The Endophytic System of Mediterranean *Cytinus* (Cytinaceae) Developing on Five Host Cistaceae Species

CLARA DE VEGA*, PEDRO LUIS ORTIZ, MONTSERRAT ARISTA and SALVADOR TALAVERA

Departamento de Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, Apdo-1095, 41080 Sevilla, Spain

Received: 28 May 2007 Returned for revision: 10 July 2007 Accepted: 20 July 2007 Published electronically: 5 September 2007

**Background and Aims** One of the most extreme manifestations of parasitism is found in the genus *Cytinus*, a holoparasite whose vegetative body is reduced to an endophytic system living within its host root. There are two species of *Cytinus* in the Mediterranean, *C. hypocistis* and *C. ruber*, which parasitize various genera of Cistaceae, one of the most characteristic families of the Mediterranean scrublands. The aim of this work is to describe the endophytic systems of *C. hypocistis* and *C. ruber*, and their tissue relationships with their host.

**Methods** Roots from five different hosts infected with *C. hypocistis* and *C. ruber* were harvested, and examined by anatomical techniques under light microscopy to elucidate the characteristics of the endophytic system of *Cytinus*, and to determine if differences in endophytic systems occur between the two species and in response to different hosts.

**Key Results** The endophytic structure is similar in both *Cytinus* species irrespective of the host species. In the initial stages of the endophyte, rows of parenchymal cells spread through the host pericyclic derivatives and phloem, and begin to generate small nodules in the outermost region of the host xylem. Later the nodules anastomose, and bands of parasitic tissue are formed. The host cambium continues to develop xylem tissue, and consequently the endophyte becomes enclosed within the xylem. The bands of parasitic tissue fuse to form a continuous sheath. This mature endophyte has well-developed vascular system with xylem and phloem, and forms sinkers with transfer cells that grow through the host xylem.

**Conclusions** The endophytic system of *Cytinus* develops in all host root tissues and reaches its most mature stages in the host xylem. It is more complex than previously reported, showing parenchyma, xylem and phloem tissues. This is the first report of well-developed phloem in a holoparasitic endophytic species.

**Key words:** Cistaceae, Cytinaceae, *Cytinus hypocistis*, *Cytinus ruber*, endophyte, Mediterranean region, parasitic plant, sieve elements, sinker, transfer cell.

**INTRODUCTION**

One of the most extreme manifestations of parasitism is found in the families of endoparasites Rafflesiaceae, Mitrastemonaceae, Apodanthaceae and Cytinaceae. These perennial plants, without chlorophyll, are obligate parasites, and depend on their hosts to obtain water and nutrients (Kuijt, 1969). All show a reduction in their morphological characters, with scale-like leaves and absence of external roots, and their vegetative body is reduced to a haustorial or endophytic system, often compared with that of a fungal plectenchyme. These endophytes live within the roots or stems of their hosts (Kuijt, 1969; Meijer, 1993), and emerge from the hosts only during the reproductive period, when the inflorescences arise. Because of this characteristic lifestyle, these endophytic holoparasites were long considered to constitute a single family, the Rafflesiaceae. However, differences in the morphology of flowers, ovaries and seeds, together with data from recent molecular phylogenetic studies, indicate that they are distinct families, even belonging to different orders (Bouman and Meijer, 1994; Barkman et al., 2004; Nickrent et al., 2004; Davis et al., 2007).

The structure of the haustorial system in these reduced endophytic holoparasites is poorly known, and there are detailed data only for the genus *Pilostyles* (Dell et al., 1982; Kuijt et al., 1985). The few existing data indicate that these endophytes are composed of parenchymal cells, with xylem elements being observed only occasionally (Fraysse, 1906; Kuijt, 1969; Dell et al., 1982; Forstmeier et al., 1983; Kuijt et al., 1985). Functional phloem has never been observed in the endophytes of these families studied to date, although Kuijt et al. (1985) have described a vestigial phloem tissue in *Pilostyles thurberi* (Apodanthaceae). Within the host, the endophytic system can grow intercellularly between the cambial zone and the phloem (Mitrastemonaceae), through the cambial zone (Rafflesiaceae, Apodanthaceae), through the phloem (Apodanthaceae), or between the cambial and the xylem (Cytinaceae) (Kuijt, 1969; Meijer and Schlauer, 2002).

The distribution of the families of the holoparasitic endophytes is mainly tropical or subtropical (Molau, 1995), and in the Mediterranean region there are only two species, *Cytinus hypocistis* and *Cytinus ruber* (Cytinaceae) both of which parasitize exclusively roots of Cistaceae, one of the most characteristic families of shrubs in the Mediterranean flora. *Cytinus hypocistis* has yellow flowers and parazites white or yellow-flowered *Cistus*, *Halimium*, *Helianthemum*, while *C. ruber* has white or pinkish-white flowers and parazites pink-flowered *Cistus*.

* For correspondence. E-mail cvega@us.es

© The Author 2007. Published by Oxford University Press on behalf of the Annals of Botany Company. All rights reserved.

For Permissions, please email: permissions@oxfordjournals.org
Although Mediterranean Cytinus have been known since ancient times, and even mentioned by Teofrasto (Villar, 1997), their life cycle, floral biology, process of infection, and relationships with the hosts remain practically unknown. The anatomy of the endophytic system of C. ruber is unknown and that of C. hypocistis poorly documented, with almost all the existing studies going back a century or more, e.g. Solms-Laubach (1867); Frayse (1906); and also Forstmeier et al. (1983). The aim of this work is to describe the endophytic system of C. hypocistis and C. ruber, and examine the relationships they establish with the host tissues. Moreover, given that the endophytes of some parasitic plants can adopt different forms depending on the host they parasitize (Thoday, 1960, 1963), the development of the endophyte of Cytinus was studied on five of its most common host species. This work falls within a broad study on the Mediterranean Cytinus which has shown that their taxonomic status is uncertain, since genetic races or cryptic species defined by host identity seem to exist (C. de Vega, unpubl. res.). However, in the absence of a new recognized classification, the two traditionally recognized species, C. hypocistis and C. ruber, are dealt with here.

MATERIALS AND METHODS

Cytinus hypocistis (L.) L. and C. ruber (Fourr.) Fritsch. are perennial holoparasites that appear exclusively on roots of Cistaceae species. The vegetative body of both parasites is contained entirely within the root of their host plants, and only in spring do the inflorescences burst through the host tissues. According to Heinricher (1917) the developmental time for Cytinus to produce an inflorescence requires over 3 years.

Roots of Cistus albidus L., infected with Cytinus ruber, and roots of Cistus ladanifer L., Cistus salviifolius L., Halimium halimifolium (L.) Willk. and Halimium ocyoides (Lam.) Willk., infected with Cytinus hypocistis, were collected from natural populations in south-west Spain. Root collection was made during the flowering of Cytinus, given that infestation is apparent only in this period.

Small pieces of these roots were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for approx. 1 d at room temperature, and further incubated in a vacuum oven for 1 h at room temperature to improve the fixation. The tissues were subsequently dehydrated in an ethanol series, embedded in Historesin (glycol methacrylate; Leica, Heidelberg), and serially sectioned at 2–4 μm, using a rotary microtome. Sections were stained with 0.12% toluidine blue and 0.05% basic fuschin (Junqueira, 1990), and mounted with Eukitt (Kindler, Freiburg, Germany). For observation of sieve elements, sections were stained with 0.05% aniline blue in 0.05 M phosphate buffer (pH 8.5) and then examined under a fluorescence microscope. Under these conditions, sieve tubes were visible due to fluorescent callose deposits.

All images were taken with a Leica DC300 camera attached to a Zeiss Axiophot light microscope. Voucher specimens of the host plants were deposited in the herbarium of the University of Seville.

RESULTS

Macroscopic morphology of the infection

Year after year, Cytinus inflorescences burst through the same zone of the host root (observations made in 50 populations over 5 years). Thus, a ‘reproductive zone’ of Cytinus on the host root can be referred to, that is generally found very close to the trunk. On the infected roots, both isolated inflorescences of Cytinus and groups of up to 22 inflorescences may occur on a relatively small portion of the root (Fig. 1A). Around the zone of emergence of each inflorescence a cup-shaped structure is formed that originates from host tissue that ruptures, enabling the inflorescence to exit (Fig. 1B). These structures remain as permanent scars on the roots.

Structure of the endophytic system and relationships with the host tissues

The anatomical structure of the roots of the five species of Cistaceae studied is similar, showing the typical characteristics of dicotyledonous secondary roots. The outermost layer of the root is a periderm, and internally a large zone of pericyclic derivatives with abundant fibres and scarce intercellular spaces is found. The phloem does not show fibres or sclereids, and appears separated from the xylem by a narrow cambial zone. The xylem occupies the...
greater part of the root, and the vascular elements are heterogeneous in size.

The development and structure of the endophytic system of *Cytinus* are similar in both species studied in their five host species. Thus, the following description is general, and independent of the host being parasitized. The endophytic system of *Cytinus* grows in all the tissues of the host roots, although it reaches the most mature and complex stages in the host xylem. In the initial stages, parenchymal cells of the parasite are observed growing intercellularly among the pericyclic and phloem cells of the host. These parenchymal cells form single-rowed filaments that spread tangentially through the pericyclic tissues and phloem of the host, and radially towards the centre of the root (Fig. 2A, B). The parenchymal cells of *Cytinus* are easily distinguished from those of the host by their large nuclei and thickened walls (Fig. 2B). When these parasitic radial filaments cross the host cambial zone, they begin to generate small nodules of parasitic tissue in the outermost region of the host xylem, contiguous with the cambial zone (Fig. 2C). Initially, these nodules have only parenchymal cells recognizable by their dense cytoplasm, which stains intensely (Fig. 2C). However, they soon thicken, and appear as a differentiated central cambial zone surrounded by parenchyma tissue (Fig. 2D). As the nodules anastomose, bands of parasitic tissue are formed (Fig. 2D). These bands show a clear differentiation of two layers of parenchymal tissue enveloping a central cambial zone (Fig. 2D). As the host cambium continues its activity, producing xylem inwardly, the endophyte bands at this stage are separated from the host cambial zone by its xylem, but connections are maintained with the radial filaments from which the nodules originated (Fig. 2D). As the endophyte bands separate from the host cambial zone, the parasite cambium begins to form vascular tissues, with phloem externally and xylem internally, and the number of cell strata in the parenchymal layers increases (Fig. 2E). The sieve elements of the endophyte are larger

**FIG. 2.** Different stages in the development of the endophytic system of *Cytinus hypocistis* and *C. ruber*. (A) Cross-section of a root of *Cistus salviifolius*. Arrows indicate the parasitic tissue. (B) Magnification of the area marked in red in (A), with the tissue of *C. hypocistis* coloured pink. Note the parasitic cells crossing the host pericyclic tissues and phloem. These cells are easily distinguished from those of the host by their large nuclei and thickened walls (Fig. 2B). When these parasitic radial filaments cross the host cambial zone, they begin to generate small nodules of parasitic tissue in the outermost region of the host xylem, contiguous with the cambial zone (Fig. 2C). Initially, these nodules have only parenchymal cells recognizable by their dense cytoplasm, which stains intensely (Fig. 2C). However, they soon thicken, and appear as a differentiated central cambial zone surrounded by parenchyma tissue (Fig. 2D). As the nodules anastomose, bands of parasitic tissue are formed (Fig. 2D). These bands show a clear differentiation of two layers of parenchymal tissue enveloping a central cambial zone (Fig. 2D). As the host cambium continues its activity, producing xylem inwardly, the endophyte bands at this stage are separated from the host cambial zone by its xylem, but connections are maintained with the radial filaments from which the nodules originated (Fig. 2D). As the endophyte bands separate from the host cambial zone, the parasite cambium begins to form vascular tissues, with phloem externally and xylem internally, and the number of cell strata in the parenchymal layers increases (Fig. 2E). The sieve elements of the endophyte are larger
than those of the host, and are more scattered (Fig. 3A, B). As observed under fluorescence, no direct connections were seen between the phloem of the endophyte and that of the host. The sieve elements of the endophyte are long, with thin walls, and a weakly staining cytoplasm lacking a nucleus. The simple sieve plates found in the cross-walls of *Cytinus* sieve elements are easily observed with fluorescence microscopy, after staining with aniline blue, as are its lateral sieve areas (Fig. 3C, D). The elements of the endophytic xylem vessels have walls with reticulated or helicoidal secondary thickening (Fig. 3E and F, respectively).

As the cambium of the host deposits new layers of xylem on the parasitic tissue, the endophytic tissue becomes progressively more deeply embedded in the host xylem than the other xylem endophyte. At this stage, nodules formed under the host cambial zone begin to form new endophyte bands (Fig. 3G). The repetition of this process gives rise to alternate strata of parasitic and host tissues (Fig. 3H). As the bands of endophyte become encapsulated within the host xylem, they fuse to form a continuous sheath (Fig. 3I).

Even before the endophyte forms this sheath, radial processes, called sinkers (Kuijt, 1977), also begin to differentiate from the parasite cambial zone. The sinkers increased in length with growth from the endophyte cambial zone, and they seem to develop in conjunction with xylem development. A sinker is generally single-rowed (Fig. 4A) and extends through intercellular spaces until it encounters mature host xylem where its terminal cell wedges itself between xylem cells given rise to a pointed end (Fig. 4B, C). The sinkers are generally formed of thick-walled parenchymal cells and where these cells are contiguous with host vessels the wall is thickest against the vessel (Fig. 4D). These parenchyma cells may have differentially thickened walls that occur in contact with host vessels (Fig. 4E), and so they can be considered as transfer cells, particularly since such thickening of the sinker walls is absent when they are abutting host fibres and parenchyma. Occasionally isolated xylem cells of the parasite are intercalated between sinker parenchymal cells, but they are easily recognizable by their helicoidal thickenings and by their radial growth in the root (Fig. 4F). In no case were phloem elements detected in the sinkers. Sometimes a sinker seems to invade the elements of the host xylem vessels, penetrating into their lumen and crossing several successively (Fig. 4G–I).

In the flowering period of *Cytinus*, the mature endophyte grows towards the outside of the host root, causing a massive distortion in its tissues, to form the inflorescence (Fig. 4J). From the continuous sheath of the endophyte, the bud from which the inflorescence originates begins to develop, formed by elongated parenchymal cells with very large nuclei (Fig. 4K, L) and by vascular filaments arranged cylindrically close to the periphery (Fig. 4L–N). Each vascular filament consists of xylem on its inner face and phloem on the outer (Fig. 4M). The phloem tissue of the inflorescences includes sieve elements similar to those described above, and associated parenchymatic cells (Fig. 4M, N). The latter are easily distinguishable because of their intensely stained protoplasts, their large nuclei, and their position beside the sieve elements.

**DISCUSSION**

The endophytic systems of *Cytinus ruber* and *C. hypocistis* are alike, and develop similarly irrespective of the host parasitized. The endophytic system of these species produces lateral filaments that extend tangentially through the pericyclic derivatives and phloem of the root host. At the same time, this endophyte grows radially, crossing the cambial zone of the host and colonizing its xylem region. Thus, the endophytic system of both species of *Cytinus* grows in all the host root tissues. Most endophytic holoparasites develop only in the host cortex and the outermost zone of the phloem (Brown, 1912; Kuijt, 1969; Rutherford, 1970; Kuijt et al., 1985), and only in *Pilostyles hamiltonii* (Apodanthaceae) do some cellular filaments of the endophyte penetrate into the xylem (Dell et al., 1982). In contrast, the endophyte of *Cytinus* reaches its highest degree of complexity in the root xylem of its hosts. This type of development has not been reported previously in any species of holoparasitic endophyte. The fact that nodules of *Cytinus* develop in the cambial zone and grow non-intrusively into the xylem raises the possibility of a developmentally synchronized growth of *Cytinus* and its host, as has been reported in other parasites (Srivastava and Esau, 1961; Lye, 2006).

One of the most important findings of the present study is the detection of well-developed phloem in the endophyte of *Cytinus*, with companion cells and sieve elements similar to those of the autotrophic angiosperms. Previously it had been reported that the endophyte of *C. hypocistis* comprised...
only a cortical outer layer, a cambial zone, and a medullary inner layer with xylem conductive elements (Solms-Laubach, 1867; Fraysse, 1906; Forstmeier et al., 1983). This was probably because fluorescence was not used in those studies and, despite the size of the phloem elements, they are barely perceptible without this technique (see Fig. 3A and B). Likewise, phloem has not been reported previously in any of the studies carried out on species of Hydnoraceae, Mitrastemonaceae or Rafflesiacae.

Sieve elements were detected in Pilostyles turberi (Apodanthaceae; Kuijt et al., 1985), but they were classed as a vestigial tissue. This study of Cytinus species is the first time that the presence of well-developed phloem has been reported in a holoparasitic endophyte species. Moreover, reports of sieve elements in holoparasitic species whose vegetative bodies mainly develop outside the host are few, e.g. Orobanchus (Dörr and Kollmann, 1975, 1995), Epifagus (Walsh and Popovich, 1977) and Aeginetia (Rajanna et al., 2005), although the existence of phloem in hemiparasitic angiosperms has been documented on many occasions (for reviews, see Kuijt, 1977; Bhandari and Mukerji, 1993). Direct contact was not detected between the phloem of Cytinus and that of the host, a type of contact infrequent in other parasites (Tate, 1925; Dörr, 1972; Dörr and Kollmann, 1995; Birschwilks et al., 2006).

The endophyte of Cytinus produces sinkers that thoroughly permeated the host xylem, parallel to the xylem rays but not associated with them. In contrast, the sinkers of many hemi- and holoparasites grow closely associated with the host medullary rays (e.g. Cohen, 1954; Srivastava and Esau, 1961, Rutherford, 1970). It has been reported that the sinkers of C. hypocistis were comprised solely of parenchymal tissue (Fraysse, 1906; Forstmeier et al., 1983), as in other endophytic holoparasite species (Rutherford, 1970; Dell et al., 1982; Kuijt et al., 1985). In this work, it has been observed that the sinkers of both species of Cytinus also have tracheal elements like those seen in hemiparasitic angiosperms (e.g. Srivastava and Esau, 1961; Sallé, 1979). Despite the tracheal elements detected in the sinkers of Cytinus, no direct parasite xylem–host xylem contact was observed. Nor were contacts observed between the xylem of the mature endophyte bands of Cytinus and the host xylem. This seems to be a common circumstance, because although xylem–xylem contacts have been reported in other parasitic angiosperms (e.g. Calvin, 1967; Kuijt, 1977; Sallé, 1979; Heide-Jørgensen and Kuijt, 1995; Pate, 1995; Calladine and Pate, 2000), a luminal continuity was actually seen in very few cases (e.g. Pate et al., 1990; Dörr, 1997).

Thus, in Cytinus, direct xylem–xylem or phloem–phloem contacts have not been observed. It has been proposed that the transfer of nutrients between parasitic plants and hosts involves the parenchymal cell walls of the haustorium, which could act as transfer cells, forming an apoplastic continuum between the cells of the parasite and those of the host (Coetzee and Fineran, 1987; Kuo et al., 1989; Gedalovich-Shledetzky and Kuijt, 1990; Heide-Jørgensen and Kuijt, 1993; Pate, 1995). Transfer cells occur in most angiosperms, both parasitic and non-parasitic, and they have a central role in nutrient distribution, facilitating the apo-symplastic transport of solutes (Fineran and Calvin, 2000; Christensen et al., 2003; Offler et al., 2003). Contacts between the endophyte of Cytinus and its host also seem to occur via parenchymal cells. The parenchyma cells of the bands and sinkers with thickened walls may act as transfer cells and, if so, they would play a crucial physiological role in the host–parasite interface, facilitating the absorption, transport and distribution of photoassimilates from host to parasite. The development of differentially thickened walls in the parenchyma cells of the sinkers contiguous with vessels of the host xylem suggests an active loading of water and solutes by Cytinus from its host. Furthermore, it is possible that the cells of the endophyte of Cytinus situated among the host phloem cells capture photoassimilates from them. Subsequently, the metabolites acquired could be transported, either symplastically or apoplastically, from the portion of the endophyte situated in the pericyclic tissues and phloem of the host, via sinkers, to the bands of mature endophytic tissue embedded in the host xylem. Thus, although the host tends to enclose Cytinus in its xylem, the parasite may achieve its nutrient requirements because of these connections.

Occasionally sinkers of Cytinus appear to penetrate the host xylem cells. An intrusion of the sinkers into the host...
xylem vessels has also been reported in other parasitic angiosperms (Kuijt, 1977; Dörr, 1997; Calladine and Pate, 2000), and it has been postulated that this penetration might be produced by both mechanical pressure and enzymatic activity (Press and Whittaker, 1993; Dörr, 1997). Such intrusive sinkers of Cytinus could take water and mineral nutrients from the host, although this uptake would be prevented if host vessels were occluded by tyloses. Tylosis formation is considered a normal phenomenon in many species but it can also be induced by mechanical injury and by diseases (Fahn, 1977). In fact, Fraysse (1906) found tyloses in roots of Cistaceae infected with C. hypocistis. However, tyloses were not observed in vessels contiguous with sinkers cells in any of the material used in the present study.

Finally, for the first time, continuity has been demonstrated here between the vascular tissues (xylem and phloem) of the endophyte of Cytinus and those of its inflorescences, ensuring the flow of water and nutrients necessary for flowering and fruiting. Although holoparasitic angiosperms lack chlorophyll, and need to obtain water and nutrients from their hosts, they sometimes show a small capacity to assimilate carbon independently of the host (Renaudin et al., 1986; Stewart and Press, 1990). Thalouarn et al. (1986), using radioisotope techniques, have demonstrated that inflorescences of C. hypocistis detached from the host are capable of non-photosynthetic carbon fixation using external CO₂ in small amounts – up to 12% of the carbon received from the host. Thus, C. hypocistis may obtain from its inflorescences, extra nutrients that qualitatively complement those supplied by the host, and which can be easily transported thanks to the continuity of the conductive elements described in this work.

CONCLUDING REMARKS

The endophytic system described for both species of Cytinus is more complex and extends deeper into the host tissues than previously reported. The endophytic system grows in all host root tissues, the youngest tissues being located in the host pericyclic tissues and phloem and the oldest tissues in the host xylem, with connections being established between the different developmental stages of parasitic tissues by means of sinkers. The mature endophytic system has a well-developed vascular system with xylem and functional phloem. The latter, as observed in Cytinus, is described for the first time in any member of endophytic, holoparasitic parasite. Sinkers formed from the mature endophyte grow through the host xylem, and their cells with thickened walls may act as transfer cells. Although the current study presents a number of very novel features of the endophytic system of Cytinus, little is known still about its relationship with the host species from the first stages of development. Since seed germination has never been observed in any of the families of endophytic holoparasitic plants, the initial establishment and development of Cytinus on the roots of its host species remains an open and exciting question.

ACKNOWLEDGEMENTS

We thank Dr R. G. Albaladejo for help with the field sampling, Dr D. G. Simão and Dr R. Camo-Oliveira for technical assistance and Dr P. E. Gibbs for checking the English text. This study was carried out in the Laboratory of Morphology, Microscopy and Image Processing of the Institute of Biology-UFU, MG, Brazil. This work was supported by a PhD grant from the Spanish MEC to C. de Vega and by two projects from the Spanish CICYT (REN2002-04354-C02-02 and CGL 2005-01951).

LITERATURE CITED


