CARYOLOGIA Vol. 53, no. 1: 9-29, 2000

Penetration and early colonization in basidiospore-derived infection on needles of *Pinus pinea* L. by *Cronartium flaccidum* (Alb. et Schw.) Wint.

NICOLA LONGO*, SIMONETTA POGGIOLESI, BIANCAMARIA NALDINI and GABRIELE TANI

Dipartimento di Biologia Vegetale, Universita di Firenze, via la Pira 4, 50121 Firenze, Italia.

Abstract — The behaviour and the morphology of the infection structures of the monokaryotic phase of *Cronartium flaccidum* were studied on needles of *Pinus pinea* seedlings inoculated with the rust basidiospore. It was found that the penetration and early colonization structures of *C. flaccidum* in the monokaryotic phase maintained the morphological and functional significance of the typical monokaryotic ones, even if some aspects of their behaviour seemed to recall those of the dikaryon. A possible hypothesis as to the reason for the dynamics of penetration carried out on the markedly cutinized needles of pine by *C. flaccidum* in the monokaryotic phase is discussed. It can be concluded that in *C. flaccidum* in the monokaryotic phase it is the nuclear set which determines the morphology and function of the structures involved in the infection process; this is true even if the histological characteristics of the host organ which this rust species has evolved to infect in nature, condition its way of penetration.

Key words: *Cronartium*, monokaryotic infection structures, rust penetration.

INTRODUCTION

The germ tubes of rust fungi infect their hosts by means of two ways of penetration: directly through the intact wall of the epidermal cell and indirectly through the stomatal apertures. From the most of literature cited the first way of penetration results peculiar to the basidiospore germ tubes of the monokaryotic phase, whereas the second one is characteristic of the aeciospore and urediniospore germ tubes of the dikaryotic phase.

As a result of a direct penetration, an intraepidermal infection structure (vesicle and infection hypha) 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).

Moreover, PATTON and JOHNSON (1970) in their studies on *Cronartium ribicola* J. C. Fisher ex Rabenh. monokaryotic phase in *Pinus strobus* L. needles found that the penetration of this rust fungus takes place indirectly through the stomatal apertures producing a substomatal vesicle, despite the fact that the latter was a basidiospore-derived infection.

However, subsequent studies on the penetration of the basidiospore germ tubes of several rust fungi on their hosts (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 direct penetration for these spores and considered the indirect penetration of (i) *C.ribicola* on *P.strobus* (PATTON and JOHNSON 1970) and (ii) *Cronartium comandrae* Pk. on *Pinus banksiana* Lamb. (BERGDHAL and FRENCH 1985) peculiar events which needed to be clarified.

Infact, GOLD and MENDGEN (1984), referring to two other cases of basidiospore-derived indirect penetration (GRILL *et al.* 1978 for *Crys-omyxa abietis* on *Picea abies;* BAUER 1983 for *Coleosporium* spp. on *Pinus* spp.), hypothesized that basidiospore-derived direct penetration was characteristic of rust fungi on angiosperms, whereas basidiospore-derived indirect penetra-

* Corresponding author: fax ++39 055 2757398; e-mail nlongo@unifi.it.

tion was characteristic for gymnosperms. According to these authors, the difference be tween the two ways of penetration could be as cribed to the heavily cutinized, even young leaves of gymnosperms.

On the other hand, LONGO *et al.* (1988, 1991, 1997), studying the basidiospore-derived penetration *oiMelampsorapinitorqua* (A Br.) Rostr. and *Melampsora laricitremulae* Kleb. respectively on *Pinus sylvestris* L. and *Larix decidua* Mill., observed that these two fungi penetrated directly whichever organs they would infect; such was the case also for *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Cumm.) Burds. & Snow on *Pinus elliotii* Engelm. var. *elliottii* (MILLER *et al.* 1980) and *Endocronartium harknessii* (J. P. Moore) Y. Hirat, on *Pinus contorta* Dougl. var. *latifolia* Englm. (HOPKIN *et al.* 1988). Furthermore, LONGO *et al.* (1991) emphasized the indirect penetration of *C. ribicola* on *P. strobus* (PATTON and JOHNSON 1970) and of *C. comandrae* on *P. banksiana* (BERGDHAL and FRENCH 1985) whichever organs were infected.

From these reports it may be seen that the same gymnosperm organs are penetrated directly or indirectly by basidiospore germlings depending on the rust species. Therefore, it may also probably depend on the rust species mode of penetration into the organs usually infected in nature: i.e., a direct penetration by M. *pinitorqua*, *C. quercuum* f.sp. *fusiforme* and *E. harknessii* on growing shoots (where too few or no stomata are present); and an indirect pen etration by *C. ribicola* and *C. comandrae* on needles.

The aim of the observations of this work is to provide further information on the early phases of the monokaryotic stage infection of the Uredinales in general and, more specifically, of those which involve the conifers. Indeed, since such infection phases are still characterised by many question marks, it would be interesting to clarify what links the "direct" and "in direct" penetration way with the type of spore (monokaryon and dikaryon) and/or with the histological characteristics of the organ which becomes infected.

In the context of these questions, it seemed useful to add to the observations on the basidiospore-derived infection, already known for other species, the study of the behaviour and of the morphology of the infection structures of the monokaryotic phase of *Cronartium flaccidum* (Alb. et Schw.) Wint. in order to compare these structures with those of the same phase in other rust species and with what is typical of the "indirect" penetration of the dikaryotic phase; the infection process of such a phase in the Literature is considered to be, in all its aspects, more evolved when compared to that of the monokaryotic one (GOLD and MENDGEN 1991; MENDGEN and DEISING 1993; HEATH 1995; MENDGEN *et al.* 1996; MENDGEN 1997).

C. flaccidum is a macrocyclic and heteroecious rust which forms spermogonia and aecia on some species of the genus *Pinus*, the uredinia and telia on some angiosperms such as *Vincetoxicum hirundinaria* Medicus and *Peonia* spp.

The basidiospore-derived infection begins on the primary and secondary needles of the pine (WILSON and HENDERSON 1966) and the haploid mycelium, proceeding in the colonization of mesophyll, forms stromata in the transfusion tissue giving rise to yellowish spots, which constitute the first symptom, on the pine, of the rust infection (RADDI 1976; RAGAZZI and MORIONDO 1979, 1980). Following the forma tion of the stromata, the rust hyphae invade the tracheids, reaching the brachyblasts (NALDINI and LONGO unpublished data) to then form spermogonia and aecia in the young stem.

RAGAZZI *et al.* (1987) and RAGAZZI and DEL-LA VALLE FEDI (1992), after some observations on germlings of *C. flaccidum* on needles of *Pinus pinaster Kit.* and *Pinus nigra* Arn. subsp. *laricio* (Poiret), concluded that such rust fungus seemed to behave like *C. ribicola* (PATTON and JOHNSON 1970), given that the basidiospore germ tubes penetrated the stomata and not through the wall of the epidermal cells. The same authors however, underlined that nothing could be concluded as to how the germ tube it self proceeded after having invaded the stomatal aperture.

On the other hand, it should be noted that as regards the basidiospore-derived infection of *E. harknessii* on *P. contorta* (HOPKIN *et al.* 1988), and of *C. quercuum* f. sp. *fusiforme* on *P. elliottii vat. elliottii* (MILLER *et al.* 1980), the authors reported that in some cases the germ tube introduced itself into the stomatal antechamber apparently starting an "indirect" type of penetration; then, however, it immediately penetrated the anticlinal wall of one of the subsidi ary cells of the stoma, where it produced an in-

tracellular structure typical of the "direct" type of penetration through the wall of the epider mal cell. Thus, these observations were not exhaustive enough to clarify the *C. flaccidum* penetration dynamics.

MATERIALS AND METHODS

Artificial inoculations were' carried out on seedlings of *Pinus pinea* L., pine species which is reported to be highly susceptible to *C. flaccidum* (RADDI *et al.* 1979), with basidiospores of this rust produced from germinating teliospores on leaves of *Vincetoxicum hirundinaria* Medicus.

Cotyledons, primary and secondary needles were inoculated suspending the leaves of the *Vincetoxicum* on the whole potted seedlings.

The inoculated seedlings were kept in a greenhouse, at a nocturnal temperature of 13° — 16°C and a diurnal temperature of 24° — 26°C. Small glass holders covered with a film of distilled water were placed in the containers of the inoculated plants so as to check periodically the quantity of basidiospores released from the germinating teliospores in order to decide the period and the frequency of the samplings. Checking took place approximately every 24 hours, starting with the moment of inoculation. To carry out the samplings, observations with fluorescence optics of the inoculated needles were made to check, not only the quantity of basidiospores present on the surface of the needle, but also the number of germ tubes which appeared to introduce themselves into the stomatal antechamber. The material was sampled continuously from the third to the tenth day following inoculation. The repeated sampling of the material was necessary since the length of time taken by the germ tubes to penetrate the internal part of the needle and form the first infection structures, was not known. About two months following inoculation, a further sampling of the primary needles was carried out: the material was taken where the yellowish spots showing the first symptoms of the rust infection occurred.

Fluorescence light microsopy

This was used for (a) whole mounts of fresh material treated with Calcofluor and (b) cryosectioned fresh material treated with Nile-Red (BECCARI and MAZZI 1966; GREENSPAN *et al.* 1985).

(a) In order to highlight the basidiospores with their germ tubes (PATTON and JOHNSON 1970; DELL-AVALLE FEDI and RAGAZZI 1991), the inoculated needles were divided into pieces measuring 30-40 mm in length, treated with Calcofluor 0.05% in distilled water for 10 minutes, and observed with epifluorescence optics using a Leica microscope equipped with a mercury arc lamp. With U.V. light exciter filter BP-340-380 nm, dichroic beam splitter RKP-400nm and barrier filter LP-430nm the basidiospores and the germ tubes emitted bright blue fluorescence.

(b) In order to highlight the cutin in the internal tangential walls of the primary needle epidermal cells, sections obtained using a Cryocut microtome American Optical were treated with Nile-Red 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-510nm and barrier filter LP-520nm. The cutin in the cell walls was detected on the basis of the fluorescence emitted.

Bright field light microscopy

Small fragments from (a) needles previously treated with Calcofluor and observed using epifluorescence optics and (b) needles with the first symptoms of the infection were fixed in F.A.A. for 5 days; dehydrated in ethyl alcohol 95% for Ih; infiltrated and embedded in "Historesin" (glycolmetacrylate).

The sections, 2.5 (µm thick, and obtained with a glass knife using an ultramicrotome Reichert Om U3, were stained with Toluidine Blue 0.1% + Na carbonate 0.1% in distilled water for 5 minutes and observed using Leitz Orthoplan and Leica optic microscopes.

Trasmission electron microscopy

Small fragments of needle from the samplings taken from the third to the tenth day, treated with Calcofluor and observed using epifluorescence optics, were prefixed in a solution of glutararaldehyde 2.5% + paraformaldehyde 4% in a phosphate buffer 0.015M pH 6.9 (KARNOWSKY 1965) diluted 1:1 in the same buffer. The dilution of the prefixative solution was necessary since the prefixation was varied from 16h to 60h. The variable duration of sampling prefixation was due to the need to collect, for each sampling, a sufficient number of specimens which might present probable penetrations. The prefixed specimens were then fixed in a solution of glutaraldehyde 2.5% + paraformaldehyde 4% in phosphate buffer 0.15M pH 6.9 for l-2h; postfixed in OsO4 2% in phosphate buffer 0.15M pH 6.9 for 2h; dehydrated with the ethanol series; infiltrated in Spurr (SPURR 1969) + propylene oxide and embedded in Spurr.

The needles with the first symptoms of infection were prefixed for about 12h, then fixed, post-fixed,

dehydrated and embedded as the preceding material.

Serial sections were cut using a Reichert Ultracut E with a diamond knife. They were then stained with lead citrate (REYNOLDS 1963) and observed with a Philips EM 300.

Scanning electron microscopy

Small fragments of needle from the samplings taken from the third to the tenth day, treated with Calcofluor and observed using epifluorescence optics, were prefixed in a solution of glutaraldehyde 2.5% + paraformaldhyde 4% in phosphate buffer 0.15M pH 6.9, diluted 1:1 in the same buffer, for a period of between 6h and 7 days; fixed in a solution of glutaraldehyde 2.5% + paraformaldhyde 4% in phosphate buffer 0.15M pH 6.9 for 4-5h; postfixed in OsO_4 1% in phosphate buffer 0.15M pH 6.9 for 12-14h at 4°C; dehydrated in an acetone series; critical point dried; fractured with epidermal stripping (HUGHES and RIJKEMBERG 1985); gold sputter coated, and observed with a Philips S.E.M. 505.

RESULTS

Prepenetration

On the surface of the inoculated needles, the basidiospores appear roundish to oval, 8-12 m in diameter (Figs. 1, 2), with a smooth wall. Their germ tube is generally single, unbranched with quite a uniform diameter. It grows and develops at random, without moving directly towards the stomata (Fig. 1,2).

The penetration of the germ tubes into a stomatal antechamber is casual. Indeed, many have been observed, variable in length, and only some of them penetrate into the chamber itself. In some cases, a germ tube appears initially to lodge itself in the stomatal antechamber and then exit again continuing its growth without penetrating the stomatal aperture (Fig. 1). Several germ tubes can enter the same chamber, but only one of them penetrates the stomatal aperture.

Once inside the stomatal antechamber, the germ tube which will eventually penetrate the stomatal aperture moves towards it and remains more or less attached to the walls of the subsidiary cells which lie above the stomatal guard cells (Fig. 3).

Penetration

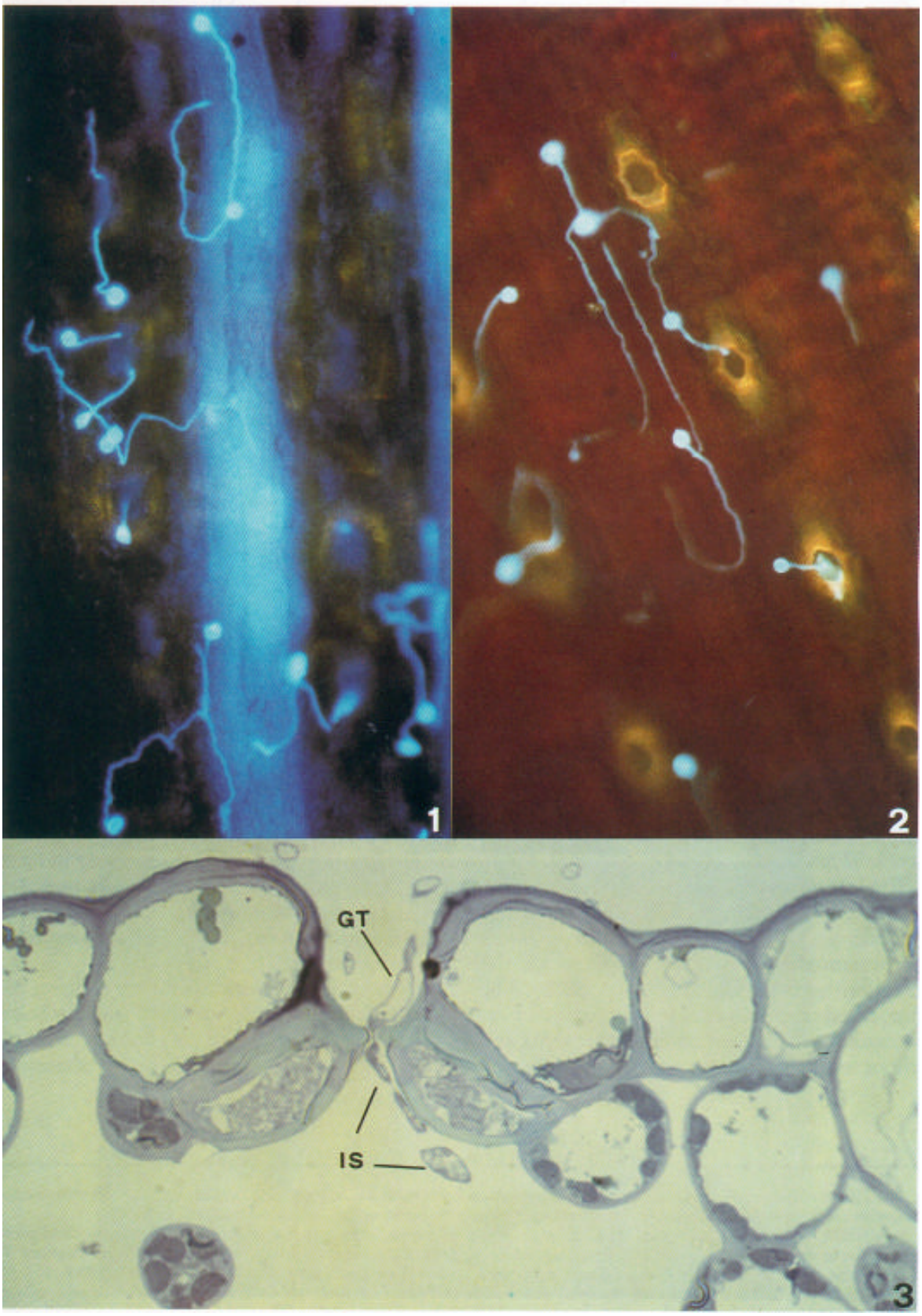
It is the uppermost tip of the germ tube which, without giving rise to particular structures, effects the penetration of the stomatal aperture (Figs. 2, 3). At this point, the germ tube decreases in diameter: indeed, when appearing in the substomatal chamber, its diameter is about half the size it was at the stomatal antechamber level. At the stomatal aperture level, the germ tube takes on a flattened appearance, ribbon-shaped (Figs. 3, 4) and occupies a limited area, at times peripheral, of the aperture itself. In the same stoma, the germ tube appears even more flattened if it is in the more peripheral area and less so if it involves the more central portion of the aperture itself. Preferred stomatal aperture penetration areas by the germ tube have not been observed.

The germ tube undergoes its maximum reduction in diameter at the level of the "ledges" of the stomatal aperture, where the "lips" appear folded towards the inner portion of the stoma which is penetrated by the tube itself (Fig. 4).

The ultrastructure of the germ tube, at the level of the stomatal aperture, is that typical of a hypha of the vegetative mycelium, with a cytoplasm which is rich in organelles and glycogen granules (Fig. 5).

Figs. 1-17 — All the Figures refer to *Pinus pinea* needles. *Abbreviations* — D: distal portion of germ tube; E: endoplasmic reticu-lum; FH: the first haustorium; FS: fungal stroma; GT: germ tube; H: haustorium; I: intercellular hypha; IH: infection hypha; IS: infection structure; M: extrahaustorial matrix; Mb: extrahaustorial membrane; N: nucleus; P: proximal portion of germ tube; PC: parenchimal cell; S: septum; SbC: subsidiary cell; SC: stomatal cell; V: vesicle.

Figs. 1,2 — Epifluorescence optics of Calcofluor stained basidiospores with germ tubes on the epidermis of a cotyledon (Fig. 1) and a secondary needle (Fig. 2). The germ tubes show a random behaviour; appressoria are not produced (x 256). Fig. 3 — L.M. of a Toluidine Blue stained thin transverse section of a cotyledon. Stomatal penetration carried out by germ tube without appressorium; the germ tube is flattened at the stomatal aperture (x 1280).


1
2
GT
IS
3

Once the sides of the stomatal aperture have been overcome, reaching the substomal chamber, the hypha which originates directly from the germ tube becomes the "infection struc ture"; this term refers to the hyphal portion which develops inside the substomatal chamber before giving rise to intracellular colonization (Figs. 6, 7).

The infection structure, continuing its development in the substomatal chamber in order to reach the chlorenchyma, may move towards the central portion of the chamber itself or it may remain more or less attached to one of the two stomatal guard cells, along the cuticular layer which extends from the epidermis as far as the beginning of the lower tangential wall of the stomatal guard cells.

The infection structure, in its proximal portion, has an ultrastructure which is very like that described for the distal portion of the germ tube across the stomatal aperture (Fig. 5).

Following this initial tract, increasing in diameter, the hypha gradually gets bigger so as to form a fusiform structure, similar to a " substomatal vesicle", which represents the median part of the infection structure (Fig. 6). This fusiform structure appears in the proximal re gion of the substomatal chamber, usually at the point of thickening of lignin presented by the lower tangential walls of the stomatal guard cells. The "vesicle" is always occupied by a large vacuole in central position and by a nucleus in proximal position with respect to the vacuole (Fig. 5).

The infection structure, more or less distally to its fusiform median portion, tends to reduce its diameter again (Fig. 6): in this area one finds the formation of a septum beyond which the "infection hypha" originates (Figs. 7, 8). The latter, filled with vacuoles in its proximal portion, without the formation of further septa and without ever branching, moves towards the nearest parenchyma cell until it comes into contact with the cell wall (Figs. 6, 7,8).

Initial phases of colonization

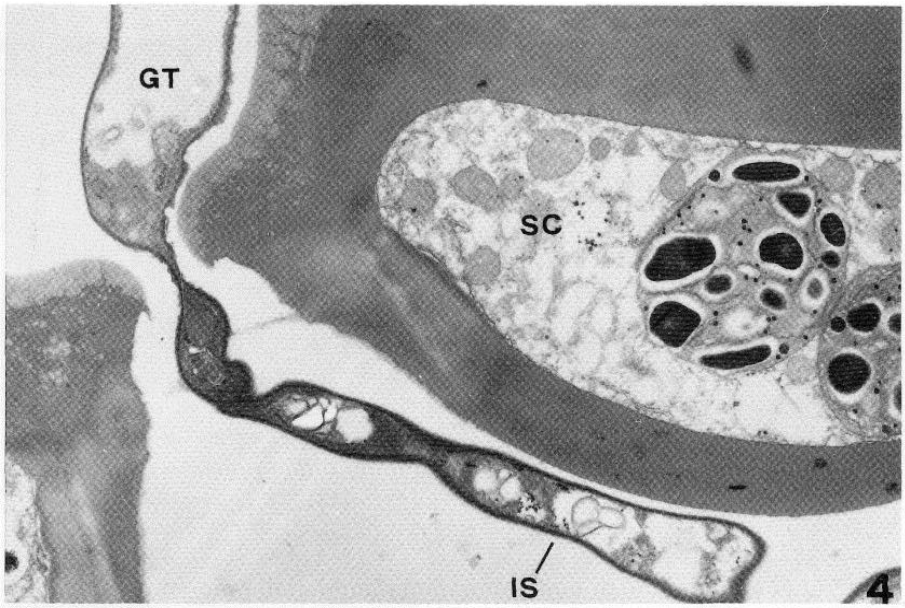
In its distal portion the infection hypha, given the lack of other septa above the first haustorium, takes on the role of a haustorial "mother cell" without however differentiating itself as a completely separate compartment (Figs. 7, 8). A penetration peg originates from the haustorial "mother cell", in the wall of the host cell inside which the first haustorium differentiates (Fig. 8).

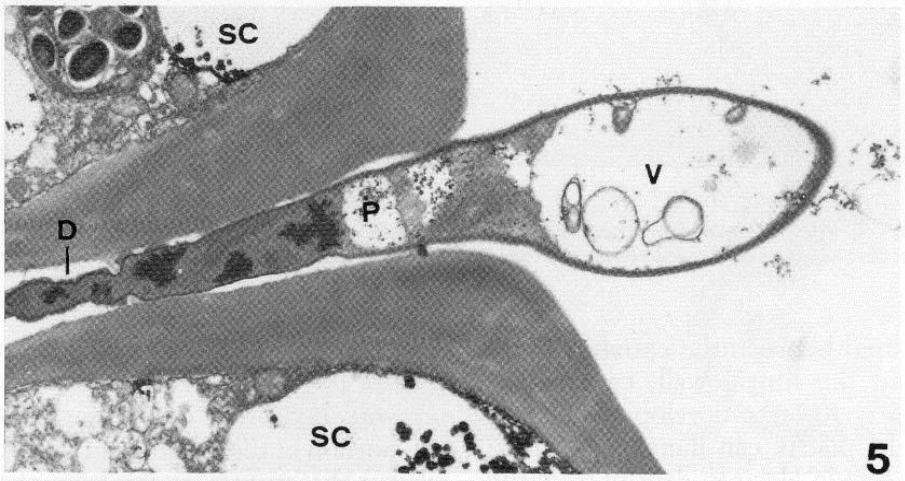
The mother cell is surrounded by amor phous material which noticeable increases in thickness in the region in contact with the host cell walls (Fig. 8). The mother cell wall does not present any specific thickenings, or neo-forma- tions of layers in the penetration point and, crossing with all its thickness the host cell wall, appears continuous with the haustorial wall. The same happens with the mother cell plasma membrane which is continuous with that of the haustorium (Fig. 9).

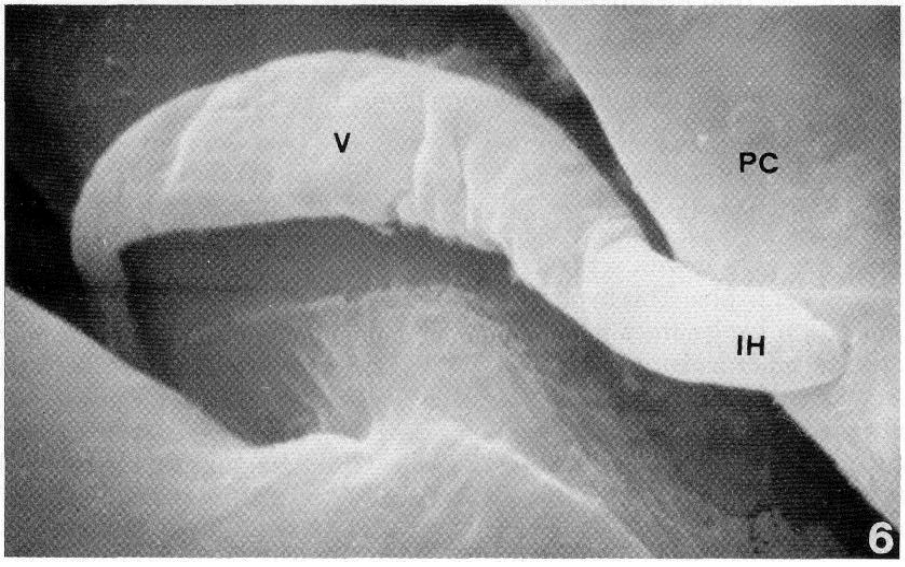
In the host cell wall, the first haustorium presents a penetration hole (Fig. 9) which can measure up to 0,5 μm. In the portion closest to the region of penetration, the haustorium takes on a more or less cylindrical form; developing further in the host cell, it very gradually increases in diameter so as to reach even 3 μm at quite a distance from the region of penetration: in this way, it takes on the typical appearance of a hypha, growing at times noticeably in length with a twisted pattern. A septum is present at the base of the haustorium, but always at a discrete distance from the region of penetration into the host cell (Fig. 10).

Along the haustorium outline, in direct contact with the cytoplasm of the host cell, the extrahaustorial membrane continuous with the host plasmamembrane and the extrahaustorial

Fig. 4 — T.E.M. of the penetration of Fig. 3. Note the germ tube flattened at the ledges of the stomatal aperture. Below the stomatal aperture, in the substomatal chamber, the hypha from the germ tube becomes the "infection structure" (x 8900). Fig. 5 — T.E.M. of a stomatal penetration on a cotyledon. In sequence from left to right note: the ultrastructure of the distal portion of germ tube at the stomatal aperture; proximal and middle portions of the infection structure in the substomatal chamber. The middle portion of infection structure, the "substomatal vesicle", presents a characteristic large central vacuole (x 8000). Fig. 6 — S.E.M. of a stomatal penetration observed on the underside of the stripped epidermis of a cotyledon. The germ tube exit from the stomatal aperture forming the infection structure in the substomatal chamber. The middle portion of the infection struc ture, the substomatal vesicle, and the distal portion, the "infection hypha", are visible. The infection hypha comes into contact with the parenchyma cell wall (x 5800).


GT
SC
IS
4


SC
V
P
D
SC
5


V
PC
IH
6

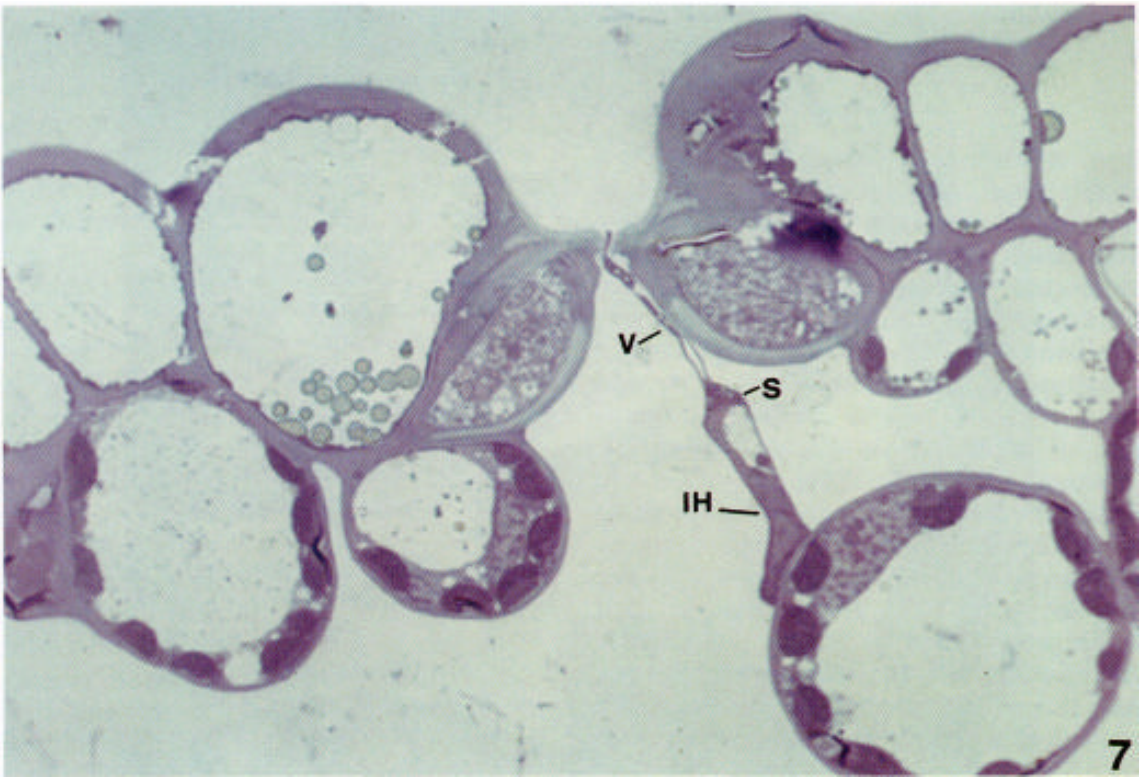


Fig. 7 — L.M. of a Toluidine Blue stained thin transverse section of a cotyledon. The infection structure in the substomatal chamber presents a septum between the substomatal vesicle and the infection hypha which comes into contact with a parenchyma cell (x 1280).

matrix interposed between the extrahaustorial membrane and the fungal wall, can be observed. Depending on the development of the haustorium, the matrix can increase in thickness and decrease in electron density from the proximal portion to the distal one of the haustorium itself. Close relationships between the extrahaustorial membrane and the endoplasmic reticulum of the host cell are evident (Fig. 11).

The infection hypha which gave rise to the first haustorium does not terminate in its growth, but continues through the leaf mesophyll and, after dividing itself with septa, continues as an intercellular hypha. This hypha, behaving as the infection hypha from whence it derives, gives rise to other haustoria (Fig. 12), even more than one in the same host cell. A second haustorium may form in the same parenchyma cell which has already been penetrated by the first haustorium.

The characteristics of the haustoria which are subsequent to the first, even also of the haustoria of the following colonization, are: the penetration hole is smaller in size when compared to that of the first haustorium; the diameter of the haustorium increases as the latter is growing in the host cell so much so that the basal portion of the haustorium takes on a conical shape; the septum at the base of the haustorium, is clearly nearest the region of penetration, than observed in the first haustorium (Figs. 12, 13).

Colonization during early symptoms on the needles

The symptoms of the previous infection and colonization by the rust appear, after about two months following the inoculation, with the presence of chlorotic spots on the surface of the needle. Inside the latter, in correspondence to the spots on the surface, a very advanced hyphal colonization can be noticed; this interests the entire thickness of the chlorenchyma and extends towards the central part of the needle where the hyphae organize themselves in a very thick and widely extended stroma (Fig. 14).

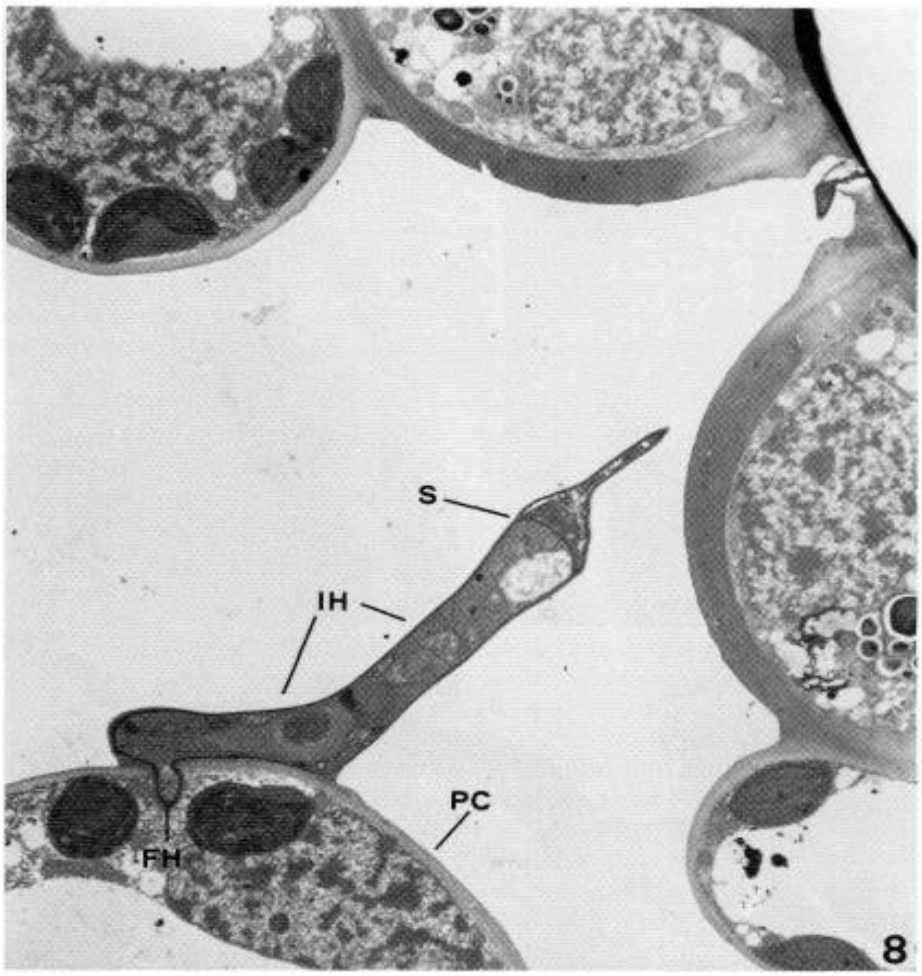


Fig. 8 — T.E.M. of the infection structure of Fig. 7. Note the septum between the vesicle and the infection hypha; the whole distal portion of the infection hypha acts as "mother cell" of the first haustorium in the parenchyma cell (x 4600).

Despite the fact that this stage of colonization is characterised by the formation of numerous haustoria in the cells of the mesophyll, it is particularly interesting to point out at the epidermis level the presence of haustoria exclusively in the subsidiary cells surrounding the stoma (Fig. 15).

At this point it is important to highlight that the internal tangential wall of the subsidiary cells, through which the haustoria penetrate, appears different to that of the other epidermal cells: indeed, besides appearing extremely thin (Fig. 16), with fluorescence optics observation of the sections stained with Nile-Red, it seems to lack the cutin, while the cutin appears to be clearly present in the internal tangential walls of the other epidermal cells (Fig. 17).

The morphological characteristics of the haustoria in the stomatal subsidiary cells are those already described for the haustoria in the cells of chlorenchyma, which form subsequently to the first.

DISCUSSION

Some of the most significant aspects related to the basidiospore germling penetration and the infection structures of *C. flaccidum* on the needles of *P. pinea* (Fig. 18 C), are as follows.

The penetration is always of the "indirect" type through the stomatal aperture, but with non-oriented germ tube growth and without

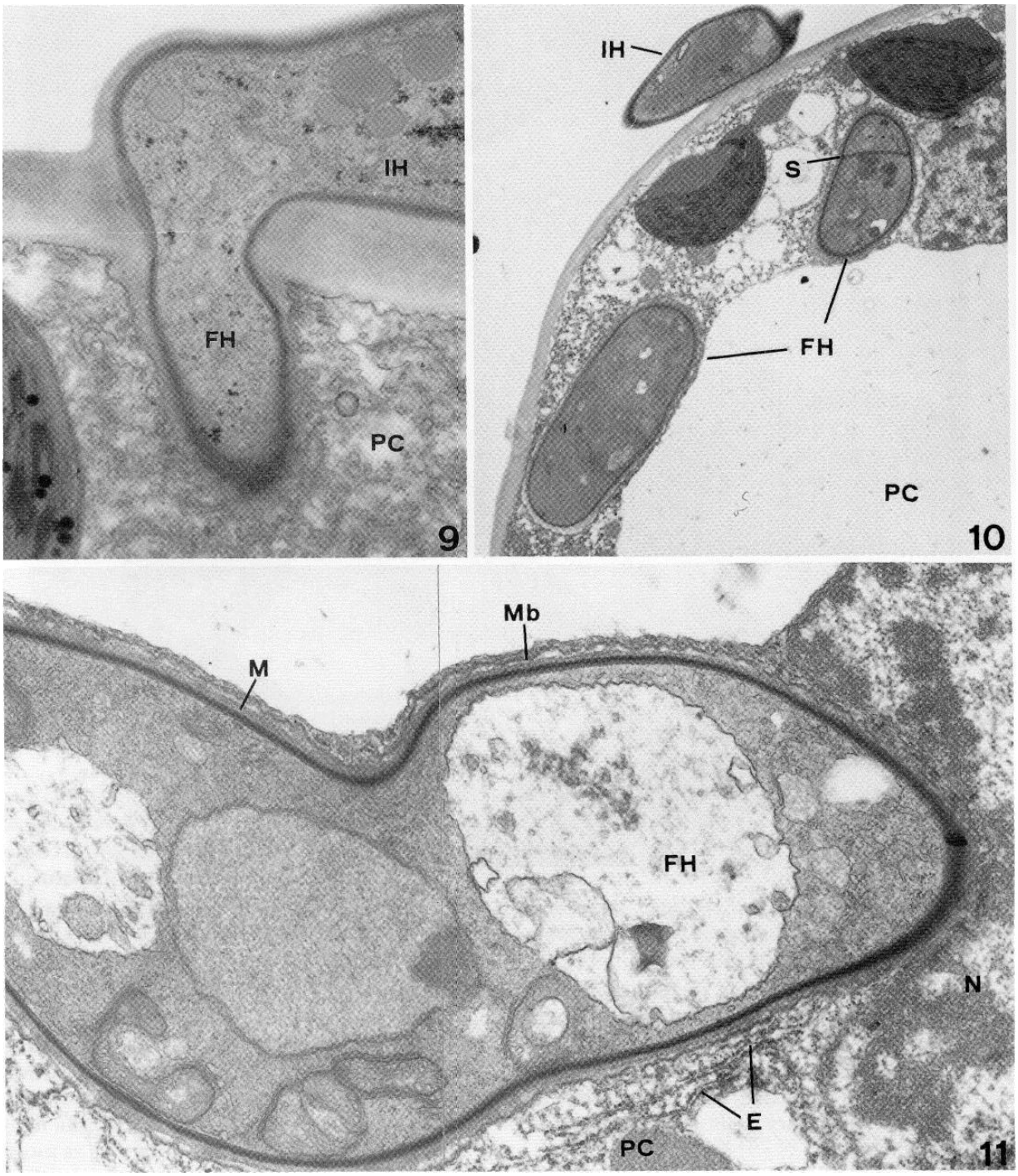


Fig. 9 — T.E.M. of the penetration by the developing first haustorium through the parenchyma cell wall of a cotyledon. The "mother cell" wall, without ultrastructural modifications, is continuous with the haustorial wall. See the wide penetration hole in the parenchyma cell wall (x 33200).
Fig. 10 — T.E.M. of two sectioned portions of the first haustorium in a parenchyma cell of a cotyledon. Note the septum in distal position respect to haustorium penetration zone (x 6300).
Fig. 11 — T.E.M. of a third portion of the first haustorium of Fig. 10. Note the haustorium ultrastructure, the extrahaustorial matrix and membrane, the relationships of haustorium with host endoplasmic reticulum and nucleus (x 23000).

the formation of appressorium-like adhesion structures. A "peg" of infection cannot be identified, rather it is the apical part of the germ tube which penetrates into the stomatal aperture and, inside the substomatal chamber, gives rise to a hypha which becomes the infection structure. The latter gradually gets bigger taking on a spindle-like shape, then forms a septum and gives rise to the infection hypha which, without branchings or further septa, grows towards the nearest parenchyma cell. The distal portion of the infection hypha, taking on the role of the haustorial "mother cell", penetrates into the wall of the parenchyma cell thus giving rise to the first haustorium. This shows a markedly hyphal appearance characterised by a wide penetration hole in the host cell wall, a cylindrical shape at least in its basal part and a septum at a discrete distance from the point of penetration. The extrahaustorial matrix is present from the point of penetration.

The infection hypha then continues its growth in the chlorenchyma, forming further septa and branching out into intercellular hyphae which give rise to the subsequent haustoria. The latter, when compared to the first haustorium, have a narrower penetration hole and a more basal septum.

In the advanced colonization stage of the rust, in the primary needles, haustoria are present in the stomatal subsidiary cells, the only epidermal cells colonized by the fungus in the monokaryotic phase.

When one compares the characteristics described for *C. flaccidum* with the equivalents of other rusts which have the same way of penetration during the monokaryotic phase as well as with those considered in the literature cited to be typical of the monokaryotic and dikaryotic penetration (Fig. 18 A, B, C), the following remarks can be made.

The morphology of the basidiospores of *C. flaccidum* has shown itself to be similar to that already observed in the same rust by RAGAZZI *et al.* (1987); the only difference lies in their size which is greater when compared to that reported by those authors.

The growth of the germ tubes on the surface of the needle is at random and, therefore, non oriented towards the stomatal apertures. The Literature considers such behaviour to be typical of the monokaryotic infection phase, as reported for many rusts (GRAY *et al.* 1983; GOLD and MENDGEN 1984; BERGDAHL and FRENCH 1985; HOPKIN *et al.* 1988; LONGO *et al* 1994, 1997), as well as for *C. flaccidum* on *P. pinaster* and *P. nigra* subsp. *laricio* (RAGAZZI and DEL-LA VALLE FEDI 1992). Despite the fact that some of these authors (i.e. GOLD and MENDGEN 1984) have observed a growth of germ tubes towards the anticlinal walls of the epidermal cells, their whole behaviour is always to be considered as being casual.

On the contrary, in the dikaryotic infection phase, the germ tubes usually have an oriented pattern towards the stomata triggered by the morphological characteristics of the epidermis surface and, more specifically, by those of the stomata (Hocn and STAPLES 1991; TERHUNE *et al.* 1991); the exceptions seem to be *Cronartium ribicola* J.C. Fisch. Ex Rabenh. on *Kibes* spp. (Woo and MARTIN 1981) and *Melampsora laricipopulina* Klebahn and *Melampsora medusae* Thuem on *Populus* (SPIERS and HOPCROFT 1988) where a germ tube behaviour which is apparently non oriented has been observed.

In *C. flaccidum*, as already observed by RAGAZZI and BELLA VALLE FEDI (1992), also the penetration of the germ tubes into the stomatal antechamber is casual; moreover, in accordance with BERGDAHL and FRENCH (1985) for *C. comandrae*, several tubes can enter the same chamber, even if only one penetrates the stomatal aperture.

Once inside the stomatal antechamber, the germ tube of *C. flaccidum* grows towards the stomatal aperture without forming appressorium-like structures which are differentiated from the germ tube; the lack of an appresso-rium has already been observed for the same rust by RAGAZZI and DELLAVALLE FEDI (1992) and also reported for *C. ribicola* (PATTON and JOHNSON 1970) and *C. comandrae* (BERGDAHL and FRENCH 1985); the same way of penetration as in *C. flaccidum* has been described for the monokaryotic phase of these two rust species.

The appressorium of the Uredinales (LITTLEFIELD and HEATH 1979; HARDER and CHONG 1984; HOCH and STAPLES 1991) is highly differentiated in the dikaryotic infection phase. In this phase the appressorium is separated by a septum from the germ tube from which it originates. In the monokaryotic phase, the appressorium, which is generally present, is instead a not well differentiated structure, and only appears

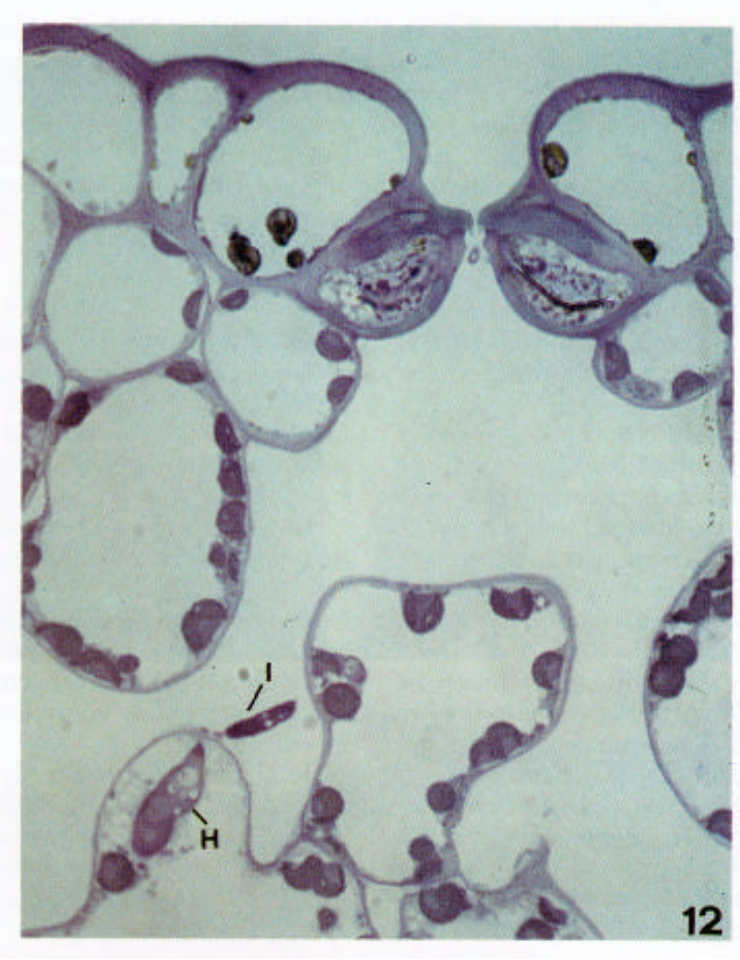


Fig. 12 — L.M. of a Toluidine Blue stained thin transverse section of a cotyledon. General view of epidermis with a stomatal penetration and mesophyll parenchyma showing intercellular colonization with one of the haustoria occurred after the first (x 1000).

as a swelling of the terminal portion of the germ tube and is usually not separated by septa from the latter (MILLER *et al.* 1980; GRAY *et al.* 1983; GOLD and MENDGEN 1984; HOPKIN *et al.* 1988; LONGO *et al.* 1991, 1994; MORIN *et al.* 1992).

Given that in *C. flaccidum* the appressorium is lacking, so too a differentiated infection peg, it is the apical portion of the germ tube itself which penetrates into the stomatal aperture.

In the dikaryotic phase the infection peg is described as a thin outgrowth, which derives from the innermost layer of the appressorium wall, which grows apically introducing itself into the stomatal aperture (LITTLEFIELD and HEATH 1979).

Also the infection peg of the monokaryotic phase derives directly from the appressorium, but it is not differentiated like that of the dikaryotic one (GOLD and MENDGEN 1984; MENDGEN 1997); it appears very thin at the point of penetration into the cuticle of the epidermal cell, then it expands in the wall and enters the host cell by invaginating its plasma membrane.

In *C. flaccidum* the germ tube, growing inside the stomatal aperture, decreases in diam eter and becomes clearly flattened, finally faces the substomatal chamber; a similar aspect is also described in *C. ribicola* (PATTON and JOHNSON 1970) and in *C, comandrae* (BERGDAHL and FRENCH 1985).

At this point, it is possible to conclude that *C. flaccidum*, as far as the aspects related to the basidiospore-derived prepenetration are concerned, differs from what has been observed in both the monokaryotic and dikaryotic phases of the rusts in general and that the way of penetra tion of its monokaryotic phase, as observed in

P. pinea, is always of the indirect type, as had already been hypothesized by RAGAZZI and DEL-LA VALLE FEDI (1992) for other species of the genus *Pinus*. This is analogous to what happens in C *ribicola* (PATTON and JOHNSON 1970) and in *C. comandrae* (BERGDAHL and FRENCH 1985), but not to what happens in *E. harknessii* (Hop-KIN *et al.* 1988) and *C. quercuum* f. sp. *fusiforme* (MILLER *et al.* 1980) where, in some cases, the penetration begins in an indirect manner and involves the stomatal antechamber, but continues and ends in a direct manner through the anticlinal walls of the subsidiary cells of the stoma. After having reached the substomal chamber, the germ tube of *C. flaccidum* gives rise to a hypha which grows towards the underlying chlorenchyma. The fusiform portion of this hypha corresponds to the monokaryotic intraepi-dermal vesicle and to the dikaryotic substo-matal one. PATTON and JOHNSON (1970) and BERGDAHL and FRENCH (1985) describe, for *C. ribicola* and *C. comandrae* respectively (both of which penetrate indirectly in the monokaryotic phase), a substomatal vesicle similar to that observed in *C. flaccidum*, at least as far as the shape is concerned. Diversely, however, to what has been reported for *C. comandrae,* in *C. flaccidum* a septum has never been found in the proximal part of the "vesicle" just as no multi- septate vesicles have ever been observed.

In the monokaryotic infection with direct penetration, the intraepidermal vesicle, described in detail for *Uromyces appendiculatus* (Pers.) Unger var. *appendiculatus* by GOLD and MENDGEN (1984), for *Puccinia xanthii* Schw. by MORIN *et al.* (1992), for *Melampsora pulcherrima* (Bub) Maire by LONGO *et al.* (1994), for *M. pimtorqua* and *M. larici tremulae* by LONGO *et al.* (1997), is made up of a thin neck which derives from the penetration peg and of a widened portion with a large central vacuole. In accordance with what has been observed in the substomatal vesicle of *C. flaccidum*, also in the intraepidermal one there is no septum to separate the infection peg from the vesicle; only in *C. quercuum* f. sp. *fusiforme* a septum as been observed between the infection peg and the neck of the vesicle (GRAY *et al.* 1983).

In the dikaryotic infection phase (LITTLE-FIELD and HEATH 1979; HARDER and CHONG 1984) with indirect penetration, a septum always separates the substomatal vesicle from the

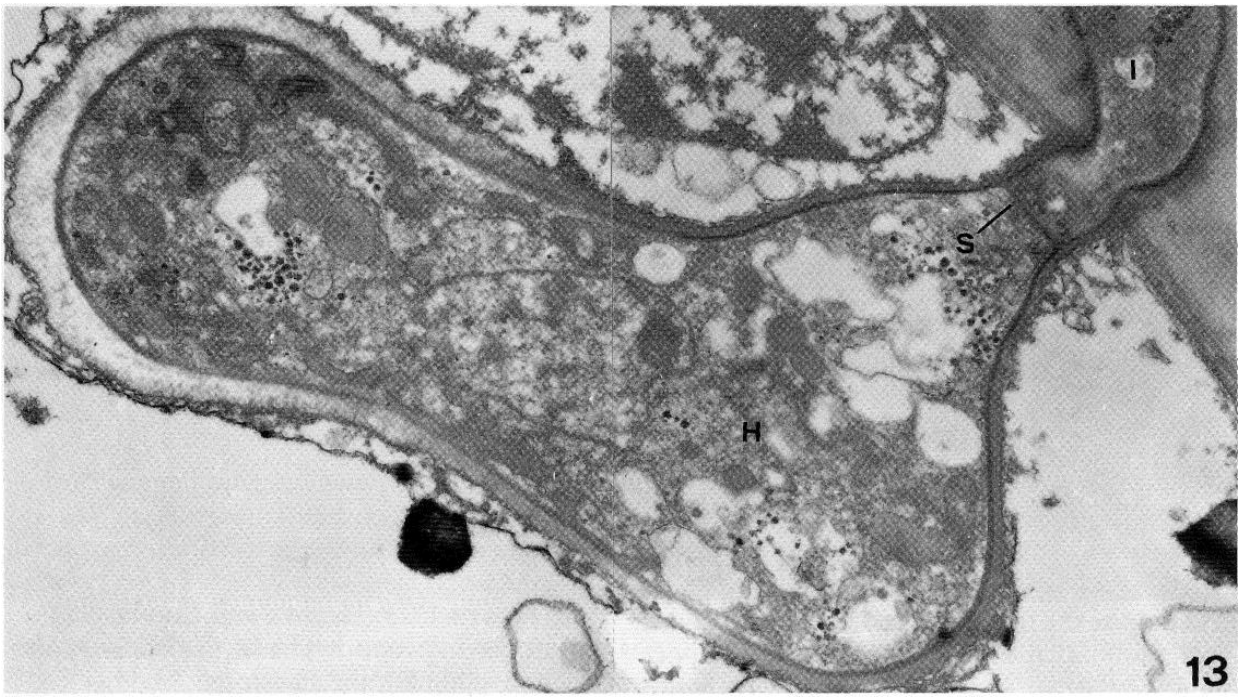


Fig. 13 — T.E.M. of another haustorium occurred after the first in a primary needle. Note the reduced penetration hole and the proximal position of the septum as in Fig. 12 (x 15300).

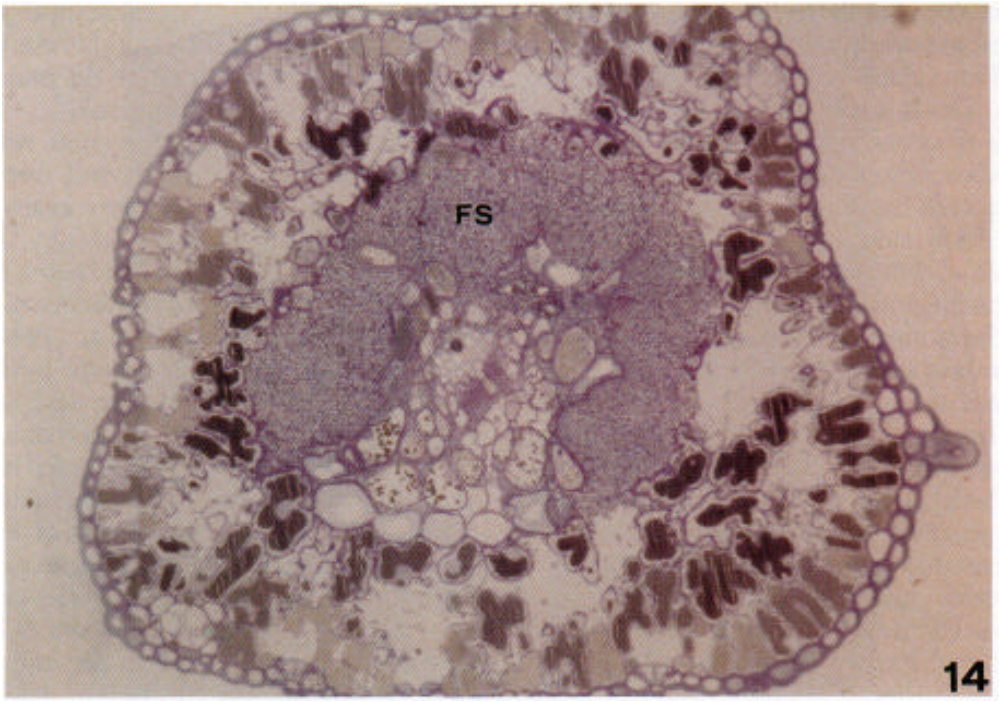


Fig. 14 — L.M. of a Toluidine Blue stained thin transverse section at level of a yellow spot on infected primary needle. Fungal stroma within the transfusion tissue (x 128).

infection peg, as described in detail by LITTLEFIELD and HEATH (1979), DAVIES and BUTLER (1986), SHAIN and JARLFORS (1987), MIMS *et al.* (1989), Hu and RIJKEMBERG (1998). The size and shape of substomatal vesicle may be, depending on the different rust species, roundish, ovoidal, spindle-shaped, lobed or even multi-septate and branched (NiKS 1986).

A septum located immediately distal to the vesicle, as observed in *C. flaccidum*, is present in the monokaryotic intraepidermal vesicle, and behind it, the intracellular infection hypha, contrary to what instead occurs in *C. flaccidum*, can further form other septa and branch out, thus continuing its growth with the production of intracellular hyphae and haustoria with hypha-like behaviour in the neighbouring host cells, as well as hyphae in the intercellular spaces. The infection hypha of the intraepidermal infection structure is surrounded by an extrahyphal matrix which is typical of all the intracellular structures; instead, its vesicle always appears matrix-less despite the fact that it too is an intracellular structure (GOLD and MENDGEN 1984; MORIN *et al.* 1992; LONGO *et al* 1994, 1997). The entire structure of infection of *C. flaccidum* is matrix-less as are all the intercellular structures, be they monokaryotic or dikaryotic.

The septum directly below the vesicle is not usually present for the dikaryotic substomatal vesicle; from the latter, as from the monokaryotic vesicle, one or more infection hyphae originate depending on the different morphology of vesicles, which in its turn depends on the species of rusts (SOTOMAYOR *et al.* 1983; NIKS 1986). These hyphae can also form septa and branch out before developing the haustorial mother cell and the first haustorium in a parenchyma cell, as occurs in *P. porn* (Sow.) Wint. (DAVIES and BUTLER 1986) and in *Pucdnia recondita* Rob. Ex Desm. f. sp. *tritid* (Hu and RIJKEMBERG 1998).

One of the characteristics of the dikaryotic phase, is the presence of a septum in the terminal portion of the infection hypha so as to separate this from the haustorial mother cell. Such a septum, as well as the mother cell at the penetration point, shows a marked ultrastructural specialization (HEATH *et al.* 1975; LITTLEFIELD and HEATH 1979; HARDER and CHONG 1984).

In the infection hypha of *C. flaccidum*, the septum outlining the haustorial "mother cell" has not been found: in this rust, the apical portion of the infection hypha penetrates into the parenchyma host cell without thickenings or

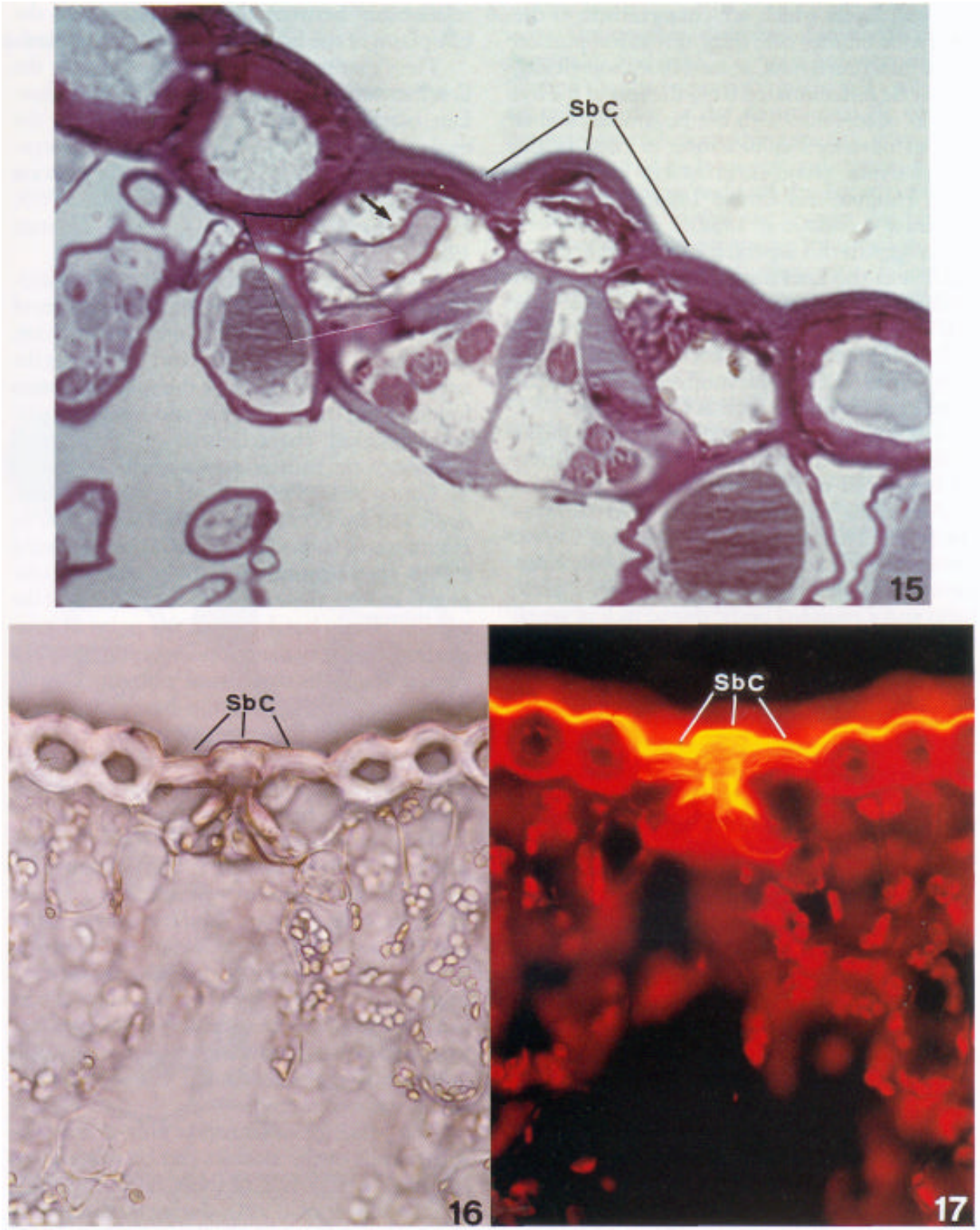


Fig. 15 — L.M. of a Toluidine Blue stained transverse section of a primary needle. General view of epidermis with a stoma showing an haustorium (arrow) in a subsidiary cell (x 1280).
Figs. 16, 17 — L.M. of a Nile-Red stained cryosection of a fresh primary needle. General view of epidermis with a stoma which is observed: in Fig. 16 with bright field and in Fig. 17 with epifluorescence optics. In Fig. 16: see the inner tangential wall of the two subsidiary cells which appear thinner than that of the other epidermal cells. In Fig. 17: the red fluorescence, which showes the wall cutinization, is not detectable in the inner tangential wall of the two subsidiary cells (x 512).

new wall layers which are characteristic of the dikaryotic mother cell; thus, this apical portion of the infection hypha, at least morphologically, cannot be differentiated from the rest of the hypha, as occurs also with the haustorial mother cells of advanced colonization in rusts in the monokaryotic phase (LITTLEFIELD and HEATH 1979; HARDER and CHONG 1984) including *C. flaccidum* (LoNGO *et al*, 1982).

As regards this aspect, it is worth remember ing that in the Literature detailed observations carried out during the initial phases of colonization and thus pertaining to the first haustorium and its mother cell, are not reported for the rusts which penetrate indirectly during the monokaryotic phase, such as *C. flaccidum.*

The first haustorium of *C. flaccidum* shows differences when compared to typical haustoria, be they di- or monokaryotic.

According to the literature cited (LITTLEFIELD and HEATH 1979; HARDER and CHONG 1984, 1991; HARDER 1989), the dikaryotic haustorium is made up of a thin neck with a neck band and a rounded body; due to lack of septa, the haustorium is continuous with its mother cell.

Differentiated neck and body were not found in the monokaryotic haustorium; instead, this is characterised by a hypha-like behaviour and a septum is always present at its base (LITTLEFIELD and HEATH 1979; HARDER and CHONG 1984, 1991; HARDER 1989). These morphological characteristics described for the two types of haustoria in the Literature cited, were observed for the dikaryotic and monokaryotic haustoria of *C. flaccidum* during the advanced colonization, already studied by LONGO and BRUSCAGLIONI (1986) on leaves of *Vincetoxicum hi-rundinaria* Medicus and by LONGO *et al.* (1982) on primary needles and young stems of some species of the genus *Pinus.*

Even if in the first haustorium of *C. flaccidum* in monokaryotic phase one finds the same characteristics as in the haustoria typical of this phase, it has some particular aspects: a) the dis tal position of the septum with respect to the point of penetration into the host cell; b) a pen etration hole which is particularly wide; c) a more or less cylindrical shape in its basal por tion which makes it take on an even more hypha-like behaviour.

In the first haustorium of *C. flaccidum* the extrahaustorial membrane and matrix and the relationship between these structures and the cytoplasm of the host cell, appear to be typical.

The "mother cell" which gave rise to the first haustorium is not terminal and this behaviour is typically found in all rusts during the monokaryotic phase, while during the dikaryotic phase the haustorial mother cells are always terminal (LITTLEFIELD and HEATH 1979; HARDER and CHONG 1984, 1991; HARDER 1989).

It was interesting to observe that, in *C. flaccidum,* both the haustoria of the initial phases of colonization which develop after the first one, as well as those in the subsidiary cells during the advanced colonization, lack the specific aspects found in the first haustoria, and have the same morphological characteristics of the typical monokaryotic haustoria, previously described by LONGO *et al.* (1982) for the same *C. flaccidum,* and by LITTLEFIELD and HEATH (1979), HARDER and CHONG (1984, 1991) and HARDER (1989) for many other rusts in monokaryotic phase. Indeed, these show: a) the position of the septum clearly basal behind the point of pen etration; b) a very narrow penetration hole; c) a conical shape in their basal portion. Despite their different shape at the base when compared to that of the first haustorium, it can be noted that also these haustoria maintain a hy-phal behaviour, even if not as marked as that observed in the first haustorium.

The presence of haustoria in subsidiary stomatal cells was described in the Literature for the dikaryotic phase of two species of Uredinales, *Puccinia graminis* Pers. f. sp. *tritici* Eriks. and Henn. (TIBURZY *et al.* 1990) and *Hemileia vastatrix* Berk, and Br. (COUTINHO *et al.* 1993). Indeed, these two rusts in the substomatal chamber produce an infection structure from whence the first haustoria in the subsidiary cells originate directly, while the other epidermal cells are not colonized.

The presence of haustoria only in the subsidiary cells of the stomata can be related to a specific characteristic of these cells. According to THERUNE *et al.* (1991), during the dikaryotic phase of the *U. appendiculatus* on *P. vulgans,* the chance that haustorial mother cells can differentiate at the level of the internal tangential wall of the epidermical cells, can be related to the degree of hydrophoby of those walls: the success of the development of the haustoria

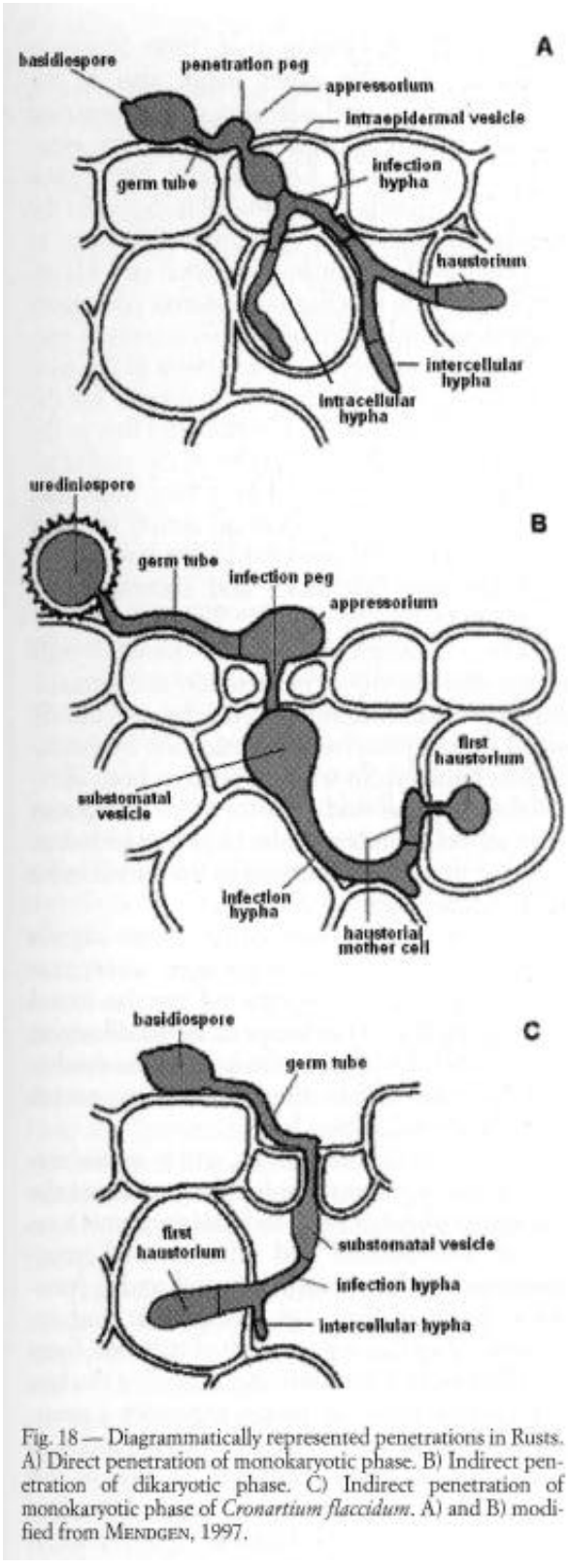


Fig. 18 — Diagrammatically represented penetrations in Rusts. A) Direct penetration of monokaryotic phase. B) Indirect penetration of dikaryotic phase. C) Indirect penetration of monokaryotic phase of *Cronartium flaccidum*. A) and B) modified from MENDGEN, 1997.

could, therefore, depend upon the lack or scarsity of substances which when present modify the walls of the epidermal cells, by making them impermeable. As far as the subsidiary cells, the same Authors then describe, always with reference to *P. vulgaris*, "...a cuticular covering extending slightly beyond the guard cell - subsidi ary cell junction in the substomatal chamber", but without adding anything specific as regards the presence of hydrophobic substances in the internal tangential walls of the latter cells; on the other hand, in this last respect the Litera ture does not offer exhaustive information.

In *C. flaccidum*, fluorescence following staining of sections of *P. pinea* primary needles with Nile-Red has indicated that the internal tangential walls of the subsidiary cells do not seem to contain cutin, while the latter is present in the walls of the other epidermal cells, includ ing the stomatal ones.

Following on the hypothesis of TERHUNE *et al.* (1991) it can be concluded therefore that in *C. flaccidum* the presence of the haustoria in the subsidiary cells of the stomata could be correlated to the scarsely hydrophobic nature of their internal tangential wall; instead, on the basis of the above, the formation of haustoria in the other epidermal cells seems not to occur be cause the presence of cutin.

CONCLUDING REMARKS

The literature cited covering the two types of prepenetration, penetration and early colonization respectively of the dikaryotic and monokaryotic phases of the Uredinales, has highlighted the fact that behaviour and structures are substantially different for the two phases.

The characteristics of the indirectly penetrating dikaryotic phase on the host surface are: germ tubes which are oriented towards stomata; appressorium from which the tube itself is separated by a septum; an infection peg which penetrates into the stomatal aperture.

The steps of this part of the infection proc ess are mediated by a series of both physical and chemical stimuli, which are sent by the host sur face (HocH and STAPLES 1991; MENDGEN *et al.* 1996; EPSTEIN and NICHOLSON 1997) and more specifically by the stomata (TERHUNE *et al.* 1991); these stimuli, which guide the behaviour of the fungal structures on the host surface are all the better, the stronger the adhesion of the fungus to the host surface. Such adhesion

comes about as a result of mucilaginous exhudates produced by the fungus and containing numerous lythic enzymes, especially the cutinase, which are considered to give a significant contribution to the adhesion itself and, thus, to the subsequent processes of rust structure differentiation (MENDGEN *et al.* 1996; HOWARD 1997; MENDGEN 1997). More specifically, according to MENDGEN *et al.* (1996) and EPSTEIN and NICHOLSON (1997), the action of such enzymes modifies the cuticular surface of the host from hydrophobic to hydrophylic as that of the fungus is, and thus plays a positive role in the adhesion of the latter. The hydrophobic nature of the host surface thus would seem to condition the entire prepenetration process of the fungus. In indirect penetration, of marked importance it is the role of the adhesion of the appressorium which must maintain a correct position on the guard cells of the stoma (EPSTEIN and NICHOLSON 1997).

The characteristics of the dikaryotic phase after the penetration through the stoma are: substomatal vesicle divided from the infection peg by a septum; infection hypha with a distal septum which divides it from the haustorial mother cell which is terminal originating the first haustorium.

In the substomatal chamber, the signals which regulate the interaction between the fungus and the host occur all along the entire rust structure from the vesicle to the first haustorium which, in the dikaryotic phase, is the first intracellular structure (MENDGEN *et al.* 1988; HEATH 1989, 1995).

The characteristics of the directly penetrating monokaryotic phase on the host surface are: germ tubes with a non-oriented pattern on the leaf surface; slightly differentiated appresso-rium; penetration peg which penetrates the epidermal cell wall.

The signals which occur in the monokaryotic phase between the host surface and the fungus during prepenetration are not as well known as those of the dikaryon (MENDGEN 1997); nonetheless, the presence of some type of signal which guides the rust structures has been hypothesized by some authors (DESPREZ-LOUSTEAU and EE MENN 1989; GOLD and MENDGEN 1991; EONGO *et al.* 1994) also because, in some rusts, a preferential pattern of germ tubes along the anticlinal walls of the epidermal cells has been observed (GOLD and MENDGEN 1984; HOPKIN *et al.* 1988; MORIN *et al.* 1992). On the other hand, also in the monokaryon, a good adhesion of the fungus to the host surface is considered important; especially, according to EPSTEIN and NICHOLSON (1997), as regards the relationship between the appressorium and the epidermis of the host to be penetrated. According to GOLD and MENDGEN (1991), the mucilaginous matrix commonly present around external rust structures is useful, not only to favour the adhesion of the rust, but, above all, as an enzymatic reserve for the penetration. It must be remembered that in the direct penetration the pressure of the turgor inside the appressorium plays a very important role, and thus the mechanical action together with the enzymatic one enables the host wall to be perforated (MENDGEN and DEISING 1993; HOWARD 1997).

The characteristics of the monokaryotic phase after the direct penetration are: intraepi-dermal vesicle continuous with the peg, but divided by a septum from the infection hypha; infection hypha from whose branches both intracellular hyphae and haustoria in contiguous cells, as well as intercellular hyphae which then give rise to further haustoria in the parenchyma cells, can originate.

Also in the monokaryotic phase signals which regulate the host-parasite interaction once penetration has occurred can be found but, diversely to what happens in the dikaryon, all of them take place at the level of the epidermal cell in which the penetration occurs (HEATH 1989, 1995).

As a result of this analysis, and in consideration of the fact that the infection process of the dikaryotic phase of the Uredinales presents a series of well defined and differentiated structures both from a morphological and a functional point of view (as the number and arrangement of the septa which divide one from the other clearly demonstrate) as well as the fact that each of these structures expresses a peculiar interaction with the host, such a process is more evolved when compared to that of the monokaryotic phase (GOLD and MENDGEN 1991; MENDGEN and DEISING 1993; HEATH 1995; MENDGEN *et al.* 1996; MENDGEN 1997).

Taken as a whole, the monokaryotic infection process of *C. flaccidum* presents a similarity, even if only apparently, with what is typical of the rusts in dikaryotic phase. Indeed, also *C.*

flaccidum shows an indirect penetration, the formation of a substomatal infection structure as well as of a first haustorium in a parenchyma cell. However, this is a similarity of a behavioural type because the morpho-functional characteristics which emerge in such an infec tive process are essentially those of a monokaryotic phase. And in point of fact: the germ tubes on the host surface do not have a specific orientation and only by chance do they penetrate into a stomatal aperture without dif ferentiating an appressorium and an infection peg; the infection structures in the substomal chamber are characterised by a single septum between the vesicle and the infection hypha; all the haustoria, including those in the subsidiary cells of the stoma, are typical monokaryotic structures, especially the first one which, on account of its particular morphology, presents an even more marked hyphal behaviour. Moreover, the peculiar aspects of the latter can be observed also for the monokaryotic intracellular structures of M. *pinitorqua* (LoNGO *et al.* 1988, 1991), which deriving directly from the infec tion hypha in the epidermal cell, colonise both the epidermal and the parenchymal contiguous cells. The first haustoria of *C. flaccidum* could therefore be considered the extension of the substomatal infection hypha of which it maintains the behaviour even if, covering itself with extrahaustorial membrane and matrix, it takes on the function of exchange of substances between host and parasite. This also takes place in the intracellular infection hypha which derives from the intraepidermal vesicle in monokaryotic direct penetration; indeed, such a hypha, diversely to what happens with the vesicle, is covered by a matrix so as to indicate its haustorial function.

The penetration of the monokaryotic phase of the *C. flaccidum* differs from both that of a dikaryon and that of a typical monokaryon since it is accomplished by the germ tube with out the differentiation of an appressorium and, therefore, of a true penetration peg. In calling to mind that the function of the appressorium is that of attaching the rust to the host, it's lack ap pears consequent if one considers the fact that *C. flaccidum* neither penetrates directly through the intact host epidermis (monokaryotic characteristic), nor does its indirect penetration through the stoma (dikaryotic characteristic) occur with an oriented behaviour of the germ tube towards the stoma itself.

It is interesting to consider the similar behaviour found between *C. flaccidum* and the rusts in a dikaryon phase towards cutinized surfaces. Indeed, with reference to the dikaryon phase, the hydrophobic nature of the cutinized walls of the host cells, which extends from the external surface of the epidermis as far as the substomal chamber given the continuity of the cuticle, seems to stimulate the penetration proc ess (MENDGEN *et al.* 1996; EPSTEIN and NICHOL-SON 1997) and the development of the intercel lular infection structures at the level of the substomatal chamber (TERHUNE *et al.* 1991). With reference to the intracellular colonization, the hydrophobic nature of the cutinized internal tangential wall of the epidermal cells prevents the differentiation of the haustorial mother cells and therefore the formation therein of the haustoria (TERHUNE *et al.* 1991 for *U. appendiculatus* on *P. vulgaris).*

With regard to the monokaryotic phase of *C. flaccidum* we do not know what signals occur between the host cell walls and the structures of prepenetration and of infection, nor do we know the role played by the enzymatic load in the exchange of such signals. We have seen, however, that this species in the monokaryotic phase, as the rusts in a dikaryotic phase, does not penetrate through the epidermal cell walls, but produces the first intracellular structures in the parenchymal cells, in whose walls the cutin is scarse or absent, while it never does so in the epidermal cells which, instead, are impregnated with cutin in all their walls.

As a proof of what above considered, the fact of having found in *C. flaccidum* the haustoria only in the stomatal subsidiary cells, and the fact of having revealed that their internal tangential wall through which the haustoria pen etrate seems to be lacking in cutin, are of the ut most importance.

Following all considerations, a possible hypothesis as to the reason for the dynamics of penetration carried out by *C. flaccidum* in the monokaryotic phase could be as follows. The basidiospore germ tubes, having to infect "in nature" organs such as the secondary needles of *Pinus* spp. which are markedly cutinized, but also rich in stomata, and not being able to exercise a suitable mechanical action which in monokaryotic penetration is always associated

with the enzymatic one, in order to fit themselves on this substratum, modified their way of penetration from the direct one, which is typical of the monokaryotic phase of the Uredinales, to that of the indirect type. Nevertheless, the penetration structures of this rust in the monokaryotic phase have maintained the morphological and functional significance of the typical monokaryotic ones, even if some aspects of their behaviour seem to recall those of the dikaryon.

Hence, one can conclude that in *C. flaccidum* in the monokaryotic phase it is the nuclear set which determines the morphology and function of the structures involved in the infection process; this is true even if the histological characteristics of the host organ which this rust species has evolved to infect in nature, condition its way of penetration.

Acknowledgments — The authors wish to thank Dr. A. Paolillo, Mr. Pietro Di Falco and Mr. Fiorenzo Drovandi for their technical assistance and helpful cooperation. Research grants by the University of Florence are also acknowledged.

REFERENCES

BAUER R., 1983 — *Experimentell - ontogenetische und karyologische Untersuchungen an Uredinales.* Doctoral dissertation, Universitat Tubingen, Tubingen, Federal Republic of Germany.

BECCARI N. and MAZZI V., 1966 — *Manuale di tecnica microscopica.* Societa Editrice Libraria, Como.

BERGDAHL D.R and FRENCH D.W., 1985 — *Penetration of the primary tissues of Pinus banksiana by Cronartium comandrae.* In: J. BARROWS- BROADDUS and H.R. POWERS (Eds.), Proc. IUFRO Rusts of hard pines Conf., Oct. 1984, pp. 179-192, Athens, Univ. Georgia.

BUSHNELL W.R. and ROELFS A.P., 1984 — *The cereal rusts.* Vol. 1. Academic Press, Orlando.

COUTINHO T.A., RIJKEMBERG F.H.J. and VAN ASCH M.A.J., 1993 — *Development of infection structures by Hemileia vastatrix in resistant and susceptible selections ofCoffea and in Phaseolus vulgaris.* Can. J. Bot, 71: 1001-1008.

DAVIES M.E. and BUTLER G.M., 1986 — *Development of infection structures of the rust Puccinia porn, on leek leaves.* Trans. Br. Mycol. Soc., 86: 475-515.

DELLAVALLE I. and RAGAZZI A., 1991 — *Behaviour of Cronartium flaccidum basidiospores on different organs of the same pine species*. In: Y. HIRATSUKA *et al.* (Eds.), "Rusts of Pine". Proc. IUFRO Rusts of Pine Conf., Sept. 1989, Banff, Alberta. Inf. Rep. Nor - X - 317: 281-286.

DESPREZ-LOUSTEAU M. and LE MENN R., 1989 — *Epicuticular waxes and Melampsora pinitorqua Rostr. preinfection behaviour on maritime pine shoots.* Eur. J. For. Path., 19: 178-188.

EPSTEIN L. and NICHOLSON R., 1997 —*Adhesion of spores and hyphae to plant surfaces.* In: G.C. CARROL and P. TUDZYNSKI (Eds.), "The Mycota", Vol V, (part A): 11-25. Springer, Berlin.

GOLD R.E. and MENDGEN K., 1984 — *Cytology of basidiospore germination, penetration, and early colonization of Phaseolus vulgaris by Uromyces appendiculatus var. appendiculatus.* Can. J. Bot., 62: 1989-2002.

—, 1991 — *Rust basidiospore germlings and disease initiation.* In: G.T. COLE and H.C. HOCH (Eds.), "The fungal spore and disease initiation in plants and animals", pp. 67-99. Plenum Press, New York.

GRAY D.J., AMERSON H.V. and VAN DYKE C.G., 1983 — *Ultrastructure of the infection and early colonization of Pinus taeda by Cronartium quercuum f. sp. fusiforme.* Mycologia, 75: 117-130.

GREENSPAN P., MAYER E.P. and FOWLERS S.D., 1985 — *Nile red: a selective fluorescent stain for intracellular lipid drop lets.* J. Cell. Biol., 100: 965-973.

GRILL D., LACKNER E. und SCHARNER M., 1978 — *Untersuchungen an mit Chrysomyxa abietis befallenen Fichten-nadeln.* Phyton, 19: 71-82.

HARDER D.E., 1989 — *Rust fungal haustoria - past, present, future.* Can. J. Plant Path., 11: 91-99.

HARDER D.E. and CHONG J., 1984 — *Structure and physiology of haustoria.* In: W.R. BUSHNELL and A.P. ROELFS (Eds.), "The cereal rusts", Vol I: 431-476. Academic Press, Orlando.

—, 1991 — *Rust haustoria.* In: K. MENDGEN and D.E. LESEMANN (Eds.), "Electron microscopy of Plant Pathogens", pp. 235-250. Springer, Berlin.

HEATH M.C., 1989 —*A comparison of fungal growth and plant responses in cowpea and bean cultivars inoculated with urediospores or basidiospores of the cowpea rust fungus.* Physiol. Mol. Plant Path., 34: 415-426.

—, 1995 — *Signal exchange between higher plants and rust fungi.* Can. J. Bot., 73: 616-623.

HEATH M.C. and HEATH I.B., 1975 — *Ultrastructural changes associated with the haustorial mother cell septum during haustorium formation in Uromyces phaseoli var. vignae.* Protoplasma, 84: 297-314.

HOCH H.C. and STAPLES R.C., 1991 — *Signaling for infection structure formation in fungi.* In: G.T. COLE and H.C. HOCH (Eds.), "The fungal spore and disease initiation in plants and animals", pp. 25-46. Plenum Press, New York.

HOPKIN A.A., REID J., HIRATSUKA Y. and ALLEN E., 1988 — *Initial infection and early colonization of Pinus contorta by Endocronartium harknessii (western gall rust).* Can. J. Plant Path., 10:221-227.

HOWARD R.J., 1997 — *Breaching the outer barriers. Cuticle and cell wall penetration.* In: G.C. CARROL and P. TUDZYNSKI (Eds.), "The Mycota", Vol V, (part A): 43-60. Springer, Berlin.

Hu G.G. and RIJKENBERG F.HJ., 1998 — *Scanning electron microscopy of early infection structure formation by Puccinia reconditaf.sp. tritici on and in susceptible and resistant wheat lines.* Mycol. Res., 102: 391-399.

HUGHES F.L. and RIJKEMBERG F.HJ., 1985 — *Scanning electron microscopy of early infection in the medial stage of Puccinia sorghi in Zea mays.* Plant path., 34: 61-68.

KARNOVSKYM.S., 1965 —*A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy.* J. Cell. Biol., 27: 137A.

LITTLEFIELD LJ. and HEATH M.C., 1979 — *Ultrastructure of the rust fungi.* Academic Press, New York, S. Francisco. London.

LONGO N. and BRUSCAGLIONI L., 1986 — *Ultrastructural observations on the dikaryotic haustorium of Cronartium flacci-*

dum (Alb. et Schw.) Wint. in Vincetoxicum himndinaria Medicus. Caryologia, 39: 51-64.

LONGO N., MORIONDO F. and NALDINI LONGO B., 1982 — *Ultrastructural observations on the hostpathogen interface in infections of Cronartium flaccidum on fine.* Caryologia, 35: 307-326.

LONGO N., NALDINI B., DROVANDI F., GONNELLI T, and TANI G., 1994 — *Penetration and early colonization in basid-iospore-derived infection of Melampsora pulcherrima (Bub) Maire on Mercurialis annua L.* Caryologia, 47: 207-222.

LONGO N., NALDINI B., PAOLILLO A., DROVANDI F., TANI G. and GONNELLI T., 1997 — *Morphological aspects of early host-parasite interactions in infections ofMelampsora pinitorqua and Melampsora larici-tremulae on Pinus sylvestris. Implications in the taxonomical relationship of the two rust fungi.* Caryologia, 50: 35-57.

LONGO N., NALDINI LONGO B., TANI G. and DROVANDI F., 1988 — *Osservazioni sull'infezione basidiosporica di Melampsora pinitorqua Rostr. (Uredinales) in alcuni ospiti del gen. Pinus.* Giorn. Bot. Ital., 122 (suppl. 1): 155.

—, 1991 — *Penetration and early colonization in basidiospore-derived infection ofMelampsora pinitorqua Rostr. on Pinus. Structural and ultrastructural observations.* In: Y. HIRATSUKA *et al.* (Eds.), "Rusts of Pine". Proc. IUFRO Rusts of pine Conf., Sept. 1989, Banff, Alberta. Inf. Rep. Nor - X - 317:120-127.

MENDGEN K., 1997 — *The Uredinales.* In: G.C. CARROL and P. TUDZYNSKI (Eds.), "The Mycota", Vol V (part B): 79-85. Springer, Berlin.

MENDGEN K. and DEISING H., 1993 — *Infection structures of fungal plant pathogens - a cytological and physiological evaluation.* New Phytol., 124: 193-213.

MENDGEN K., HAHN M. and DEISING H., 1996 — *Morphogenesis and mechanism of penetration by plant pathogenic fungi.* Annu. Rev. Phytopathol., 34: 367-386.

MENDGEN K., SCHNEIDER A., STERK M. and FINK W., 1988 — *The differentiation of infection structures as a result of rec ognition events between some biotrophicparasites and their hosts.*J. Phytopath., 123: 259-279.

MILLER T., PATTON R.F. and POWERS H.NJN., 1980 — *Mode of infection and early colonization of slash pine seedlings by Cronartium quercuumf. sp. fusiforme.* Phytopathology, 70: 1206-1208.

MIMS C.W., TAYLOR J. and RICHARDSON E.A., 1989 — *Ultrastructure of early stages of infection of peanut leaves by the rust fungus Puccinia arachidis.* Can. J. Bot., 67: 3570-3579.

MORIN L., BROWN J.F. and AULD B.A., 1992 — *Teliospore germination, basidiospore formation and the infection process of Puccinia xanthii on Xanthium occidentale.* Mycol. Res., 96: 661-669.

NIKS E.R., 1986 — *Variation of mycelial morphology between species and formae speciales of rust fungi of cereals and grasses.* Can. J. Bot., 64: 2976-2983.

PATTON R.F. and JOHNSON D.W., 1970 — *Mode of penetration of needles of eastern white pine by Cronartium ribicola.* Phytopathology, 60: 977-982.

RADDI P., 1976 — *Indagine sui tipi di macchie fogliari e sul loro significato in discendenze di Pinus pinaster infette da Cronartium flaccidum.* Rivista Patologia vegetale, 12: 91-98.

RADDI P., MITTEMPERGHER L. and MORIONDO F., 1979 — *Testing of Pinus pinea and P. pinaster progenies for resistance to Cronartium flaccidum.* Phytopathology, 69: 679-681.

RAGAZZI A. and DELLAVALLE FEDI L, 1992 — *Penetration of Cronartium flaccidum into pine needles.* Eur. J. For. Path., 22:278-283.

RAGAZZI A., FAGNANI A. and DELLAVALLE FEDI L, 1987 — *Te-lial and basidiospore stages of Cronartium flaccidum: light and scanning electron microscopy observations.* Phytopath. medit, 26: 81-84.

RAGAZZI A. and MORIONDO F., 1979 — *Suscettibilitd del Pinus sylvestris L. e del Pinus nigra Arn. alia ruggine vescicolosa Cronartium flaccidum (Alb. et Schw.) Wint.* LTtalia forestale e Montana, 34: 121-129.

—, 1980 — *Results of Cronartium flaccidum (Alb. et Schw.) Wint. inoculations on eight-year-old plants of Pinus pinea L.* In: H.R. POWERS *et al.* (Eds.), "Rusts of hard pines". Proc. IUFRO Meeting, Sept. 1979, Florence. Phytopath. Medit., 19:51-56.

REYNOLDS E.S., 1963 — *The use of leadcytrate stain in electron microscopy.}.* Cell. Biol., 17: 208-212.

SHAIN L. and JARLFORS U., 1987 — *Ultrastructure of eastern cottonwood clones susceptible or resistant to leaf rust.* Can. J. Bot., 65: 1586-1598.

SOTOMAYOR LA., PURDY L.H. and TRESE A.T., 1983 — *Infection of Sugarcane leaves by Puccinia melanocephala.* Phytopathology, 73: 695-699.

SPIERS A.G. and HOPCROFT D.H., 1988 — *Penetration and infection of poplar leaves by urediniospores of Melampsora laricipopulina and Melampsora medusae.* New Zeal. J. Bot, 26: 101-111.

SPURR A.R., 1969 — *A low-viscosity epoxy resin embedding me dium for electron microscopy.* J. Ultrastr. Res., 26: 31-43.

TERHUNE B.T., ALLEN E.A., HOCH H.C., WERGIN W.P. and ERBE E.F., 1991 — *Stomatal ontogeny and morphology in Phaseolus vulgaris in relation to infection structure initia tion by Uromyces appendiculatus.* Can. J. Bot., 69: 477-484.

TIBURZY R., NOLL V. and REISENER H.J., 1990 — *Resistance of wheat to Puccinia graminis f.sp. tritici: histological investi gation of resistance caused by S2* 5 *gene.* Physiol. Mol. Plant Path., 36: 95-108.

WILSON M. and HENDERSON D.M., 1966 — *British rust fungi.* Cambridge University Press, Cambridge.

Woo J.Y. and MARTIN N.E., 1981 —*Scanning electron micros copy of Cronartium ribicola infecting Ribes leaves.* Eur. J. For. Path., 11:7-15.

Received 28 March 2000; accepted 25 May 2000