

Induction of mature stromata in *Rosellinia necatrix* and its taxonomic implications

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A method for the induction of stromata with mature perithecia in Portuguese isolates of *Rosellinia* obtained from diseased apple trees is described. Comparison of teleomorphic features with those of Californian material and with the type description of *R. necatrix* indicate that they are the same. The relationship of this taxon to *Rosellinia bothrina* is given further consideration.

Keywords: taxonomy, cultures, pathology.

Rosellinia necatrix PRILL. or more usually its anamorphic state *Dematophora necatrix* HART. is well known from the literature as a serious root infecting pathogen of many plant species (KHAN, 1959; SIVANESAN & HOLLIDAY, 1972; ROGERS, 1979). According to SZTEJNBERG & MADAR (1980) *R. necatrix* can infect over 170 species of plants from 63 genera and 30 families and has a worldwide distribution. It also has the apparent ability to succeed in both temperate and tropical climates. TEIXEIRA DE SOUSA (1985) reported on the differences in susceptibility of a range of plant species noting that several herbaceous plants were also infected and could be regarded as a potential source of inoculum in nature.

FRANCIS (1985) in considering the true identity of *R. necatrix* suggested that it is probably more prevalent in temperate areas with the closely related species, *R. bothrina* (BERK. & BR.) SACC. (= *R. arcuata* PETCH) being more usual in the tropics. However, the situation is more complex since in the same article FRANCIS (1985) commented on the difficulty in separating these two species stating “The similarity between the American specimens named as *R. necatrix* and the type material *R. bothrina* is striking and the only morphological difference that I can find is the presence of a sheath on the spores of *R. bothrina* and its absence from the spores of the *R. necatrix* material”.

The significance of the reference to, and the importance of, the American specimens becomes clear once it is realized that *R.*

necatrix does not usually appear to produce its ascigerous state in nature and that the fungus was first recognized as the anamorphic *Dematophora necatrix* in 1883 by HARTIG. The first report of ascocarps associated with *D. necatrix* belongs to VIALA (1891) and later PRILLIEUX (1897; 1904) reported it on diseased vine roots in France. Clear and detailed teleomorphic descriptions including spore measurements were given by both authors.

According to FRANCIS (1985) there were no further reports on the ascocarps until HANSEN & al. (1937) described those occurring on apple roots infected with *D. necatrix* which had been kept for 2 years in a moist chamber in their Californian laboratory. Germinated ascospores gave rise to conidial *D. necatrix* and thus the connection was established. Comparison of their material with descriptions given by VIALA (1891) and PRILLIEUX (1897; 1904) satisfied HANSEN & al. that their fungus was indeed *R. necatrix*. The true identity of *R. necatrix* is however still not clear. FRANCIS was unable to locate ascospore type material and as she pointed out a very large number of the identifications reported from all over the world were based solely on vegetative characters (FRANCIS, 1985). These included the development of a white mycelial fan on the diseased roots, the presence of pear-shaped swellings adjacent to septa in the hyphae, and sometimes the appearance of synnematosus *D. necatrix*. It should be noted that we have observed the development of white fan like mycelia in many other xylariaceous fungi and its presence should therefore not be taken as a definitive identification of *R. necatrix*. Furthermore many xylariaceous taxa produce an anamorph which can be assigned to the form genus *Geniculosporium* CHESTERS & GREENHALGH and it would not be unreasonable to view the *Dematophora* state simply as a synnematosus form of *Geniculosporium*. Indeed, *Rosellinia buxi* FABRE can produce coremia when grown on a sawdust soil medium (GREENHALGH & CHESTERS, 1968) or on oatmeal agar (ROGERS & MALMGREN, 1977) and as stated by GREENHALGH & CHESTERS (1968) the coremia "develop as erect, stiff and very dark bristle-like bodies up to 2.5 mm high, composed to regular pigmented hyphae. The external hyphae diverge from the main axis in the upper two-thirds of the structure and branch repeatedly to form the individual conidiophores". *Hypoxyton mammatum* (WAHL.) MILL., an important pathogen of *Populus tremuloides*, usually produces conidial pillars (ROGERS & BERBEE, 1964) and these and the bristle-like coremia in *R. buxi* are superficially very similar to *D. necatrix*. Interestingly, SEIFERT (1990) decided not to differentiate between mononematosus and synnematosus *Geniculosporium*. In contrast the presence or absence of pear-shaped swellings might be a more useful feature since we have not detected them in other species of *Rosellinia* including *R. aquila* (FR.) DE NOT., *R. britannica* L.E. PETRINI & al., *R.*

buxi, *R. desmazieresii* (BERK. & BR.) SACC., *R. mammaeformis* (PERS: FR.) CES & DE NOT., and *R. thelena* (FR.) RABENH.

The fundamental problem concerns the apparent inability of *R. necatrix* to produce ascocarps and as FRANCIS (1985) suggested long term experiments are required to induce their production. Only then will it be possible to correlate ascocarp characteristics and disease symptoms of different hosts from different parts of the world and resolve this identity 'crisis'.

Although there is no short cut to perithecial production we can report on a reliable technique for induction of ascocarps of *Dematophora necatrix* and can confirm that their cardinal features conform to those described by VIALA (1891), PRILLIEUX (1897; 1904) and HANSEN & al. (1937) for *R. necatrix*.

Methods

Isolates of a fungus identified on the basis of vegetative features and disease symptoms as *R. necatrix* were obtained from infected roots of apple trees growing in orchards of the Estacaõ Nacional de Fruticultura de Vieira Natividade at Alcobaça, Portugal. Roots which were infected and partially killed by the fungus were washed with water to remove the soil and then with a 10% sodium hypochlorite solution for 1 minute. The roots were then subjected to serial washing with sterile water on 10 occasions of approx. 30 seconds duration for each washing. The outer layer of root tissue was then removed to reveal the mycelial 'fan' which was transferred to potato dextrose agar (Difco) in 9 cm Petri dishes. Other isolates were obtained by placing roots, which has been treated with sodium hypochlorite followed by serial washing as described previously, in plastic bags which were then inflated and sealed. After 10–13 days mycelium had developed on the roots and plastic surface and could easily be transferred to PDA.

The original isolates were grown on PDA to provide a suitable inoculum and this was made in the form of 1 cm disks taken from the edge of the growing colonies with a cork borer. Inoculations were made into 250 ml flasks containing oats, wheat, potato, wood pieces and water in approximately equal proportions. The wood pieces used were from apple branches and were usually 2–3 cm long and 0.5–1 cm diam. The flasks were then sterilized by autoclaving at 120 C for 20 min and inoculated with the *R. necatrix* isolates. Within 3 weeks profuse growth had occurred and the wood pieces were well colonized. At this stage 3 pieces of the wood were used to inoculate living apple tree seedlings of 3 years maturity which were growing in 20 l clay pots. The inoculum was placed adjacent to the roots and covered with soil and the pots were 'incubated' outdoors under natural con-

ditions. The susceptible apple varieties Golden Delicious and Starling were used.

Results

After 30–40 days post inoculation the apple seedlings were dead and at this stage the tops were removed. On alternate days the pots were watered and in August when air temperature reached 30–35 °C coremia developed on the root collar at soil level. During the winter the pots received natural irrigation by rain and later were artificially watered to maintain moisture levels. In the summer of the second year coremia-bearing conidiophores again developed. Irrigation was continued on alternate days and during the winter (December/January) black coloured stromata developed in place of the coremia at the collar of the dead tree stems (Figs. 1 and 2a). Sometimes ascospores were ejected at this time and examination of perithecial contents revealed typical ascospores of *R. necatrix* (Figs. 2b and 3a) measuring 35–44 x 5.4–7.2 µm which closely fitted those dimensions quoted by FRANCIS (1985) for the American material (i.e. 36–46 x 5.5–6.3 µm).

Ascospores were germinated on PDA or Malt Extract Agar (Fig. 3b) and, via the production of 2 germination tubes, developed as mycelial cultures. These cultures were then inoculated into living apple trees using the same technique as previously described and within 30 days the apple trees were dead.

Discussion

Using the technique described herein it has been possible to produce mature ascocarps of a root infecting *Rosellinia* species. Comparison of its teleomorphic features with those of the material of HANSEN & al. (1939) as described by FRANCIS (1985) proved them to be the same. The ascocarps formed during the current investigations were 1–2 mm diam., dark coppery brown in colour with a slightly flattened top and small but conspicuous black conical papilla. In a number of cases the papillae were observed to be surrounded by a slightly sunken annular area. The ascospore dimensions agree very closely with those given by FRANCIS (1985) and are also in the range of 30–50 x 5–8 µm listed by SIVANESAN & HOLLIDAY (1972) although their source was not cited. The straight germination slit at 10–15 µm is therefore always much shorter than the spore which, when fresh, has a hyaline epispore as was noted by HANSEN & al. (1937) for their material.

FRANCIS (1985) in discussing the relationship of *R. necatrix* to *R. bothrina* stated that “the similarity between the American specimens

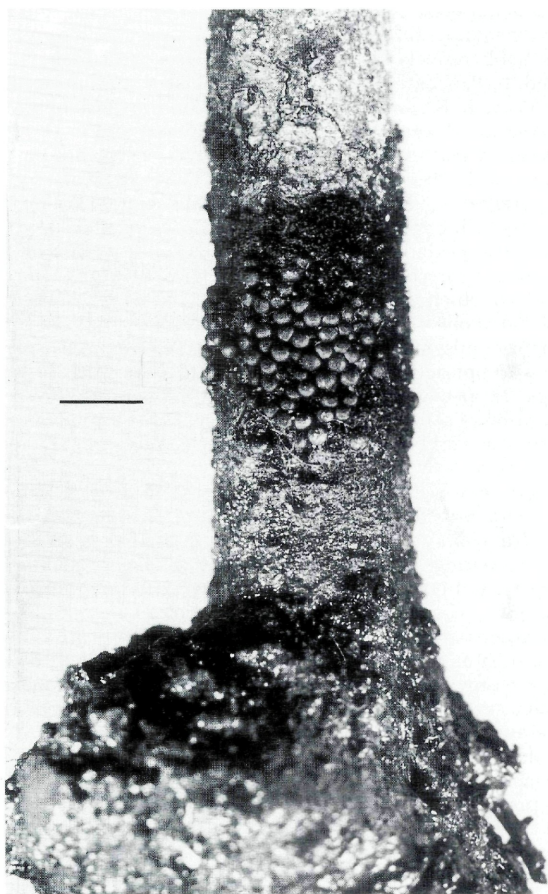


Fig. 1. – Stromata on collar of inoculated apple tree; bar 1 cm.

named as *R. necatrix* and the type of *R. bothrina* is striking and the only morphological difference that I can find is the presence of a sheath on the spores of *R. bothrina* and its absence from the spores of *R. necatrix* material". The occurrence of an epispore in the Portuguese material now suggests that this is not a reliable difference but more probably reflects the condition and age of the ascospores when examined. In *R. necatrix* there are pear-like swellings on the hyphae but according to SIVANESAN & HOLLIDAY (1972) these are absent from *R. bothrina* (as *R. arcuata*) and they considered this to be a useful distinguishing feature. PETCH (1923), however, in his account of *R. arcuata* on tea bushes stated that "With the aid of a microscope, it is always possible to identify a tea root disease caused by *Rosellinia* from the mycelium alone. In the species of *Rosellinia* parasitic on tea, the hyphae, especially the darker hyphae on the exterior of the strands, bear pear-shaped swellings. These occur at one end of the sections into which the hyphae are divided by the septa, and as the inflated end of one section joins on to the narrow end of the next, the structure reminds one of an old-fashioned condenser. As a rule these swellings do not occur before every septum". Whether or not these swellings are species specific for *R. necatrix* remains to be seen. On consideration of other features as well there is the strong possibility that *R. necatrix* and the *R. bothrina* as discussed by PETCH (1923) are really the same.

We are, however, reluctant to reduce *R. necatrix* to synonymy with *R. bothrina* for the time being. Since *R. necatrix* has been reported from many different countries, both temperate and tropical, and on a wide range of hosts we believe it would be prudent to induce ascocarp formation in a selection of these isolates from different geographical locations and from different hosts before new taxonomic alterations are proposed. Furthermore the application of other methods such as the examination of secondary metabolite profiles which have provided valuable taxonomic data in other Xylariaceous taxa (WHALLEY & EDWARDS, 1987) could aid taxonomic deliberations.

The apparent inability of *R. necatrix* to produce ascocarps in nature also deserves some comment since other species of *Rosellinia* usually can and in *R. desmazieresii* (BERK. & BR.) SACC. ascospores are readily produced on inoculated *Salix* branches (FRANCIS, 1985). We suggest that for *R. necatrix* the lack of ascocarps in nature results from a number of interactions, both physical and nutritional. The importance of water is demonstrated by the need to continue irrigation of dead trees (in the pots) and the requirement for water to induce fructification. Under field conditions in Portugal diseased trees are usually under dry conditions for much of the year. Furthermore there is evidence that the natural reserves of the host tissue should be exhausted or at least greatly depleted before ascocarps

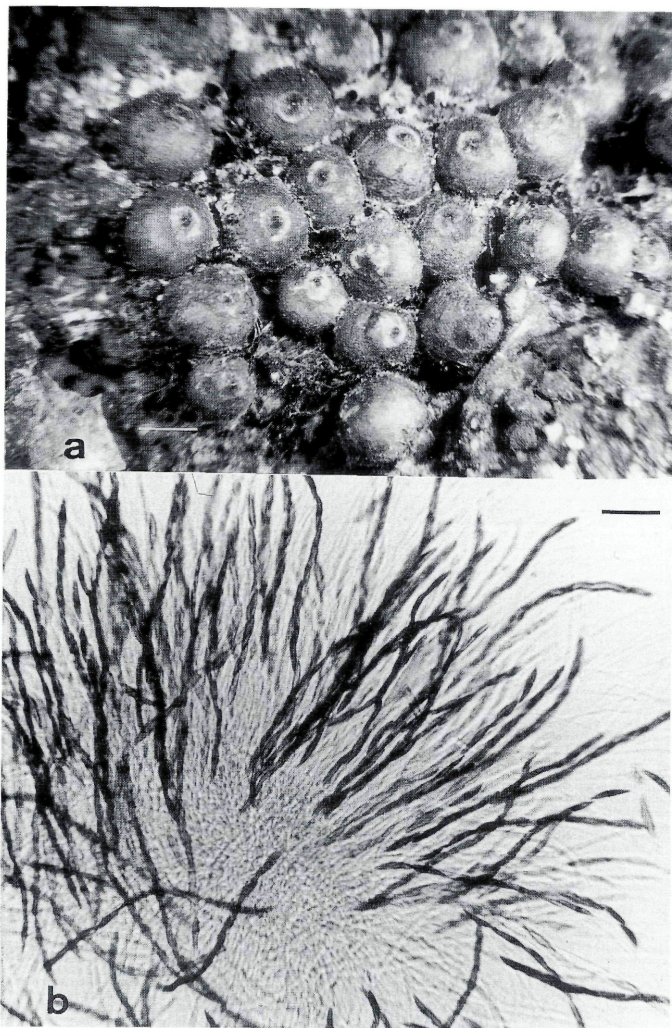


Fig. 2. - a. Stromata; bar 1 mm; - b. ascospores; bar 50 μ m.

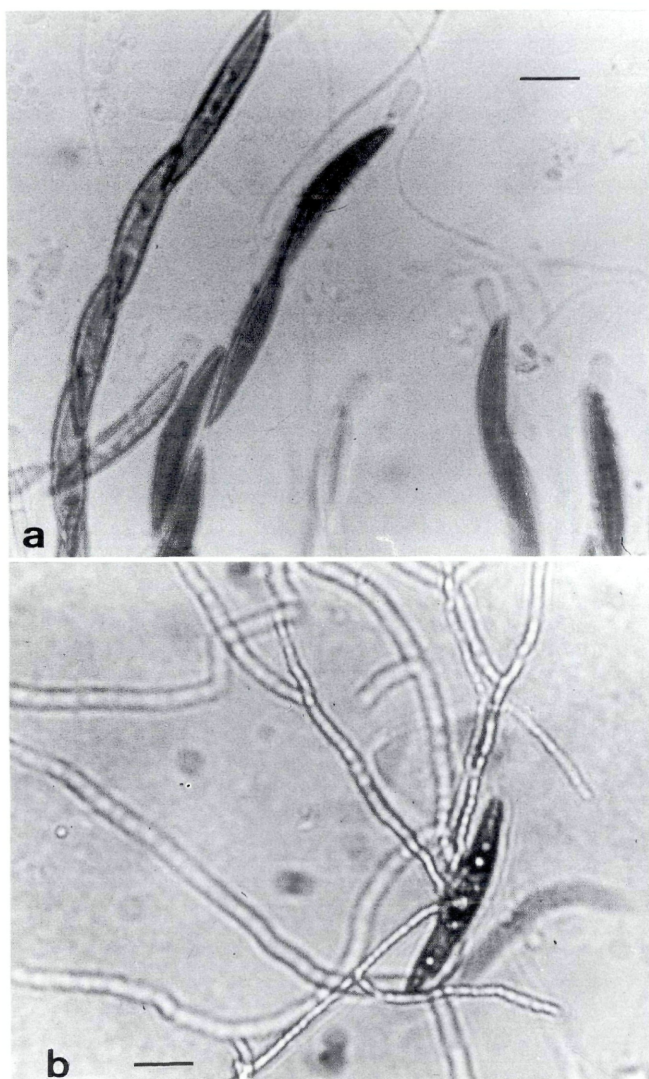


Fig. 3. - a. Ascospores; bar 10 μm . - b. germinating ascospores; bar 10 μm .

appear. It is interesting to note that PETCH (1923) also remarked on lack of perithecial material stating "Emphasis was laid on the fact that the fructification of *Rosellinia* was rarely found, and therefore its extension must be brought about by the spread of the mycelium. But *Rosellinia arcuata* has at least two kinds of fructification, a conidial stage and a perithecial stage; and while the perithecia are rare and scarcely ever found on the tea bush in the field, ...". He also recognized their long development period: "After the bush has been dead for a long time, the second form of fructification, the perithecium, appears" and "...ascospores are a negligible factor in dealing with the disease in tea, because the dead bushes are seldom left standing long enough to produce them". It is also now apparent that species of *Rosellinia* are not frequent in natural forests in the tropics as has recently been noted for Sulawesi (ROGERS & al., 1987) and Queensland, Australia (A.J.S. WHALLEY, unpublished). It is therefore tempting to postulate that *R. necatrix* is adapted for the conditions provided by cultivation and the practice of monoculture where, following normal procedure, diseased trees and bushes are removed long before perithecia have chance to develop.

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