape seedling penetration on SA27774 accession (transmission). F, Optical section of E showing intense fluorescence (red channel) in the host cells and in the parasite intrusive cells. G, The same section as F through the green channel. H, Overlay of E, F, and G showing the localization of the fluorescence in tissues. I, Darkened crenate broomrape tubercle on SA4327 accession of *M. truncatula* (transmission). J, Optical section of I showing intense fluorescence (red channel) in the haustorium and distal parts of the tubercle. K, The same section as J through the green channel. L, Overlay of I, J, and K showing the localization of the fluorescence in tissues. M, Optical section of a darkened crenate broomrape tubercle on SA4327 accession showing intense fluorescence (red channel) in the host xylem vessels (arrow). N, The same section as M through the green channel. O, Overlay of M and N with a transmission image showing the localization of the fluorescence in tissues. P, Optical section of a normal crenate broomrape tubercle on SA4327 accession. No fluorescence (green channel) can be detected. ps, Parasite seedling; pao, parasite attachment organ; hr, host root; pic, parasite intrusive cells; hc, host cortex; hcc, host central cylinder; pha, parasite haustorium; pt, parasite tubercle. Scale bar: 80 μm in A to D, I to L, and P; 40 μm in E to H and M to O.
attachment organ of some parasites in contact with the host root (Fig. 6, A–D). Regarding incompatible interactions on the late resistant accession (SA4327), accumulation of phenolics was observed in the haustoria and tubercles of the parasite (Fig. 6, I–L) and within host xylem vessels connected with the parasite haustorium (Fig. 6, M–O). No presence of phenolics compounds was detected in compatible interactions on both accessions SA4327 (Fig. 6P) and SA4087 (data not shown).

Cell Viability Assay

Trypan blue staining was used in fresh hand cut sections to check the viability of the cells in compatible and incompatible interactions (Fig. 7). In compatible interactions (Fig. 7, A and B) and uninfected roots (Fig. 7E) all the cells exclude the dye, confirming they were alive. However, the parasite intrusive cells and those corresponding to the attachment organ (Fig. 7, C and D) in incompatible interactions were clearly stained by the dye, indicating no viable cells. Moreover, parasite cells located in the distal part of the tubercle in incompatible interactions on the late resistant accession were also stained (Fig. 7F) and consequently were not viable.

Identification of Phytoalexins

Thin-layer chromatography (TLC) plates showed the presence of known phytoalexins in the methanolic extracts of inoculated plants from both soluble and cell wall-bound phenolics fractions (Fig. 8). Medicarpin and maackiain were found in the soluble phenolic fraction of inoculated plants of both *M. truncatula* accessions. Scopoletin was identified in the cell wall-bound phenolic fraction of inoculated plants of *M. truncatula* accession SA4327. Pisatin was not detected in any of the extracts. Several other compounds appeared on the TLC plate corresponding to soluble phenolics from inoculated plants but they did not correlate with any of the phytoalexins used as standards. The retention factor of the known phytoalexins was 0.158 for scopoletin, 0.526 for pisatin, 0.595 for maackiain, and 0.632 for medicarpin.

DISCUSSION

Mechanisms of resistance against crenate broomrape were characterized in two genotypes of *M. truncatula*. One of them, SA27774, shows an early expression of the resistance to this parasitic plant and does not allow
the establishment of the parasite. On the contrary, SA4327 is infected with crenate broomrape and allows the establishment and development of parasite tubercles. However, it is not completely susceptible to the pathogen attack and a late resistance is expressed after tubercles establishment. This does not prevent completely the development of the parasitic plant, but limits the amount of individuals of the pathogen growing on it. Both kind of incompatible interactions, appearing before or after the development of the parasite haustorium, have been previously reported in resistant host to parasitic plants (Labrousse et al., 2001; Sørensen et al., 2003; Pérez-de-Luque et al., 2005b, 2006b; Echevarría-Zomeño et al., 2006). They are indicative of resistance, but the exact nature of the mechanisms underlying such resistance is not well understood. For example, stoppage of seedling penetration has been usually associated to a hypersensitive response (HR; Dörr et al., 1994; Goldwasser et al., 1997), but there is no conclusive evidence that a HR really occurs in these interactions (Rubiales et al., 2003; Pérez-de-Luque et al., 2005b) in a manner similar to that described for fungal attack (Heath, 1999; Richael and Gilchrist, 1999). For that reason, histological and histochemical studies are of great value to know what is really happening inside host tissues.

The cytological data show that the penetration of crenate broomrape in the early resistant *M. truncatula* accession (SA27774) is stopped once the parasite intrusive cells have reached the host central cylinder. To our knowledge, this is the first time that a prehaustorial mechanism of resistance against parasitic root plants is located inside the central cylinder of the host. Usually, the prehaustorial defensive mechanisms against parasitic plants have been located in the host cortex (Echevarría-Zomeño et al., 2006; Pérez-de-Luque et al., 2006a, 2007b) or the endodermis (Pérez-de-Luque et al., 2005b, 2006a, 2007b). This kind of resistance is also different to that found in rice (*Oryza sativa*) and maize (*Zea mays*) against the parasite Striga spp. (Gurney et al., 2003, 2006) where the parasite is stopped before penetrating the central cylinder, probably due to physical barriers as described in the case of *Orobanchaceae* spp. But once the parasite reached the central cylinder, a haustorium was developed connecting with the hosts vascular tissues and resistance relied on posthaustorial mechanisms such as sealing of vessels by mucilage (Pérez-de-Luque et al., 2005a, 2006b). Moreover, to our knowledge, for the first time is also described a posthaustorial defensive mechanism different from the sealing of vessels (Pérez-de-Luque et al., 2005a, 2006b) in the late resistant *M. truncatula* accession (SA4327).

The only physical barrier detected against the parasite intrusion is the thickening of host xylem walls. This was reported some time ago (Dörr et al., 1994), and our results indicate that the secondary thickening of the xylem walls is composed of some kind of lignin, or at least (because no birefringence was detected) by some kind of pollyphenol. Although some callose depositions were located, their presence was not enough or well located enough to stop broomrape penetration as has been recently found in faba bean (*Vicia faba*; Pérez-de-Luque et al., 2007b). In this last case, big callose depositions were located in host cell walls in contact with parasite tissues (i.e. at the point of the resistance) stopping the parasite penetration. However, we cannot discard a possible role of callose as a reservoir of β-glucans elicitors (Esquerre-Tugayé et al., 2000) as suggested in the case of pea (*Pisum sativum*)-crenate broomrape (Pérez-de-Luque et al., 2006a).

So the lack of strong physical barriers preventing the parasite penetration into the host must be complemented and reinforced by another type of defensive mechanism: The fluorescence points toward the presence of phenolic compounds (phytoalexins) as the mechanism responsible to stop parasite intrusion, as has been recently reported in the sunflower (*Helianthus annuus*)-crenate broomrape interaction (Echevarría-Zomeño et al., 2006). However, contrary to what has been shown in that work, in which suberization plays a crucial role stopping crenate broomrape penetration in the cortex, no physical barriers appear in *M. truncatula* roots and the parasite intrusive cells are able to pierce easily through the cortex and the endodermis, reaching the vascular cylinder. At this moment, an intense release of phenolic compounds at the infection point

Figure 8. TLC plate of the methanolic extract from root samples of crenate broomrape inoculated (i) and noninoculated (ni) *M. truncatula* plants. Ctrl, Control with standard phytoalexins (scopoletin, medicarpin, pisatin, and maackiain).
takes place through the host cells in contact with the parasite tissues and the xylem vessels. Medicarpin and maackian have been found as part of the soluble phenolics fraction in inoculated plants and both phytoalexins have been previously reported as implicated in defense against pathogens (Cachinero et al., 2002). This maybe creates an unpleasant environment for the parasite intrusive cells, contributing to their further death and avoiding the development of an haustorium. Moreover, these phenolic compounds seem to be secreted to the external part of the root, affecting broomrape seedlings attached near a previous unsuccessful penetration attempt. This is not strange, because accumulation and secretion of phytoalexins against parasitic plants has been previously reported (Goldwasser et al., 1999; Serghini et al., 2001; Échevarría-Zomeño et al., 2006). The absence of dead host cells in this case, confirmed by the viability test, supports that this defensive mechanism is not a HR. However, other mechanisms of resistance acting at the same time cannot be discarded. For example, hormonal compatibility between host and parasite is needed to develop a functional haustorium (D.M. Joel and A. Pérez-de-Luque, unpublished data) and a lack of this compatibility could affect the normal parasite growth.

In the case of the late resistance found in SA4327, the accumulation and secretion of phenolic compounds seems to be operating but at a later stage. Once the parasite has formed a haustorium and established vascular connections with the host, the last one produces phenolic compounds that are translocated through the vascular system and reach the parasite. The first evidence for this is the presence of a dark deposit in parasite vessels of the haustorium. This deposit probably corresponds with the oxidation of phenolic compounds, which usually originates dark brown components (Takahama, 2004). The fluorescence found in host vessels, haustoria, and tubercles implies that the host is poisoning the parasite established tubercles releasing phytoalexins through the vascular connections. These phytoalexins accumulate in the tubercles by the sink effect that crenate broomrape has once it has been connected with the host root. The presence of parasite dead cells in the same areas where phenolic compounds accumulate within the tubercles could indicate that the accumulation of toxic metabolites (phenolics) from the host plant is killing the parasite. The presence of soluble phenolics like medicarpin and maackian also supports the idea of excretion of phytoalexins. As in the previous case, other(s) defensive mechanism(s) cannot be discarded, including the lack of hormonal compatibility. The presence of scopoletin in the cell wall-bound phenolics fraction could be indicative of further development of physical barriers not detected at this point by cytochemical methods.

All these results are in accordance with those obtained by Dita et al. (2007), who found an activation of genes related with the synthesis of phenolic compounds also in early and late resistant accessions to crenate broomrape. The main difference was, like in this case, a matter of time: Similar genes were activated but with a time gap between them.

CONCLUSION

Recent studies have revealed that multiple factors are involved in resistance to parasitic plants. Behind the observation of incompatible interactions (unsuccessful attachment penetration and darkening of established tubercles) underlies a complex system of multiple mechanisms of resistance. To date, most of those described were based mainly in physical barriers preventing parasite penetration into the host central cylinder (prehastorial mechanisms) and blocking of host vessels disrupting the nutrient fluxes between host and parasite (posthaustorial mechanisms). In this work we presented one mechanism of resistance, accumulation of phytoalexins (phenolic compounds), that does not rely on physically stopping parasite penetration into the host: The parasite penetrates reaching the central cylinder, but it seems to be poisoned and killed before developing a haustorium. Despite other mechanisms of resistance that could be involved, a crucial difference between both accessions is the moment at which the host detects and reacts against the parasite. It determines a more effective resistance against the pathogen: The earliest the parasite is detected, the most effective are the defensive mechanisms activated, and the infection is lower.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Crenate broomrape (Orobanche crenata) was grown on accessions of Medicago truncatula showing early or late resistance to this pathogen (accessions SA27774 and SA4327, respectively).

The petri dish system described by Pérez-de-Luque et al. (2005a) and Rubiales et al. (2006) was used for in vitro cultivation of the M. truncatula plants and inoculation with crenate broomrape seeds.

M. truncatula seeds were supplied from the South Australian Research and Development Institute Genetic Resource Centre in Australia (origin: Yugoslavia for SA4327 and Morocco for SA27774). Seeds were scarified with a metal sheet and sterilized in commercial bleach (20% in sterile water) for 10 min. For synchronize germination, seeds were place at 4°C for 36 h in sterile water. During this period, we replaced sterile water two or three times to help germination. After that, seeds were rinsed with sterile water at room temperature for 3 or 4 h, changing it six or eight times. Finally, seeds were placed in petri dishes on wet glass fiber filter papers (Whatmann GF/A) and kept in darkness at 20°C for 1 to 2 d. When the radicle reached 2 cm length, seedlings were transferred to new dishes (15 cm diameter) with perlite and new glass fiber papers (Pérez-de-Luque et al., 2005a).

Crenate broomrape seeds were collected from infected faba bean (Vicia faba) plants at Córdoba during 2000. They were disinfected with formaldehyde according to González-Verdejo et al. (2005) and spread on the glass fiber paper (approximately 8 mg) where the M. truncatula roots were growing. To prevent exposure of the parasite seeds and host roots to direct light, the dishes containing test plants inoculated with parasite seeds were sealed with paraffin and covered with aluminum foil. Then, the upward growing host plants were placed vertically in trays with Hoagland nutrient solution (Hoagland and Arnon, 1950) and grown in a controlled environment chamber at 20°C ± 0.5°C with a day/night 14 h photoperiod and an irradiance of 200 μmol m−2 s−1.

At the same time plants were growing, crenate broomrape seeds were conditioned. For conditioning, parasite seeds need to be in darkness at 20°C for 10 d (Pérez-de-Luque et al., 2005a).
After conditioning period, we applied 5 mL of the synthetic stimulant GR24 (1 mg/mL; Magnus et al., 1992) on the paper with the seeds (for crenate broomrape homogenous germination induction; Rubiales et al., 2003).

**Minirhizotron Studies**

The infection process was followed using a binocular microscope (Nikon SMZ1000; Nikon Europe B.V.). Fifteen days after GR24 application, the percentage of crenate broomrape attach seedlings on *M. truncatula* roots was calculated. The total of 200 crenate broomrape seedlings close (<3 mm) to the *M. truncatula* roots were visualized in each petri dish and the number of attached seedlings was referred to the total number of seedlings. At 22 d after GR24 application, the percentage of compatible and incompatible attachments against total attachments was scored. An attachment was considered compatible when it resulted in tubercle formation. Finally, 30 d after GR24 application established bromptones were quantified and expressed as absolute value per plant. In addition, the number of darkened tubercles was recorded and expressed as a percentage respect to the total number of established tubercles per plant.

**Collection and Fixation of Samples**

Observations were taken using a binocular microscope. At 15 d after GR24 application, seedlings of crenate bromptone were sampled at random with the corresponding attached parts of host roots.

For staining methods and 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.

Fixed samples were then dehydrated in ethanol series (50%, 80%, 100%, and 100%: 12 h each) and transferred to an embedding solvent (Xylene; Panreac Quimica S.A.) and attached to adhesive-treated microscope slides (poly-l-lysine slides; Menzel GmbH & Co. KG).

**Staining Methods**

After removal of paraffin, sections were stained with different dyes: (1) Staining with 0.05% TBO in PO4 buffer (pH 5.5) during 5 to 10 min was used. In this case the dye was applied before removal of paraffin (Ruzin, 1999). This method allows the detection of phenolics as well as tannins, lignin, and suberin (Bayaen et al., 1996; Bordallo et al., 2002; Mellersh et al., 2002; Crews et al., 2003). (2) AGS (Joel, 1983): The slides were dried and mounted with DePeX (BDH Chemicals). With this staining method, carbohydrates (including cell walls and mucilage) appeared green, yellow, or blue, while lignified, de-luque et al., 2005a). (3) Phloroglucinol (2% in ethanol)-HCl (35%; Ruzin, 1999) were applied covering tissue sections for examination under the fluorescence microscope. (4) Aniline blue fluorochrome in water (Naught 534; Auxilab S.A.) and attached to adhesive-treated microscope slides (polysine slides; Menzel GmbH & Co. KG).

**Extraction of Total Phenolics and Identification of Phytoalexins**

The petri dish system was used for plant material collection from *M. truncatula* accessions showing early or late resistance (SA27774 and SA4327, respectively) to crenate broomrape as described before. *M. truncatula* roots from noninfected and infected plants were sampled 30 d after GR24 application, the date corresponding to establishment of the parasite. For noninfected plants, small root pieces (approximately 1 cm) were sampled at random. For infected plants, parts of the root with a crenate broomrape attachment were sampled, removing the parasite tissues. Then, samples were washed with tap and then distilled water, blotted dry with filter paper, frozen in liquid nitrogen, and stored at ~80°C until biochemical analysis (Perez-de-Luque et al., 2005a).

Frozen root tissue (0.04 g fresh weight) was homogenized in 1 mL methanol by using a pestle and mortar. After filtering off the solvent extract, the residue was further sequentially extracted twice with a similar volume of methanol and centrifuged twice at 15,000g for 15 min. The combined solvent extracts were dried and phenolic compounds were resuspended in 0.2 mL of methanol. The pellet residue was resuspended in 0.08 mL of 2 N NaOH and incubated at 70°C for 16 h. The suspension was cooled down, neutralized with 0.08 mL of 2 N HCl, and centrifuged (15,000g for 15 min). Then, the suspension was dried and resuspended in the same volume of methanol.

TLC analysis of the methanolic extract was performed according to Prats et al. (2003) by using Silicagel F254 plates (Merck) and diethylether:hexane (70:30 v/v) as the mobile phase. Plate was visualized under UV light (254 nm) lamp. Scopoletin (Sigma), medicarpin, pisatin, and maackiani (from Plantech) were used as standards.

**Statistical Analysis**

Minirhizotron assays were performed with two plants per petri dish and 10 petri dishes per *M. truncatula* accession (SA27774 and SA4327). Data were recorded from three randomized areas in each plant. To study possible interactions due to cultivation of plants in different dishes, each petri dish was treated as a block and an ANOVA was performed (Statistica v11.1 for Windows). No significant differences were found between blocks so each petri dish was considered as a replicate. For cytochemical and confocal studies, at least 10 petri dishes were selected at random from several petri dishes for each study. Percentages were transformed according to the formula $Y = \arcsin(\sqrt{X/100})$ prior to statistical treatment.

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Lozano-Baena et al.