Host surface tissues and basidiospore-derived infection strategies of some rust fungi

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Abstract — Some Authors belief that the basidiospore germ tubes of rust fungi penetrate angiosperms directly and gymnosperms indirectly, hypothesizing that the different strategies of basidiospore-derived penetrations could be ascribed to the heavily cutinized and thick walled epidermal cells of the leaves of gymnosperms. In this paper the surface tissues of the organs of some angiosperms and gymnosperms - which are directly and/or indirectly penetrated by basidiospore germlings of *Melampsora* spp*., Cronartium flaccidum* and *Crysomyxa abietis* - were examined, in order to: (i) evaluate the role of the cuticular and epidermal cell-wall thickness, still considered a barrier to a direct penetration, and (ii) search for other possible cyto-histological aspects of surface tissues which could explain the different penetration strategies of rust basidiospore germlings. The results show that the thickness of cuticle and epidermal cell walls of the organs onto which the monokaryotic phase of each rust species has fitted itself during evolution to penetrate is not important in determining the penetration strategy, whereas it seems that it is the presence of lignin in the surface tissues of these organs which probably limits the direct penetration of rust basidiospore germlings.

Key words: Basidiospore-derived infection, *Cronartium,* host surface tissues, *Melampsora,* rust fungi.

INTRODUCTION

Spore germ tubes of rust fungi use two ways of penetration to infect the organs of their hosts: (i) directly through the intact wall of the epidermal cell, which is the characteristic way of the monokaryotic phase, i.e. basidiospore-derived penetration; (ii) indirectly through the stomatal apertures, which is the characteristic way of the dikaryotic phase, i.e. aeciospore - and urediniospore-derived penetration. As a result of a direct penetration an intraepidermal infection structure develops, whereas an indirect penetration produces a substomatal infection structure (Littlefield and Heath 1979; Bushnell and Roelfs 1984; Hoch and Staples 1991; Mendgen et al. 1996; Epstein and Nicholson 1997; Mendgen 1997).

However, Patton and Johnson (1970) and Bergdhal and French (1985) reported that the penetration of the basidiospore germlings of *Cronartium ribicola* J.C. Fisher ex Rabenh. on *Pinus strobus* L. and of *Cronartium comandrae* Pk. on *Pinus banksiana* Lamb. occurred indirectly through the stomatal apertures, despite the fact that it involved basidiospore-derived infections. Moreover, many studies (Miller et al. 1980; Gray et al. 1983; Gold and Mendgen 1984; 1991; Hopkin et al.1988; Longo et al. 1988; 1991; 1994; 1997; Morin et al. 1992) confirmed the direct penetration of the basidiospore germ tubes of several rust fungi on their hosts, and considered the indirect penetration of basidiospore germlings of *C. ribicola* and *C. comandrae* peculiar events which needed to be clarified.

Gold and Mendgen (1984), referring to two other cases of basidiospore-derived indirect penetration, i.e. *Crysomyxa abietis* (Wallr.) Unger on *Picea abies* L. (Grill et al. 1978) and *Coleosporium* spp. on *Pinus* spp. (Bauer 1983), hypothesized that the different strategies associated with basidiospore-derived penetrations - direct on angiosperms and indirect on gymnosperms - could be ascribed to the heavily cutinized and thick walled epidermal cells of the leaves of gymnosperms.

In point of fact, Longo et al. (1994), in their studies on the monokaryotic phase of *Melampsora pulcherrima* (Bub.) Maire on *Mercurialis annua* L.,

reported a direct basidiospore-derived penetration on the leaves of this angiosperm. On the other hand, LONGO *et al*. (1988; 1991; 1997), studying the basidiospore-derived penetration of *Melampsora pinitorqua* (A. Br.) Rostr. on cotyledons and primary needles of *Pinus pinea* L., as well as on growing shoots of *Pinus sylvestris* L., and of *Melampsora larici-tremulae* Kleb. on growing needles from brachiblasts of *Larix decidua* Mill., observed that these two rust fungi directly penetrated these gymnosperms. A direct basidiospore-derived penetration was described also for *Cronartium quercuum* (Berk.) Miyabe ex Shirai f.sp. *fusiforme* (Cumm.) Burds. & Snow on hipocotyls, cotyledons and primary needles of *Pinus elliottii* Engelm. var. *elliottii* (MILLER *et al*. 1980) and for *Endocronartium harknessi* (J.P. Moore) Y.Hyrat. on hipocotyls and cotyledons of *Pinus contorta* Dougl. var. *latifolia* Englm. (HOPKIN *et al*. 1988).

Such a behaviour on gymnosperms was confirmed by LONGO *et al.* (1997), MORICCA *et al*. (2003) and RAGAZZI *et al*. (2005). Indeed, owing to the partial overlapping of the host range of *M. pinitorqua* and *M. larici-tremulae* described in previous works by LONGO *et al*. (1970; 1975), a basidiospore-derived direct penetration was shown also in the following cases of inoculations: 1) *M. larici-tremulae* on growing shoots of *P. sylvestris* (LONGO *et al.* 1997); 2) *M. pinitorqua* on growing needles from brachiblasts of *Larix decidua* (MORICCA *et al*. 2003); 3) *M. larici-tremulae* on cotyledons and primary needles of *P. pinea* (MORICCA *et al*. 2003).

On the other hand, LONGO *et al.* (1991) emphasized that the indirect basidiospore-derived penetration on gymnosperms, described by PATTON and JOHNSON (1970) for basidiospore germlings of *C. ribicola* on *P. strobus*, takes place on organs whose surface tissues have different cytological characteristics, i.e. not only on secondary needles but also on primary needles and cotyledons.

One other case of indirect basidiospore-derived penetration on gymnosperms was proposed by RAGAZZI *et al*. (1987) and RAGAZZI and DELL-AVALLE FEDI (1992) after some observations on basidiospore germlings of *Cronartium flaccidum* (Alb. et Schw.) Wint. on the surface of cotyledons, primary and secondary needles of *Pinus pinaster* Ait. and *Pinus nigra* Arn. subsp. *laricio* (Poiret) Maire. More recently, LONGO *et al*. (2000) demonstrated that the basidiospore-derived penetration of *C. flaccidum* on *Pinus pinea* was effectively indirect through the stomata, giving rise to a substomatal infection structure on all types of host needles.

From all these reports it can be seen that the same gymnosperm organs - see cotyledons and primary needles - are penetrated directly or indirectly by basidiospore germlings depending on the rust species; this behaviour probably depends on the rust species' mode of penetration into gymnosperm organs usually infected in nature: i.e., *i*) a direct penetration by *M. pinitorqua, C. quercuum* f.sp. *fusiforme* and *E. harknessii* on pine primary tissues (hypocotils, cotyledons, stems and primary needles) and by *M. larici-tremulae* on growing needles from brachiblasts of *Larix decidua*; *ii*) an indirect penetration by *C. ribicola, C. comandrae, C. flaccidum* and *Coleosporium* spp. on pine secondary needles, and by *Crysomyxa abietis* on *Picea abies* growing needles. Hence, LONGO *et al.* (2000) supposed that the reason for the dynamics of indirect penetration carried out by some rust fungi in the monokaryotic phase could be as follows. The rust fungi which evolved to infect "in nature" organs with some surface cyto-histological characteristics, such as the heavily cutinized and thick walled epidermal cells of the secondary needles of *Pinus* spp., could be prevented from exercising a suitable mechanical and/or enzymatic action. Consequently, in order to fit themselves onto a substratum with the above characteristics, it would be necessary for the basidiospore germlings to modify their way of penetration from the direct one, which is typical of the rust monokaryotic phase, to that of the indirect type. Notwithstanding, LONGO *et al.* (2000) observed that the morpho-functional characteristics of the penetration structures of *C.flaccidum* remained those of a monokaryotic phase, even if this indirect penetration recalled the mode of penetration (through the stomatal aperture) of a dikaryon.

The aims of this work are: (i) to examine the surface tissues of organs directly and/or indirectly penetrated by some of the above cited rust fungi in the monokaryotic phase, in order to evaluate the role of the cuticular and epidermal cell wall thickness, still considered in literature as a barrier to a direct penetration, and (ii) to search for other possible cyto-histological aspects of surface tissues which could explain the different penetration strategies of basidiospore germlings.

MATERIALS AND METHODS

The organs susceptible to be penetrated in nature and/or after artificial inoculations by basid-

iospore germlings of the rust fungi *Melampsora pulcherrima, M. larici-tremulae, M. pinitorqua, Cronartium flaccidum* and *Crysomyxa abietis* were studied, namely:

– leaves of *Mercurialis annua*;

– growing needles from brachiblasts of *Larix decidua*;

– growing shoots of *Pinus sylvestris*;

– cotyledons and primary needles of *Pinus pinea*;

– growing and grown-up secondary needles of *Pinus pinea*;

– growing needles of *Picea abies.*

The following characters of surface tissues were considered.

For the epidermis:

– cuticularization;

– thickness and cutinization of the cell-walls;

– amount of cytoplasm in the cells;

– absence or presence of lignin in the cell walls.

For the hypodermis:

– absence or presence of layers and their number;

– absence or presence of lignin in the cell walls.

Small fragments from the samplings were sectioned using an American optical cryo-cut microtome and treated using the methods hereafter referred to. In order to highlight the cutin, the sections where treated: 1) with Nile-Red (GREENSPAN *et al.* 1985) 0,01% in acetone for 5 minutes, and observed using epifluorescence optics with Blue light exciter filter BP 450-490 nm, dichroic beam splitter RKP 510 nm and barrier filter LP 520 nm; 2) with an 1:1 mixture of Fluorol-yellow 088 0,01% in polyethylene glycol Wt 400 and glycerol 90% in distilled water (BRUNDRETT *et al.* 1991), for 1 hour; in this case the sections were observed using epifluorescence optics with UV light exciter filter BP 360-480 nm, dichroic beam splitter RKP 400 nm and barrier filter LP 430 nm. In order to highlight the lignin, the sections where treated with the Phloroglucinol test (Phloroglucinol in HCl 20%) (JOHANSEN 1940).

All observations were made using Leitz Orthoplan and Leica DMRB Fluo microscopes.

RESULTS

The data obtained from the observations were summarized in Table 1. For each organ and host species examined, the rust species and its penetration strategy (as described in previous observations and considered in the Introduction) are listed in the third column of the Table.

The leaves of *Mercurialis annua* (Fig. 1- a, b, c), the growing needles from brachiblasts of *Larix decidua* (Fig. 1- d, e, f), the growing shoots of *P. sylvestris* (Fig. 1 - g, h, i), as well as the cotyledons and primary needles of *P. pinea* (Fig. 1 - l, m, n and o, p, q respectively) - all of wich are organs which are directly penetrated - have the following characteristics:

– highly visible cuticle; the cuticle appears thicker in the growing needles of *L. decidua* (Fig. 1 - d, e) and growing shoots of *P. sylvestris* (Fig. 1 - g, h);

– thin-walled epidermal cells;

– abundant cytoplasm in epidermal cells;

– absence of lignin in epidermal cell walls;

– absence of hypoderm.

The growing and grown-up secondary needles of *P. pinea* (Fig. 2 - a, b, c and d, e, f respectively) i.e., organs which are indirectly penetrated, have the following characteristics:

– highly visible cuticle; the cuticle, in both types of secondary needles (Fig. 2 – a, b and d, e), appears as thick as in *L.decidua* needles and *P. sylvestris* shoots;

– thick walled epidermal cells;

– scarce cytoplasm in epidermal cells;

– cutinization of the radial and inner tangential walls of the epidermal cells (Fig. 2 - a, b and d, e);

– presence of hypoderm, mono-layered in the growing needles (Fig. 2 - c) and bi-layered in grown-up ones (Fig. 2 - f), with lignified cell walls; specifically, the presence of lignin was noted in the cell walls of the first layer of hypoderm, and also appeared to interest the inner tangential wall and partially the radial walls of the epidermal cells.

The growing needles of *Picea abies* (Fig 2 - g, h, i) - which are indirectly penetrated in the same manner as the secondary needles of *P. pinea* - have the following characteristics:

– highly visible cuticle; the cuticle appears thin (Fig. 2 - g, h) as in the leaves of *M. annua* and cotyledons and primary needles of *P. pinea*;

– epidermal cells with slightly thickened walls;

– well visible cytoplasm in epidermal cells;

– presence of mono-layered hypoderm (Fig. 2 - i) with lignified cell walls; the localization of lignin in the surface tissues appears as that in secondary needles of *P. pinea*.

It must be noted that the thickness of cuticle and cell walls, and the amount of cytoplasm of the

Table 1 — Characteristics observed in the surface tissues of organs susceptible to be penetrated by the considered rust species, and basidiospore-derived penetration strategies of the rust species. 1, 2, 3 indicate increasing values approximately estimated; +, - indicate presence or absence.

host species	organs	rust species / penetration strategy		epidermis				hypodermis	
				cuticle thickness	wall thickness	walls cutinized	cytoplasm amount	layer number	lignin
Mercurialis annua	leaves	*Melampsora pulcherrima*	direct	1	1	-	3	-	-
Larix decidua	growing needles	*Melampsora larici-tremulae*	direct	2	1	-	3	-	-
		Melampsora pinitorqua	direct						
Pinus sylvestris	growing shoots	*Melampsora pinitorqua*	direct	2	1	-	3	-	-
		Melampsora larici-tremulae	direct						
Pinus pinea	cotyledons	*Melampsora pinitorqua*	direct	1	1	-	3	-	-
		Melampsora larici-tremulae	direct						
		Cronartium flaccidum	indirect						
Pinus pinea	primary needles	*Melampsora pinitorqua*	direct	1	1	-	3	-	-
		Melampsora larici-tremulae	direct						
		Cronartium flaccidum	indirect						
Pinus pinea	growing secondary needles	*Cronartium flaccidum*	indirect	2	3	+	1	one	+
Pinus pinea	grown-up secondary needles	*Cronartium flaccidum*	indirect	2	3	+	1	two	+
Picea abies	growing needles	*Crysomyxa abietis*	indirect	1	2	-	2	one	+

host organs were approximately estimated, since there were no measurements related to the observations.

DISCUSSION

The cyto-histological aspects observed in the surface tissues of host organs susceptible to be penetrated by the considered rusts allow some remarks: *i)* The cuticle appears thicker in the growing shoots of *P. sylvestris,* growing needles of *Larix decidua,* and growing and grown-up secondary needles of *P. pinea* than in the other organs examined. *ii)* The epidermal cells in both types of secondary needles of *P. pinea,* unlike the other organs, are poor in cytoplasm and show thick cutinized walls. *iii)* A hypoderm with lignified cell walls is present only in both types of secondary needles of *P. pinea* and in growing needles of *P. abies.* The results are interesting to evaluate an estimable involvement of the above described characteristics of host surface tissues in relation to rust penetration strategies.

Cuticle and epidermal cell walls - As reported in the Introduction, some Authors maintain that, in host-parasite interactions, the parasite penetration strategy could change from direct to indirect as a consequence of the thickness of the cuticle and epidermal cell walls of host organs which are considered as a barrier to a direct penetration
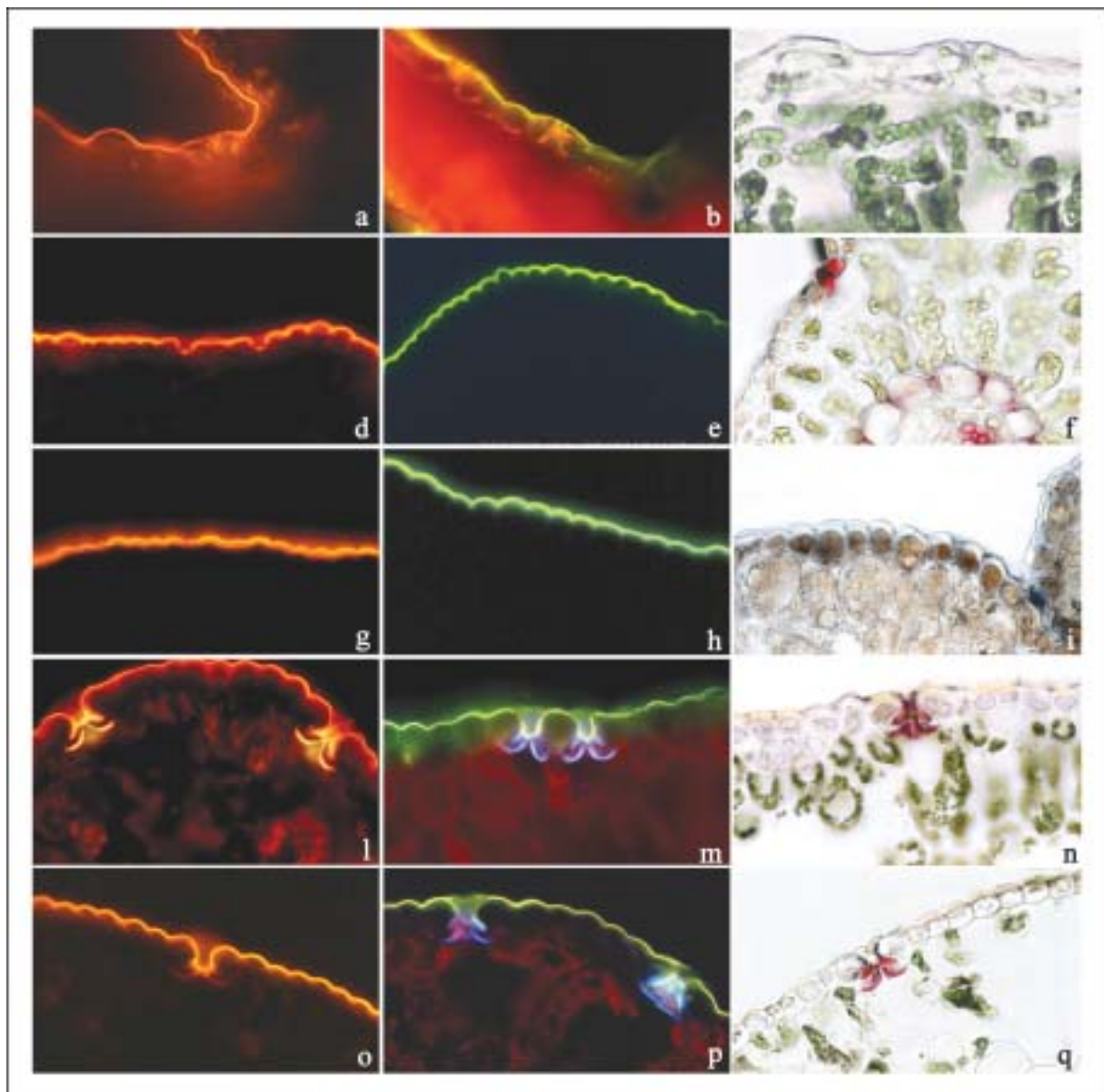
Fig. 1 — Leaves of *Mercurialis annua* (a, b, c); growing needles from brachiblasts of *Larix decidua* (d, e, f); growing shoots of *Pinus sylvestris* (g, h, i); cotyledons (l, m, n) and primary needles (o, p, q) of *Pinus pinea.* Transverse sections. The cutin is shown with Nil-Red (a, d, g, l, o) and Fluorol-yellow (b, e, h, m, p). The lignin is shown with Phloroglucinol test (c, f, i, n, q). The cuticle appears thicker in the needles of *L. decidua* (d, e) and shoots of *P. sylvestris* (g, h). The lignin is present only at the level of the stomata in the needles of *L. decidua* (f), cotyledons (n) and primary needles (q) of *P. sylvestris*, and vein of *L. decidua* (f). All pictures 200x.

(CAMPBELL 1972; DINER and MOTT 1982; GOLD and MENDGEN 1984).

With regard to this problem of plant pathogenesis, it is necessary to keep in mind that the role of fungal enzymes in degrading the surface tissues of host organs to be penetrated is clearly acknowledged. In reality, the lythic enzymes which are commonly reported by Authors - cutinases, pectinases, hemicellulases, cellulases - carry out their activity on fatty substances such as cutin and on polysaccharides/compounds which characterize the plant cell wall (TEN HAVE *et al*. 2002). The production of fungal enzymes is induced by exchange at cuticular level of signals which regulate the host-parasite interaction, e.g., the penetration of host surface (HOCH and STAPLES 1991; CHASSOT and MÉTRAUX 2005).

It must be remembered that in direct penetration typical of the rust monokaryotic phase, the role played by the enzymatic power together with
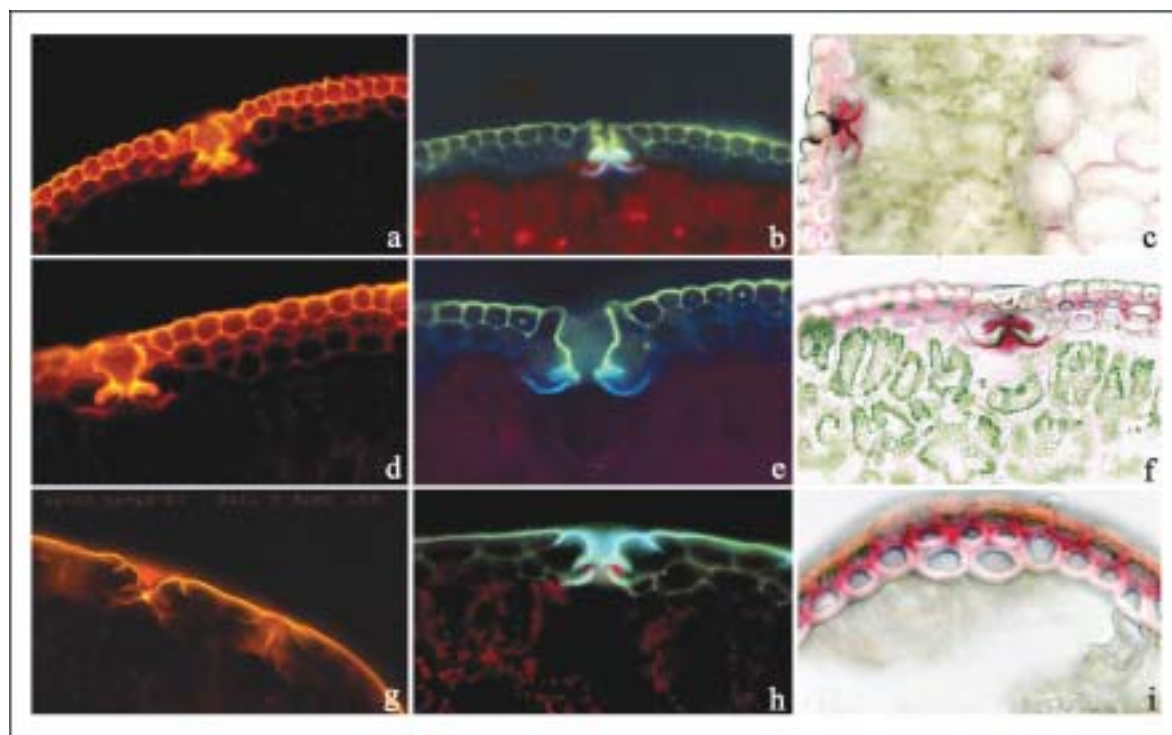
Fig. 2 — Growing (a, b, c) and grown-up (d, e, f) nedlees of *Pinus pinea*; growing needles of *Picea abies* (g, h, i). Transverse sections. The cutin is shown with Nil-Red (a, d, g) and Fluorol-yellow (b, e, h). The lignin is shown with Phloroglucinol test (c, f, i). The cuticle appears thicker in growing (a, b) and grown-up (d, e) nedlees of *P. pinea*. Note the presence of lignin in the surface tissues of growing (c), grown-up (f) needles of *P. pinea* and needles of *Picea abies* (i). All pictures 200x.

turgor pressure is very important in the penetration structures which are involved in host-rust interaction.

As regards the role of cell wall degrading enzymes in fungi with elaborate and melanised appressoria which generate high turgor pressure (such as *Colletotrycum* and *Magnaporte* species), WIRSEL *et al*. (2004) indicate that it is very likely that both, enzymes and force, are needed for initial penetration, the proportion being characteristic of each pathogen. According to GOLD and MENDGEN (1991) and HARDHAM (2001), the extracellular mucilaginous matrix commonly present around rust germlings is useful not only to favour their adhesion to host organs, but above all as an enzymatic reserve - especially cutinase - for penetration. While the turgor pressure inside the appressorium plays a role in direct penetration even if the appressorium is not melanised, the enzymatic action is very important, together with the mechanical one, so as to enable the host cuticle and cell wall to be perforated (MENDGEN and DE-ISING 1993; MENDGEN *et al*. 1996; HOWARD 1997; XU HAIXIN *et al*. 1997). A combination of enzyme activity and mechanical force in the direct host

penetration was described also for *Erysiphe graminis* by PRYCE–JONES *et al*. (1999). Moreover, TRAIL and KOLLER (1990) indicated that leaf-specific pathogens have cutinases with pH optima which are distinct from those of the stem-pathogens, whereas pathogens that infect both stems and leaves secreted both cutinase types. Thus, there is evidence that the cutinase plays a role in the expression of tissue specificity by directly penetrating fungal pathogens.

Then, if one considers the powerful role of enzymatic action in directly penetrating fungal pathogens described above, one should think that the thickness of cuticle and epidermal cell walls of host organs (either of angiosperms or gymnosperms) does not determine the penetration strategy of basidiospore germlings. That opinion is confirmed by the results of this research (see Table 1).

Indeed, with regard to the influence of the cuticle as a barrier to a direct penetration, one must observe that the basidiospore germlings of *M. pulcherrima* directly penetrate the leaves of *M. annua*, which have a thin cuticle. On the other hand, the basidiospore germlings of *M. larici-tremulae*

directly penetrate either the growing needles of *L. decidua* or the artificially inoculated growing shoots of nonhost *P. sylvestris*, both of which appear to have a thick cuticle; *M. larici-tremulae* maintains the same penetration strategy on artificially inoculated cotyledons and primary needles of *P. pinea,* which present a thin cuticle. Likewise, the basidiospore germlings of *M. pinitorqua* directly penetrate the growing shoots of *P. sylvestris*, which appear to have a thicker cuticle than that of the cotyledons and primary needles of *P. pinea,* which are directly penetrated also; *M. pinitorqua* too maintains a direct penetration when is inoculated on growing needles of *L. decidua,* which have a thick cuticle.

Amount of cytoplasm in the epidermal cells - Another aspect of the organ surface which could affect the rust penetration strategy is the amount of cytoplasm in the epidermal cells. The protoplast viability, and then the adhesion between the epidermal cell wall and the plasmamembrane, are necessary for the cells to establish the host-parasite relationship, e.g., to determine wall-associated defense responses and consequently the penetration failure (HEATH 2002), or the penetration success. The epidermal cells are always retained viable, also where they are characterized by thickened walls and a reduced amount of cytoplasm as in both the secondary needles of *Pinus pinea* (host organs of *Cronartium flaccidum*) and, though somewhat less, in the growing needles of *Picea abies* (host organs of *Crysomyxa abietis).* Consequently, the needle epidermal cells of both host species, when penetrated, can establish a host-parasite relationship. Since our results show that those organs are in fact indirectly penetrated by their specific rust fungus, also the amount of cytoplasm in the epidermal cells of host organs, as well as the thickness of cuticle and cell walls already mentioned, does not appear to determine the penetration strategy.

All these observations lead one to hypothesize that other cyto-histological aspects of host surface tissues rather than the thickness of cuticle and epidermal cell walls or the amount of cytoplasm in these cells, could be responsible for the indirect penetration carried out on their hosts by *Cronartium flaccidum* and *Crysomyxa abietis* in the monokaryotic phase.

Presence of lignin in the surface tissues - It is worth noting that both the secondary needles of *Pinus pinea* and growing needles of *Picea abies*, which are indirectly penetrated by their specific rust fun-gus, have a hypodermis which shows clearly lignified cell walls.

According to the literature on the anatomy of conifers, the surface tissues of secondary needles (epidermis and hypodermis) have cells with lignified walls (ESAU 1965). The hypodermis is the tissue which is characteristically interested by lignification, even if the degree of lignification and the tissues which are interested vary according to the different species (NAPP-ZINN 1966). Lignin infiltrates the spaces among the cell wall cellulosic microfibrils, increasing the mechanical strength and decreasing the permeability and degradability of the walls (AIST 1983). Indeed, lignin is a phenolic compound which is not easily degradable by enzymes. Moreover, enzymes such as ligninase are very restricted in their distribution (WALTON 1994) and a ligninase action by strictly biotrophic fungi such as the rusts is unknown.

The lignification process of plant cell walls, either as a natural or induced event, has a special function in the host-parasite interaction. Indeed, where the natural differentiation of cell walls includes lignification, this occurrence may well be a factor in constitutive resistance toward pathogenic fungi. In cell walls which remain unmodified, chiefly in the primary plant body, the evidence for the constitutive resistance of cell wall to penetration is weak and no one component seems to have been clearly implicated (SMART 1991). Again, it is worth remembering the evidence for lignin accumulation during the expression of the induced resistance (AIST 1983; SOUTHERTON and DEVERALL 1990). SOUKUPOVÁ *et al.* (2000), studying the influence of atmospheric pollutans on tissue lignification in needles of *Picea abies,* emphasized the role of lignified cell walls of the hypodermis in mechanical strength and in resistance to pathogenic attack.

Concluding remarks - Given what has been reported on the role of lignin, it is possible that the cell wall lignification of epidermis and hypodermis in secondary needles of *Pinus pinea* and in growing needles of *Picea abies* was responsible for: (i) the inability of basidiospore germlings of *Cronartium flaccidum* and *Crysomyxa abietis* to carry out a direct penetration, and (ii) the fitness of these rust fungi during their evolution to an indirect penetration strategy. So much so that, this same strategy is mantained by *C. flaccidum* also in the organs which are not characterized by surface tissues with lignified cell walls, such as cotyledons and primary needles of *Pinus pinea* which were artificially inoculated.

On this subject, it is demonstrated by the observations of this paper that in the surface tissues of organs which are directly penetrated by basidiospore germlings the lignin was never present, at least in the growing period during which these organs are susceptible to rust fungus infection.

Hence, the results show that the thickness of cuticle and epidermal cell walls of organs on which the monokaryotic phase of each rust species has fitted itself to penetrate could not be important in determining the penetration strategy (direct or indirect), whereas it appears that the presence of lignin in the surface tissues of these organs could effectively limit the direct mode of penetration of rust basidiospore germlings.

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