Protoplasma (1991) 162: 20–26

PROTOPLASMA

Involvement of flavonoids in the resistance of two poplar cultivars to mistletoe (*Viscum album* L.)

El Bachir Hariri[1], G. Sallé[1,*] and C. Andary[2]

[1] Laboratoire de Cytologie Expérimentale et Morphogenèse Végétale, Université Pierre et Marie Curie, Bâtiment N 2, Paris and [2] Laboratoire de Botanique, Phytochimie et Mycologie, Faculté de Pharmacie, Université de Montpellier I, Montpellier

Received August 6, 1990
Accepted October 8, 1990

Summary. Flavonoid compounds were studied in healthy and parasitized poplar branches following mistletoe (*Viscum album* L.) attack. Two poplar cultivars showing different degrees of resistance to mistletoe: sensitive "Fritzi Pauley" (FPL) and resistant "Vereecken" (VER) were used. Flavonoids were detected and localized using histofluorescence after treating frozen sections with two specific reagents. Total amounts of flavonoids were determined spectrophotometrically. Defence mechanisms were induced during penetration of the primary haustorium. They consisted of inner periderm development and flavonoid accumulation. These reactions were weaker in the FPL cultivar than in the VER one. In the latter, growth of the primary haustorium and the establishment of direct connections between the living host cells and parasite failed. The resistance of poplar cultivars to mistletoe was dependent on the production of defensive mechanisms against the pathogen.

Keywords: Flavonoids; Haustorium; Histofluorescence; Poplar cultivars; Resistance; *Viscum album*.

Abbreviations: FPL Fritzi Pauley; VER Vereecken.

Introduction

In the field of host–parasite relationships, many observations have showed that polyphenols are involved in resistance to pathogen attacks (Paupardin 1972, Royle 1976, Vance et al. 1980, Friend 1981, Agrios 1988, Debost et al. 1988, Hariri et al. 1987). According to Armillotta (1984), Sallé et al. (1984), and Hariri (1989), the resistance of some poplar cultivars to a flowering hemiparasitic plant, mistletoe (*Viscum album* L.), was related to the rapid accumulation of polyphenolic substances in the perihaustorial zone. Some parameters of healthy tissues, such as the density of polyphenol-rich parenchymatous cells per unit surface area, or the number of lignified fibres, appeared to be heavily involved in resistance to mistletoe. The former gave a good idea of the ability of the host cultivars to synthesize actively polyphenols. The most resistant (Blanc de Garonne) of the four tested cultivars (Armillotta 1984, Sallé et al. 1984) showed the greatest number of polyphenolic cells per unit surface area. The contrasting behaviour of poplar cultivars to mistletoe attack was established but no information on the nature of the polyphenolic contents given.

The aim of this work is to demonstrate flavonoid involvement in the resistance of two poplar cultivars to mistletoe.

Material and methods

Flavonoid compounds were investigated in healthy and parasitized cortical tissues of two poplar cultivars: *Populus trichocarpa* Torr. and Gray, cv. Fritzi Pauley (FPL) and *Populus nigra* L. cv. Vereecken (VER). These cultivars were chosen because they show different behaviour during artificial inoculation with mistletoe. FPL cv. is sensitive (Frochot et al. 1978, Armillotta 1984, Sallé et al. 1984, Hariri 1989) whereas VER cv. is resistant (Hariri 1989).

Detection and localisation of flavonoid in the bark of poplar branches were carried out by histofluorescence using an epi-illumination microscope (Leitz Ploemopak) with two sets of filters: 340–380 nm excitation – 430 nm suppression; 450–490 nm excitation – 515 nm suppression. Fluorochromes were highly fluorescent under UV irradiation (340 nm) and blue light (450 nm). These wavelengths were usually used with fluorescence microscopy because of their brightness. Our observations were generally performed at 450 nm but were sometimes completed at 340 nm (UV). Frozen sections (40 µm thick) were immersed in two reagents specific for fla-

* Correspondence and reprints: Laboratoire de Cytologie Expérimentale et Morphogenèse Végétale, Université Pierre et Marie Curie,

vonoids: Wilson reagent, citric acid – boric acid (5/5, w/w) in 100 ml absolute methanol; aluminium chloride/ethanol (5%, w/v). These reagents chelate flavonoids and produce yellow or yellow-green fluorescence after excitation at 340 or 450 nm.

Quantitative determination of total flavonoids was carried out using a simple spectrometric assay developed by Andary (1990) using 2-aminoethyl-diphenylborate (1% in methanol). The poplar bark extracts (0.5% with 80% methanol) were diluted 50% and then mixed with the reagent solution (100 µl for 2 ml of extract). The spectrometric evaluation was done at 404 nm. Extract absorptions were compared with absorptions of a quercetol standard methanolic solution (0.05 mg/ml) treated with the same reagent under the same conditions. Finally, the total amount of flavonoids was calculated as follows:

$$F\% = \frac{A_P \times 0.05 \times 100}{A_Q \times 2.5}$$

where A_P is the absorbance of poplar extract, and A_Q is the absorbance of quercetol standard.

Results

Localization of flavonoids in cortical tissues of healthy poplar branches: FPL and VER cultivars

Observation under epifluorescence (UV) of longitudinal radial sections of healthy FPL branches, after treatment with aluminium chloride, showed yellow fluorescence characteristic of flavonoids. This fluorescence was mainly located inside suberous cells (Fig. 1). In the thin phelloderm region, the fluorescence was weaker and localized chiefly in intercellular spaces (Fig. 1). Walls of suberous cells showed blue fluorescence whereas the fluorescence was pale green in phelloderm cell walls.

After treatment with Wilson's reagent and observation under blue light (450 nm), the suberous cell contents (Fig. 2) exhibited intense lemon-yellow fluorescence. In the phelloderm, the same type of fluorescence was only detected in certain cells (Fig. 2). In phloem parenchyma cells, no flavonoid-characteristic yellow fluorescence was observed; red fluorescence typical of chlorophylls was seen. Moreover, the Wilson reagent treatment induced yellow-green fluorescence of fibre walls (Fig. 2).

In the VER cv., after treatment of longitudinal radial sections with aluminium chloride and observation under UV, suberous cell contents showed yellow fluorescence (Fig. 3), contrasting with the blue fluorescence emitted by suberous and fibre (Fig. 3) cell walls.

After treatment with Wilson's reagent and blue light

Abbreviations used in the figures: F fibre; h haustorium; H host; P parasite; PEe external periderm; PEi internal periderm; Ph phellem; Pp phloem parenchyma; tr tracheid

Figs. 1–4. Longitudinal radial sections of healthy poplar branches treated with flavonoid specific reagent, observed under two exciting wavelengths

Fig. 1. FPL cv., aluminium chloride treatment, $\lambda = 340$ nm. Notice yellow fluorescence of the suberous cell contents (large arrows) and blue of their cell walls. In the phelloderm, whitish fluorescence is observed in intercellular spaces (small arrows)

Fig. 2. FPL cv., Wilson's reagent, $\lambda = 450$ nm. Intense lemon-yellow fluorescence of suberous cell contents (arrows), yellow fluorescence of few phellodermic cells (asterisks). No fluorescence in parenchymatous cells, except a red one due to chlorophylls. Yellow-green fluorescence of fibre cell walls (double arrowhead)

Fig. 3. VER cv., aluminium chloride treatment, $\lambda = 340$ nm. Lemon-yellow fluorescence of suberous cell contents (arrows) and blue fluorescence of the suberous and the fibre (double arrowhead) cell walls

Fig. 4. VER cv., Wilson's reagent, $\lambda = 450$ nm. Lemon-yellow fluorescence of suberous and parenchymatous (arrowheads) cell contents. The fluorescence is so intense in the suberous cells (asterisks) that it obscures their boundaries

Figs. 5 and **6.** Longitudinal radial sections of 12 months parasitized branches of poplar, treated with Wilson's reagent and observed under transmitted **(a)** or under blue light **(b)**. The brownish cell contents and yellow fluorescence of the perihaustorial zone are much more important in the resistant VER cv. **(Fig. 6)** than in the sensitive FPL cv. **(Fig. 5)**. ★ Perihaustorial zone

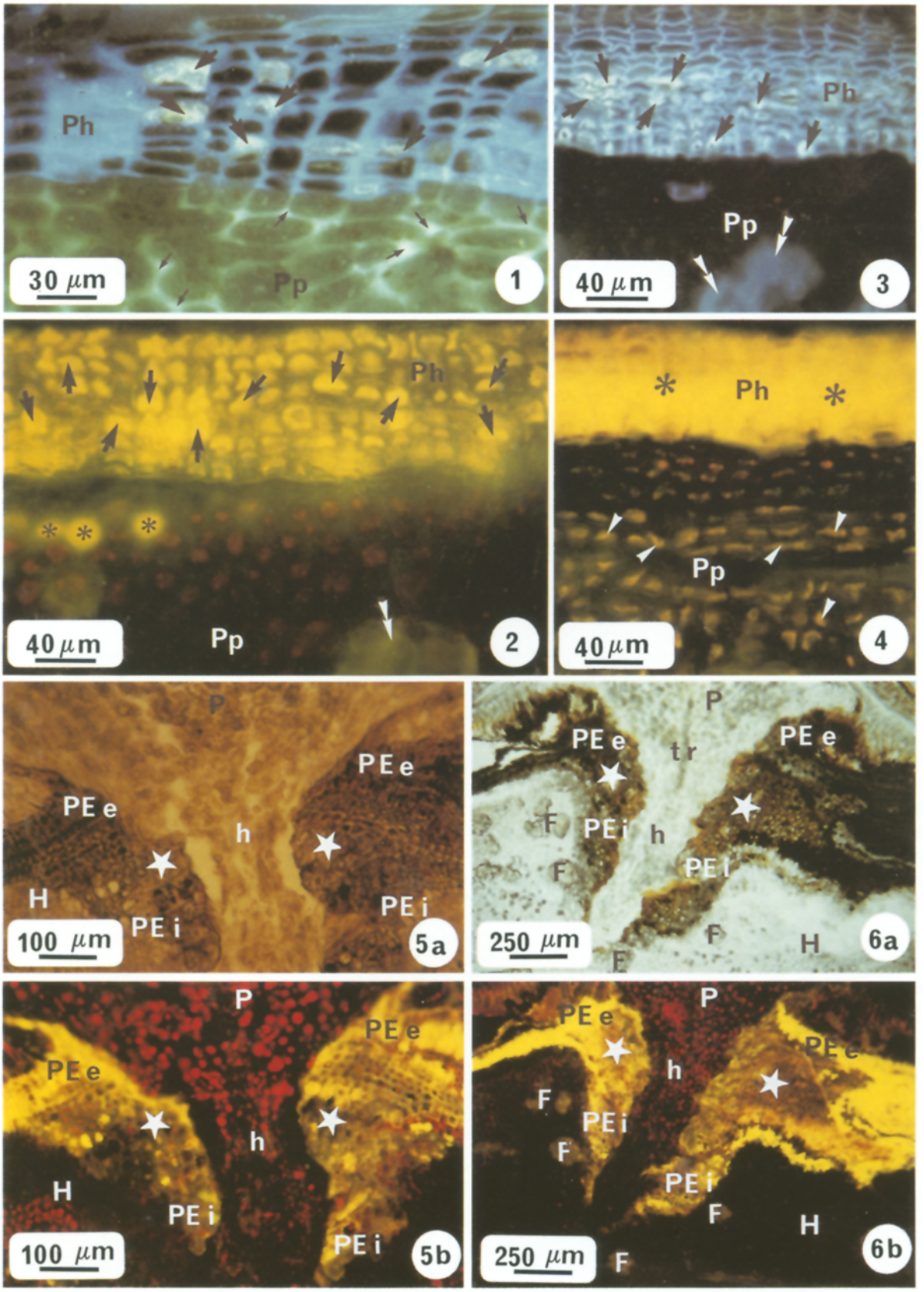
Fig. 7 a, b. Wilson's reagent. Observation under transmitted light **(a)** or under blue light **(b)**. VER cv. Presence of flavonoids in host parenchyma cells (asterisks) in contact with the tip of the growing haustorium. Note the importance of fibre clusters

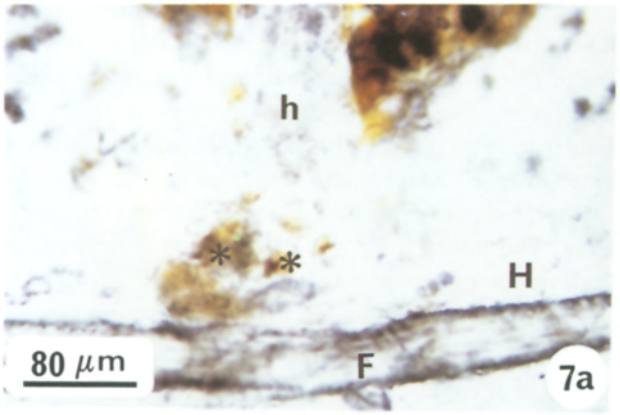
Fig. 8. Macrophotography of a successful mistletoe plantlet on the FPL cv., 19 months after inoculation. Enlargement of the host branch (asterisk) indicates the success of the parasitic installation. *l* Mistletoe leaf

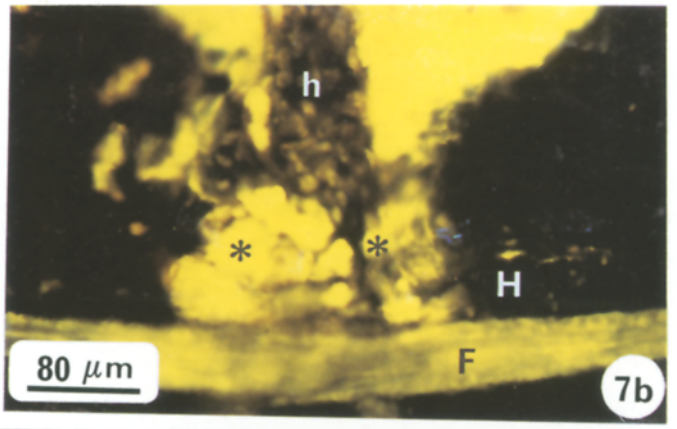
Fig. 9. Longitudinal radial section of the sample in Fig. 8. The endophytic system is well developed. *CS* Cortical strand; *h* primary haustorium; *XII* host secondary xylem

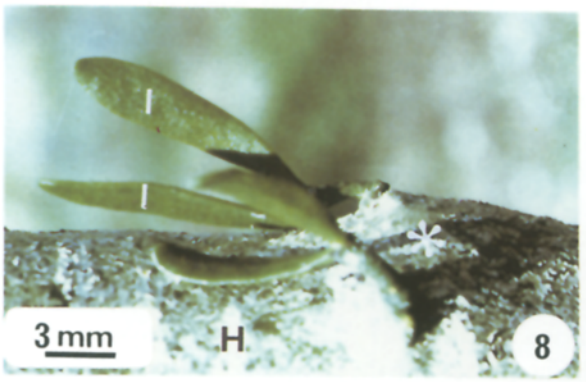
Fig. 10. Macrophotography of degenerating holdfasts (arrow) or of imprints (arrowheads) observed 19 months after inoculation on a VER cv. branch

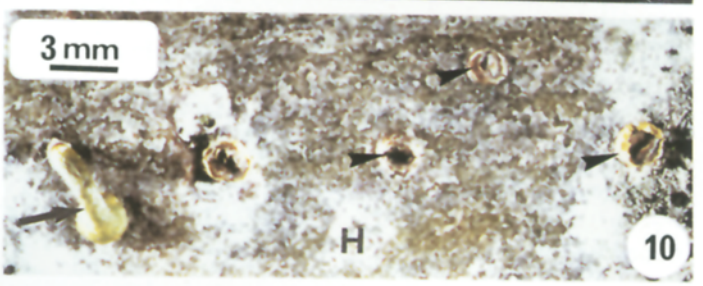
Fig. 11 a, b. Longitudinal radial sections of bark of VER cv. at the site of the holdfast imprints shown in Fig. 10, treated with Wilson's reagent and observed under transmitted **(a)** and blue light **(b)**. Mistletoe disappeared (arrow), but the remnants of the perihaustorial zone (asterisks) show intense yellow fluorescence **(b)**, indicating the presence of flavonoids

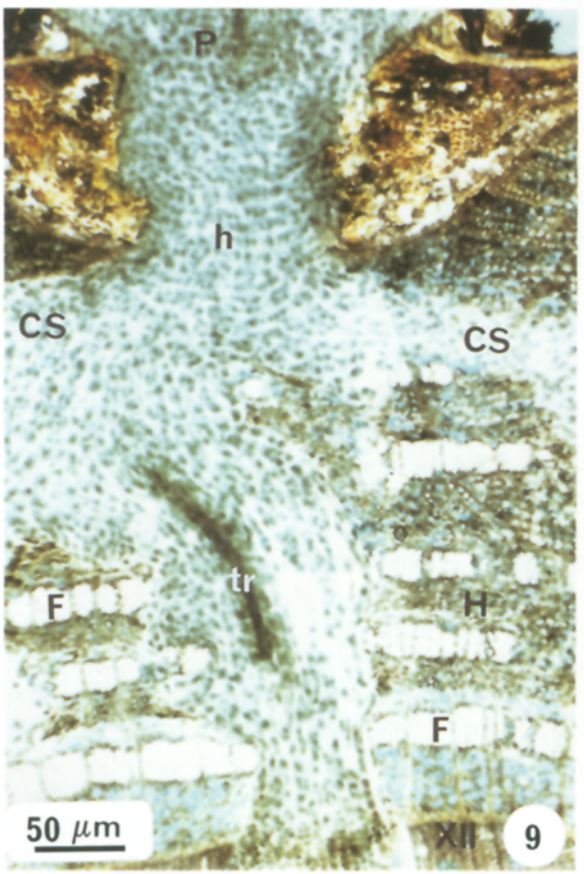

Ph
Pp
30 μm
1
Ph
Pp
40 μm
3
Ph
Pp
40 μm
2
Ph
Pp
40 μm
4
P
PEe
PEe
h
H
PEi
PEi
100 μm
5a
P
PEe
tr
PEe
F
PEi
h
F
PEi
F
H
F
250 μm
6a
P
PEe
PEe
h
F
PEi
F
PEi
H
PEi
PEi
100 μm
5b
P
PEe
PEe
h
F
PEi
F
PEi
F
H
F
250 μm
6b

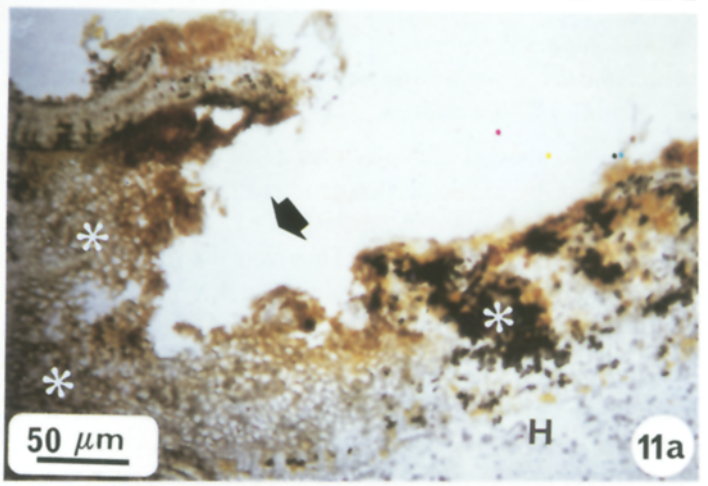

h
H
F
80 µm
7a

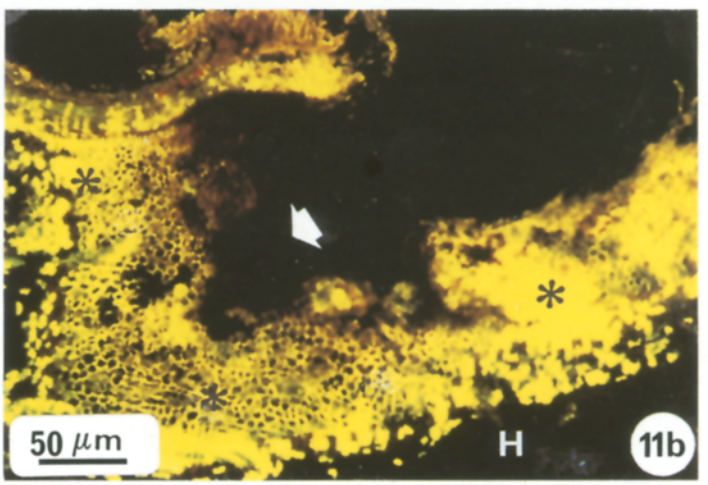

h
H
F
80 µm
7b


l
l
H
3 mm
8


3 mm
H
10


P
h
CS
CS
tr
F
H
F
XII
50 µm
9


H
50 µm
11a


H
50 µm
11b

excitation, the longitudinal radial sections showed strong lemon-yellow fluorescence of the suberous cell contents (Fig. 4) obscuring their boundaries. The same fluorescence was observed in parenchymatous cells (Fig. 4). The intensity of the fluorescence emphasized the importance of flavonoid compounds in *nigra* poplar cultivar.

Quantitative determination of total amounts of flavonoids in the bark of healthy poplar branches: FPL and VER cultivars

Under our experimental conditions, the absorbance of the quercetol standard was $A_Q = 1.35$. FPL and VER absorbances were 0.71 and 1.14 respectively. Quercetol represents the major aglycone flavonoid extract so flavonoid concentrations were expressed as g of quercetol per 100 g dry poplar bark. According to this method, flavonoid percentages were $F_{FPL} = 1.04\%$ and $F_{VER} = 1.68\%$. These results agree well with those obtained by histofluorescence.

Flavonoid fluorescence in the mistletoe/poplar association, 12 months after inoculation

In order to show the involvement of flavonoids after penetration by mistletoe, longitudinal radial sections of branches parasitized by mistletoe for 12 months were treated with Wilson's reagent. They were then observed under transmitted and under blue, $\lambda = 450$ nm, light.

Reaction of sensitive FPL cv.

Under transmitted light, the outer periderm and the perihaustorial zone had yellowish or dark brown cell contents (Fig. 5 a) while under blue light, the same regions fluoresced intensely yellow (Fig. 5 b). However, the fluorescent substances, i.e., flavonoids, were located mainly in the external periderm; the perihaustorial zone was less reactive. In Fig. 5 a and b, the inner periderm has been broken by the penetrating haustorium. Despite phellogen delamination and flavonoid synthesis, this sensitive tree had not built an efficient defensive system capable of stopping haustorial progression. The haustorium had reached the chlorophyllous host parenchyma necessary for the development of cellular exchange between the two plants.

Reaction of resistant VER cv.

Under transmitted light, longitudinal radial sections of 12 months parasitized VER branches showed the classical symptoms of resistant cultivars (Fig. 6 a) with separation of the phellogen into an external and an internal region, the latter giving rise to the inner periderm which entirely encloses the growing primary haustorium.
Under blue light (Fig. 6 b), the perihaustorial zone reacted intensely and emitted strong yellow fluorescence in the two peridermic tissues and the perihaustorial zone. The intensity of the fluorescence indicated the importance of flavonoids in these tissues. Moreover, the cortical parenchyma of the host tree showed many clusters of fibres with weak yellow-green fluorescence (Fig. 6 b). Apart from the most superficial fibres which were transverse, all others were longitudinal.
Defence mechanisms induced by the penetration of a young haustorium, i.e., numerous periderms and flavonoid accumulation, seemed to be involved in active resistance of the VER cv.
Figure 7 a and b illustrates the mechanism of resistance in a VER branch. Sometimes the primary haustorium successfully passed the perihaustorial zone and the inner periderm (Fig. 7 a and b) formed a jacket which prevented its lateral expansion. However, at the advancing haustorial front, the host parenchyma cortical cells, in contact with the parasite cells, also accumulated flavonoid compounds, as demonstrated by bright yellow fluorescence (Fig. 7 b). Moreover, the progression of the young haustorium encountered mechanical resistance due to clusters of lignified fibres (Fig. 7 a and b).

Behaviour of the mistletoe in the two cultivars, 19 months after inoculation

Mistletoe is characterized by a very low growth rate (Sallé 1977, Frochot et al. 1978), the first leaves appearing 15 to 19 months after deposition of the seed on the host branch (Fig. 8). Enlargement of the infected branch (Fig. 8) at the point of penetration indicates successful implantation of the mistletoe into the host tissues.
Observation of longitudinal axial sections of such samples (Fig. 9) showed broken perihaustorial zones and a complex endophytic system of mistletoe. This system developed a major primary haustorium with centripetal growth which passed through the host cambium and established direct connections with the xylem, and lateral cortical strands. The latter give rise to secondary haustoria. When the endophytic system reaches such a stage of development, parasitism is irreversible and the host – parasite complex non-dissociable.
In the resistant VER cv., the unsuccessful parasitic

plantlets slowly dried out and disappeared from the host surface. On artificially inoculated host branches (Fig. 10), drying hypocotyls and holdfasts were observed and also imprints corresponding to remnants of holdfasts and unsuccessful penetration attempts.

After treatment of longitudinal radial sections of these imprints (Fig. 11 a and b) with Wilson's reagent, a bright yellow fluorescence under blue light revealed important flavonoid accumulation (Fig. 11 b). This zone corresponded to an unbroken perihaustorial zone. The fluorescence was particularly intense in cells naturally coloured brown under transmitted light (Fig. 11 a). The peridermic formations around the haustorium became necrotic, building a barrier which prevented the establishment of efficient contact between the living cells of the two plants. Later, the necrotic periderm was sloughed off and secondary phloem parenchyma development filled the spaces previously occupied by the growing haustorium.

Discussion

The resistance of FPL and VER poplar cultivars to mistletoe involves both – preexisting anatomical and chemical features in the host, i.e., thickness of phellem, number of fibre clusters and cells with polyphenolic contents (Armillotta 1984, Sallé et al. 1984), and induced barriers developed during parasitic attack, i.e., inner periderms and flavonoid accumulation in the perihaustorial zone.

With respect to the preexisting resistance factors, the total amount of flavonoids, their localization and their partition in the two poplar cultivars showed some clonal variation. The sensitive FPL cv. had fewer flavonoids than the resistant one, VER. The lemon-yellow flavonoid-specific fluorescence was not very strong in peripheral FPL cv. tissues, except in the phellem where some cells were very bright after treatment with aluminium chloride. Phelloderm did not react intensely and the phloem parenchyma cells were entirely free of flavonoids. On the contrary, in healthy resistant *nigra* VER cv., abundant flavonoids were present in all tissues, i.e., phellem, phelloderm, phloem parenchyma cells and fibres. Moreover, the amount of flavonoids in the healthy cultivar was higher (1.68%) than in the sensitive one (1.04%).

Induced defence mechanisms appeared in the host during penetration of the young primary haustorium into host cortical cells, as soon as the tip of the growing haustorium reached the host phellogen. In sensitive trees of the FPL cv., the peridermic reaction was very weak, allowing parasite penetration. The inner periderm was rapidly broken off under pressure due to the actively growing haustorium. Thus, the haustorium established direct contacts with the host chlorophyllous cortical cells and developed a vigorous and efficient endophytic system.

Contrastingly, trees of the VER cv. reacted strongly during the first steps of mistletoe haustorium penetration by building several inner periderms and actively secreting flavonoids. Thus, the establishment of mechanical and chemical barriers prevented further penetration of the haustorium. Later on, unsuccessful penetration attempts of mistletoe were sloughed off by growth of the outer host tissues.

These observations suggest that flavonoids represent one component of the resistance phenomenon to mistletoe, as has been shown for other pathogens such as viruses (Cho and Goodman 1979), bacteria (Sequeira et al. 1977), and fungi (Friend 1981). It is noteworthy that in the poplar/mistletoe association, there is a clear correlation between the flavonoid content of the healthy outermost tissues and the degree of resistance, a large amount of flavonoids provides good resistance to mistletoe. Those flavonoids, particularly flavonols, which were most abundant in the resistant cultivar (Hariri et al. 1990) could contribute to pathogen rejection in two different ways: (1) they could act as toxic substances as has been observed in some host – fungi associations (Debost et al. 1988, Agrios 1988) or (2) they could play an important role as a chemical barrier. Flavonoid accumulation was not the consequence of parasite cell death: the substances were rapidly and strongly synthesized in resistant cultivars, at the very beginning of haustorium penetration, i.e., long before the death of the mistletoe. A poplar cultivar is resistant to mistletoe if it is able to produce important defensive weapons; if it reacts too slowly, it will be sensitive.

A qualitative and quantitative chemical analysis of the flavonoids of healthy tissues from several poplar cultivars is in progress in an attempt to establish a scale of resistance.

References

Agrios NG (1988) How plants defend themselves against pathogens. In: Plant pathology, 3rd edn. Academic Press, San Diego, pp 97–115

Armillotta A (1984) Etude de la résistance au gui *Viscum album*. Thèse de 3e cycle, Université Pierre et Marie Curie, Paris

Andary C (1990) Documentation chimique et pharmaceutique pour l'AMM du MERALOPS comprimés. Laboratoire Allergan-Dulcis, Monaco, France

Cho EK, Goodman RM (1979) Strains of soybean mosaic virus:

classification based on virulence in resistant soybean cultivars. Phytopathology 69: 467–470

Debost D, Senechal Y, Gohet E, Lambert C (1988) Les composés phénoliques des feuilles *d'Hevea brasiliensis* en relation avec la sensibilité au *Colletotrichum gloeosporioides*. CR Acad Sci Paris Série III 307: 293–298

Friend J (1981) Plant phenolics, lignification and plant disease. In: Reinhold L, Harborne JB, Swain T (eds) Progress in phytochemistry, vol 7. Pergamon Press, Oxford, pp 197–261

Frochot H, Pitsch M, Wehrlen L (1978) Différences de sensibilité au gui *Viscum album* L. de quelques clones de peuplier *Populus* sp. In: 103ème Congrès Nat. des Soc. Savantes, Nancy, April, 1978, 1: 371–380

Hariri EB (1989) Polyphénols et résistance au gui (*Viscum album* L.), étude histocytochimique et analytique. Thèse de Doctorat de l'Université Pierre et Marie Curie, Paris

– Andary C, Sallé G (1987) New insights on the resistance against mistletoe (*Viscum album* L.) In: Weber H, Forstreuter W (eds) Proceedings IVth International Symposium on Parasitic Weeds, Marburg, August, 1987, pp 293–294

– Sallé G, Andary C (1990) Mécanismes de résistance de quatre cultivars de peuplier en réponse à l'attaque du gui (*Viscum album* L.). CR Acad Sci Paris Série III 311: 439–444

Paupardin C (1972) Contribution à l'étude du rôle des composés phénoliques dans les phénomènes de morphogenèse manifestés par quelques tissus végétaux. Ann Soc Nat Bot Paris 13: 141–210

Royle DJ (1976) Structural features of resistance to plant disease. In: Friend J, Threlfall DR (eds) Biochemical aspects of plant–parasite relationships. Academic Press, London, pp 161–193

Sallé G (1977) Etude cytologique, cytochimique et histoautoradiographique du *Viscum album* L. (Loranthacées). Thèse d'Etat, Université Pierre et Marie Curie, Paris

– Armillotta A, Frochot H (1984) Mechanism of resistance of four cultivars of poplar against *Viscum album* L. In: Parker C, Musselman L J, Polhill R M, Wilson A K (eds) Proceeding III. International Symposium Parasitic Weeds, May, 1984, pp 22–30

Sequeira L, Gaard G, De Zoeten GA (1977) Interaction of bacteria and host cell walls: its relation to mechanisms of induced resistance. Physiol Plant Pathol 10: 43–50

Vance CP, Kirrk TK, Sherwood RT (1980) Lignification as a mechanism of disease resistance. Annu Rev Phytopathol 18: 259–288