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of *Pinus sylvestris* by *Peridermium pini* (Pers.)

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Growth of axenic cultures of *Cronartium flaccidum* on callus tissue from *Pinus nigra* var. *laricio* and *Pinus sylvestris*

By A. RAGAZZI, S. MORICCA and I. DELLAVALLE

Summary

The callus-fungal method was employed to test the response to *C. flaccidum* of the highly susceptible *P. nigra* var. *laricio* and the resistant *P. sylvestris*, and to ascertain whether results obtained with this method matched *in planta* observations. Calli were inoculated with axenic cultures of *C. flaccidum* obtained by incubating basidiospores on modified Schenk and Hildebrandt medium. Several parameters were evaluated. Colony growth was more rapid on *P. nigra* var. *laricio*. Colonies were dense on *P. nigra* var. *laricio*, but sparse on *P. sylvestris*. Aerial hyphae growth was abundant on *P. nigra* var. *laricio*, but less frequent on *P. sylvestris*. Hyphal branching began after 18 h on *P. nigra* var. *laricio* and after 45 h on *P. sylvestris*. Necrosis of the host cells set in after 24 h on *P. nigra* var. *laricio*, and after 70 h on *P. sylvestris*. The number of cells with plasmolysis was much larger in *P. nigra* var. *laricio* than in *P. sylvestris*. These results were consistent with the known resistance of the two species on whole plants.

1 Introduction

Cronartium flaccidum (Alb. et Schw.) Wint. is the causal agent of blister rust on two-needled pines which has devasted pine stands in Italy. The incidence of infection varies somewhat with the pine species but is always very high. Pines rank, as follows, in decreasing order of resistance: *Pinus pinea* L., *P. nigra* var. *laricio* Poir., *P. nigra* var. *austriaca* (Höss) Novak, *P. nigricans* var. *italica* Hochstaler, *P. pinaster* Ait., and *P. halepensis* Mill. (MORIONDO 1975).

In nature, *P. sylvestris* L. is almost always found in mixed stands with *P. austriaca*. Even when the latter species is heavily infected with blister rust, the infection is rare on adjacent *P. sylvestris*. This resistance of *P. sylvestris* has been confirmed in laboratory tests (RADDI and FAGNANI 1977; RAGAZZI and MORIONDO 1979; RAGAZZI and DELLAVALLE FEDI 1982).

With a variety of pathosystems, including some that are similar to *C. flaccidum* on pine, the callus-fungal method, in which callus tissue is inoculated *in vitro* with a pathogenic fungus, has shown to be a reliable indicator of the host-pathogen interaction and host resistance, as known or extrapolated from field data (INGRAM 1967; HARVEY and GRASHAM 1969; HELGESON et al. 1972; YAMAZAKI and KATSUYA 1987; PEI and PAWSEY 1990).

This study was undertaken to determine whether the callus-fungal method can be used with *C. flaccidum* on *P. nigra* var. *laricio* and *P. sylvestris* to investigate mechanisms of resistance and susceptibility. The results were compared with known results from experiments with whole plants.

2 Materials and methods

2.1 Callus cultures

Callus cultures of the two pine species were obtained from embryos that had been sterilized with 1 % sodium hypochlorite for 20 min and rinsed three times for 30 s each in sterile

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water. Embryo portions of 6 mm were incubated in darkness at 25 °C on an MS medium (MURASHIGE and SKOOG 1962) supplemented with 0.5 mg/l 2.4 D (dichlorophenoxyacetic acid), 2 mg/l kinetin, 30 g/l sucrose and 8 g/l Difco bacto-agar. The pH of the medium was adjusted in the same way as described below for the rust cultures. The calli produced were transferred to fresh MURASHIGE and SKOOG medium every 4 weeks for 5 months following the start of callogenesis.

2.2 Inoculum and inoculation

Basidiospores were obtained from telia excised from leaves of white swallow wort (*Vincetoxicum hirundinaria* Med.), the intermediate host of the rust, and where inoculated onto solidified medium with a sterilized hair-brush. Basidiospores were collected in three locations in Tuscany, Italy: Ospedaletto, in the province of Lucca (CFO); San Rossore, in the province of Pisa (CFP); and San Vincenzo, in the province of Livorno (CFL).

The axenic cultures were established by seeding basidiospores onto SCHENK and HILDEBRANDT nutrient medium (SCHENK and HILDEBRANDT 1972) supplemented with 0.5 mg/l 2.4 D, 2 mg/l kinetin, 30 g/l sucrose, 3 g/l Oxoid broth and 1 g/l malt-extract agar. Acidity was adjusted to pH 5.6–5.8 with 1N HCl and 1N NaOH before autoclaving at 1 atm for 15–20 min (MORICCA and RAGAZZI 1994). Cultures were incubated at 24 °C in Petri dishes sealed with Parafilm. Basidiospores began to germinate after 18–20 days and, after an additional 20 days, colonies began to develop where spore concentration was highest.

Colonies were cultured for 120 days, at which time mycelium plugs of 0.3 mm diameter were removed and placed on calli of 2 cm diameter in 9-cm diameter Petri dishes containing basic MS medium. For each pine species, 30 calli were seeded with mycelium – 10 calli per provenance. A control group of 10 calli per species was inoculated with plugs consisting of medium without fungus. Incubation was in a controlled environment, at 25 °C, in darkness.

2.3 Macroscopic and microscopic examination

Fungal cultures were inspected every 10 h and the following features noted on both the calli and the medium adjacent to the calli: colony growth, colony appearance, growth of the aerial hyphae (evaluated visually), appearance of hyphae, and length of hyphae evaluated by removing the hyphae from calli tissues under a Zeiss Lab 16 microscope (× 400; Zeiss, Oberkochen, Germany) after staining with lactophenol and cotton blue).

Calli also were evaluated for necrosis; and the number of callus cells with plasmolysis was counted on histological sections from callus samples that had been prepared by the following procedure: fixing in FAA, two ethyl alcohol baths, at 70 % and 95 %, for 30 min each, followed by: immersion for 2 h in a solution consisting of 12 ml infiltrating solution (50 ml historesin — 0.5 g activator) and 12 ml 90 % ethyl alcohol; and immersion in infiltrating solution for 4 h, and embedding in LKB historesin. Sections of 15 μm were cut using a Zeiss microtome, stained with toluidine blue at 60 °C for 1 min, and cells counted under the microscope.

3 Results

Colonies grew more rapidly on *P. nigra* var. *laricio* calli than *P. sylvestris* calli, irrespective of the provenance of the fungus. The appearance of colonies after 80 h was dense on *P.*

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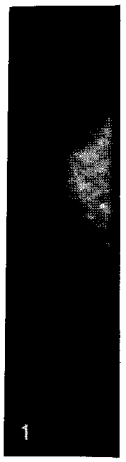

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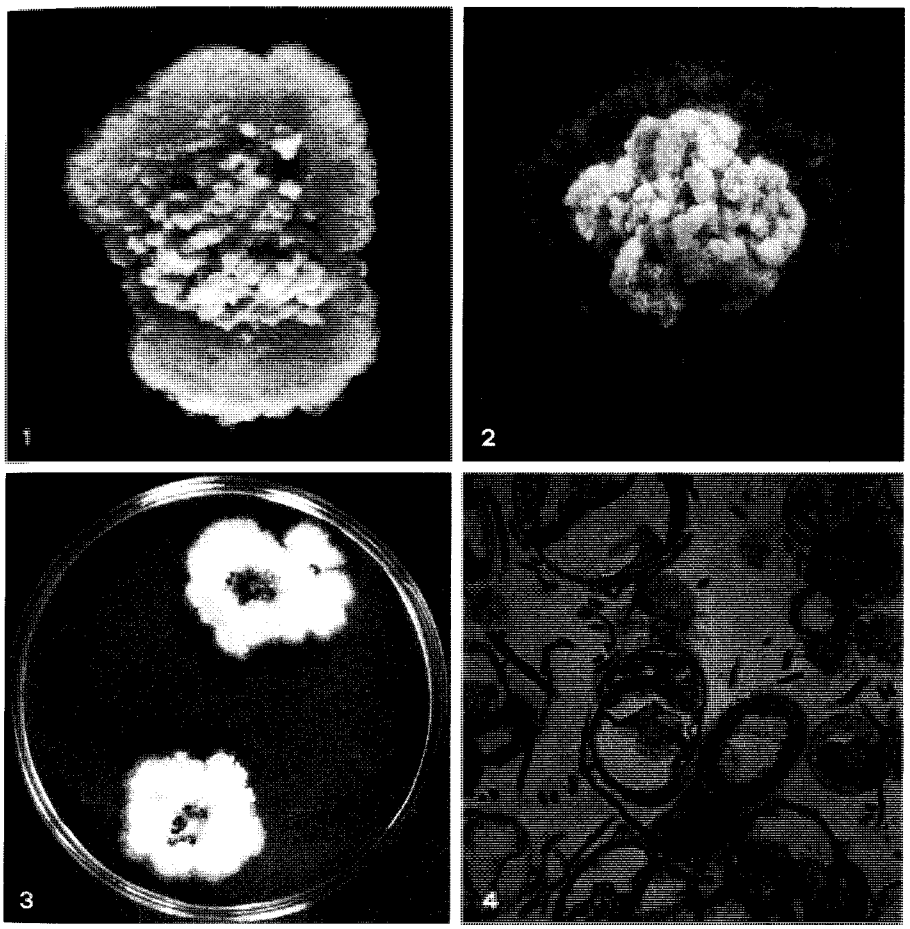
nigra var. *laricio* and sparse on *P. sylvestris* (Figs. 1, 2). Aerial hyphae were abundant on *P. nigra* var. *laricio*, less so on *P. sylvestris*.

Necrotic spotting appeared on *P. nigra* var. *laricio* calli after 24–36 h, but on *P. sylvestris* calli only after 70–75 h. Furthermore, the number of cells with plasmolysis after 80 h was much greater in *P. nigra* var. *laricio* (Table 1; Fig. 3). Analysis of variance revealed a significant difference ($p = 0.01$) between the two pine species, but not among the *C. flaccidum* provenances (Table 2).

The histological examination of *P. nigra* var. *laricio* calli after 80 h revealed, further, intercellular type of colonization which eventually separated the callus cells into groups containing different numbers of cells (Fig. 4). Intracellular haustoria were rare. Control calli exhibited many as-yet-undifferentiated cells, grouped together and fully turgid.

The hyphae branched after 18–24 h on *P. nigra* var. *laricio* (on which they were much longer), compared with 45–70 h for *P. sylvestris* (Fig. 5).



Figs. 1–4. 1. Densely growing colony of *Cronartium flaccidum* on *Pinus nigra* var. *laricio*; 2. Sparse colony of *Cronartium flaccidum* on *Pinus sylvestris*; 3. Necrotic spotting on *Pinus sylvestris* callus 70–75 h after inoculation with *Cronartium flaccidum*; 4. Intercellular type of colonization by *Cronartium flaccidum* on *Pinus nigra* var. *laricio* callus

Table 1. **Response of calli from *P. nigra* var. *laricio* and *P. sylvestris* to inoculation with *C. flaccidum***

Parameters	Pine species	
	P. nigra var. *laricio*	*P. sylvestris*
Necrosis	after 24–36 h	after 70–75 h
Cells with plasmolysis on infected callus tissue (200 cells examined 80 h after inoculation)	148	35
Cells with plasmolysis on callus tissue treated with water agar (control)	2	–

Table 2. **Analysis of variance on the number of cells with plasmolysis in calli of *P. nigra* var. *laricio* and *P. sylvestris* after inoculation with *C. flaccidum* from three provenances (ns not significant)**

Variation	df	Deviation	Variance	F
Total	5	16 003.41	–	
Among pine species	1	15 993.41	15 993.41	4649.24**
Among fungus provenances	2	3.11	1.55	0.45(ns)
Error	2	6.89	3.44	

** Significant at p = 0.01

4 Discussion

On *P. nigra* var. *laricio* calli, *C. flaccidum* colonies exhibited rapid growth, with a dense appearance, abundant hyphae, and hyphal branching starting as early as 18 h after inoculation. On calli of this species, cellular necrosis occurred after 24 h, with a high proportion of cells with plasmolysis (148 out of 200). On the *P. sylvestris* calli, fungal development and pathogenic effect were much more limited. The differences in fungal growth between *P. sylvestris* and *P. nigra* var. *laricio* calli persisted when the colonies grew out into the surrounding medium. No differences were noted among the three *C. flaccidum* provenances in any of the experiments. The fact that, on *P. sylvestris*, the hyphae tended to be twisted with limited branching, and that colonies were small, suggests that the resistance of this tree has a biochemical (fungistatic) basis: a biochemical resistance mechanism to rusts is known from other tree species (JACOBI 1982; PHILLIPS and WESTE 1991; BRONSON et al. 1992).

The behaviour *C. flaccidum* displayed on *P. sylvestris* was similar to that observed by PEI and PAWSEY (1990) on the *Peridermium pini* (Pers.) Lev. *P. sylvestris* complex, though axenic culture, in the latter case, was derived from aeciospores. They reported production of intercellular hyphae, haustoria and aerial mycelium, but only a part of the *P. sylvestris* callus was infected by the rust.

Various authors have used callus tissue to study the *Cronartium* spp./*Pinus* spp. pathosystems: YAMAZAKI and KATSUYA (1987) examined *Cronartium quercuum* Miyabe ex Shirai, and DINER et al. (1981) and HARVEY and GRASHAM (1969, 1970, 1974) examined *Cronartium ribicola* J.C. Fisch ex Rabenh. by this means. They reported the formation of numerous haustoria and also found that hyphae became twisted when they grew out into the medium surrounding the callus tissue. This is probably because the fungus then has to

d *P. sylvestris* to inoculation with *C. flaccidum*

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	P. nigra var. *laricio*	*P. sylvestris*
	after 24–36 h	after 70–75 h
	148	35
	2	–

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iation	Variance	F
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993.41	15 993.41	4649.24**
3.11	1.55	0.45(ns)
6.89	3.44	

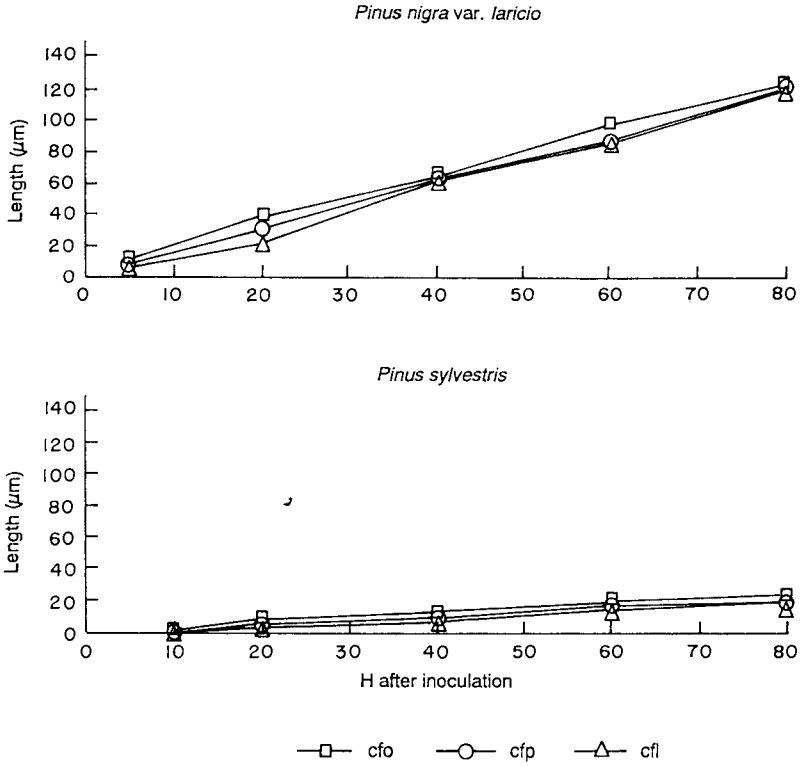

Fig. 5. Growth of *Cronartium flaccidum* on calli of *Pinus nigra* var. *laricio* and *P. sylvestris*. CFO: Ospedaletto (LU); CFP: San Rossore (PI); CFL: San Vincenzo (LI)

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hies exhibited rapid growth, with a dense hing starting as early as 18 h after inocu- ccurred after 24 h, with a high proportion *P. sylvestris* calli, fungal development and differences in fungal growth between *P.* ed when the colonies grew out into the among the three *C. flaccidum* provenances *ylvestris*, the hyphae tended to be twisted hall, suggests that the resistance of this tree al resistance mechanism to rusts is known nd WESTE 1991; BRONSON et al. 1992). *lvestris* was similar to that observed by PEI Pers.) Lev. *P. sylvestris* complex, though m aeciospores. They reported production celium, but only a part of the *P. sylvestris*

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WALKINSHAW et al. (1965), reported that *Cronartium fusiforme* Hedgc. et Hunt ex Cumm. also penetrated callus tissue intercellularly. DINER et al. (1984), examining axenic colonies of *C. ribicola* on callus tissue of *Pinus lambertiana* Dougl., found that hyphal growth was more abundant on susceptible individuals, and that hyphae grew between the cells, whereas the haustoria penetrated into the cells.

The histological examination revealed that the mycelium invaded the callus tissue intercellularly, but that, on *P. nigra* var. *laricio*, the cells nevertheless became damaged. This suggests that the fungus action was biochemical in nature and that host-pathogen recognition occurred at the cell-wall level, as has been found elsewhere (JACOBI 1982; BRONSON et al. 1992).

This study has shown that, for the *C. flaccidum* pine pathosystem, the result with the callus fungal method *in vitro* match the *in planta* situation, making this method an important tool for further research on *C. flaccidum*.

Acknowledgements

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Résumé

Croissance de cultures axéniques de Cronartium flaccidum sur des cals de Pinus nigra var. laricio et de P. sylvestris

La méthode cal-champignon a été utilisée pour tester la réponse à *C. flaccidum* de l'hôte très sensible *P. nigra* var. *laricio* et de l'hôte résistant *P. sylvestris,* et pour savoir si les résultats obtenus correspondaient à ceux observés sur plante entière. Les cals ont été inoculés avec des cultures axéniques de *C. flaccidum* obtenues après incubation de basidiospores sur le milieu modifié de Schenk et Hildebrandt. Plusieurs paramètres ont été évalués. La croissance de la colonie était plus rapide sur *Pinus nigra* var. *laricio*. Les colonies étaient denses sur celui-ci et lâches sur *P. sylvestris*. Les hyphes aériens étaient abondants sur *P. nigra* var. *laricio,* moins fréquents sur *P. sylvestris*. La ramification des hyphes commençait au bout de 18 h. sur *P. nigra* var. *laricio* et après 45 h. sur *P. sylvestris*. La nécrose des cellules de l'hôte avait lieu après 24 h. chez *P. nigra* var. *laricio* et après 70 h. chez *P. sylvestris*. Le nombre de cellules plasmolysées était beaucoup élevé chez *P. nigra* var. *laricio*. Ces résultats sont en cohérence avec la résistance connue de ces deux espèces.

Zusammenfassung

Wachstum von Reinkulturen von Cronartium flaccidum auf Callusgewebe von Pinus nigra var. laricio und P. sylvestris

In einem *in vitro* Callus-Pilz-System wurde die Reaktion von *C. flaccidum* auf Calluskulturen der anfälligen *P. nigra* var. *laricio* und der resistenten *P. sylvestris* untersucht. Die Ergebnisse wurden mit an intakten Pflanzen gemachten Beobachtungen verglichen. Die Calli wurden mit Reinkulturen von *C. flaccidum* inkubiert, die durch Inkubation von Basidiosporen auf einem modifizierten Schenk und Hildebrandt-Medium gewonnen worden waren. Auf *P. nigra* var. *laricio* wuchsen die Pilzkolonien rascher und dichter als auf *P. nigra,* und es entwickelte sich mehr Luftmycel. Die Hyphenverzweigung begann auf *P. nigra* var. *laricio* nach 18 h, auf *P. sylvestris* nach 45 h. Nekrotische Reaktionen der Wirtszellen wurden bei *P. nigra* var. *laricio* nach 24 h, bei *P. sylvestris* nach 70 h erkennbar, und erstere hatten deutlich mehr Zellen mit Plasmolyse. Die Ergebnisse stimmen mit dem bekannten Resistenzverhalten intakter Pflanzen dieser beiden Arten überein.

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