Axenic culture of the aecial state of *Cronartium flaccidum* from Italy

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Axenic cultures of *Cronartium flaccidum* were obtained for the first time by seeding aeciospores on modified Schenk and Hildebrandt's and Harvey and Grasham's media. Aeciospores came from foci at four locations in Italy. Colonies developed more readily on thickly seeded plates. High variation was observed in hyphal length and morphology, and in colony appearance, margin and morphology. The appearance and morphology of individual colonies also varied greatly over the five-month incubation period.

The macrocyclic rust fungus *Cronartium flaccidum* (Alb. & Schw.) Wint., the causal agent of blister rust of two-needled pines, occurs widely in Italy. Its spermogonia and aecia develop on Aleppo pine (*P. halepensis* Mill.), Austrian pine (*P. nigra* J. F. Arnold var. *austriaca* (Hoss.) Novak), Laricio pine (*P. nigra* var. *laricio* Poir.), Italian stone pine (*Pinus pinea* L.), maritime pine (*P. pinaster* Ait.) and more rarely on Scots pine (*P. sylvestris* L.) (primary hosts); and its uredia and telia on white swallow wort (*Vincetoxicum hirundinaria* Med.) as the intermediate host. Other plant species have been reported as intermediate hosts: *Ruellia formosa* Andr., *Gentiana* spp. and *Paeonia* spp., but these have never been found infected with *C. flaccidum* in Italy (Moriondo, 1975).

As part of a wider investigation into the biology and genetics of *C. flaccidum*, a series of trials was undertaken to culture this fungus *in vitro*. There have been recent advances in the axenic culture of other pine rusts: vegetative colonies growing separately from their hosts have been successfully established for *Cronartium fusiforme* (Hedgc. & Hunt) Cummins (Hollis, Schmidt & Kimbrough, 1972), *C. ribicola* (J. C. Fisch.) Rabenh. (Harvey & Grasham, 1974), *C. quercuum* (Berk.) Miyabe ex Shirai (Yamazaki & Katsuya, 1987), *Endocronartium harknessii* (Moore) Hirats. (syn. *Peridermium harknessii* Moore) (Allen, Blenis & Hiratsuka, 1988), and *Peridermium pini* (Pers.) Lév. (Pei & Pawsey, 1990). This paper reports the first successful attempt to obtain *C. flaccidum* in axenic culture. Our attempts to culture *C. flaccidum* axenically were prompted by the recent *in vitro* growth of the closely related mono-cyclic rust *Peridermium pini*. The closeness between the two organisms induced us to pay particular attention to media demonstrated to support growth of autoecious rust. Some characteristics of the cultures of *C. flaccidum* obtained are also described.

MATERIALS AND METHODS

Culturing experiments

Collection, storage and germination of aeciospores. Aeciospore samples were collected during May and June 1992 from systemically infected stems and branches of four pine species at four locations in Italy where there have been serious

Table 1. Media for the establishment of axenic culture of *C. flaccidum* (SH1–5: Schenk & Hildebrandt's medium, modified; HG1–5: Harvey & Grasham's medium, modified)

Substances added	SH1	SH2	SH3	SH4	SH5	HG1	HG2	HG3	HG4	HG5
Oxoid broth (g l^{-1})	3	3	3	3	4	4	4	4	4	4
Malt extract (g l^{-1})	1	1	1	1	1	—	—	—	—	—
Sucrose (g l^{-1})	30	40	—	30	30	30	30	30	—	—
Glucose (g l^{-1})	—	—	30	—	—	—	—	—	30	30
Kinetin (mg l^{-1})	2	2	2	—	2	—	2	—	—	2
2,4-D (mg l^{-1})	0·5	0·5	0·5	—	0·5	—	0·5	—	—	0·5
NAA (mg l^{-1})	—	—	—	2	—	—	—	2	2	—
BAP (mg l^{-1})	—	—	—	1	—	—	—	1	1	—

Table 2. Source of *Cronartium flaccidum* aeciospores and their germination rate

Area	Location	No. of lesions sampled (ruptured/unruptured)	Germination (%)
Murge	Bosco di Mercadante	8/7	86
S. G. Matese	Serra delle Giumente	9/5	84
Garfagnana	Monte Vosgi	9/7	81
Monticiano	Monte Quoio	7/6	79

rust outbreaks in the past: from *P. halepensis* at the Forest of Mercadante (Murge, province of Bari), from *P. nigra* var. *laricio* at Serra delle Giumente (San Gregorio Matese, province of Caserta), from *P. pinaster* at Monte Quoio (Monticiano, province of Siena), and from *P. nigra* var. *austriaca* at Monte Vosgi (Garfagnana, province of Lucca).

To have aeciospores in sufficient quantity it was necessary to take spores also from ruptured aecium-bearing lesions, accepting that this would increase the incidence of contaminant spores into the sample (Pei & Gibbs, 1991). Spores were aspirated with a cyclone collector, which then deposited them in gelatine capsules. The capsules were immediately wrapped in aluminium foil and stored in sealed photographic film tubes to protect them from the light, which reduces spore viability.

Fresh spore samples had a moisture content varying from 30 to 70% depending upon environmental conditions at the time of collection. Within 24 h after the spores were collected from the aecial pustules, they were placed in a desiccator containing silica-gel, which was evacuated to 2--2·5 mmHg. The aeciospores, which initially were clumped together because of the humidity, were kept in the desiccator at 5 °C for *ca* 1 wk, until they became powdery and their humidity was reduced to 5--10%. The humidity was measured by placing a hygrometer inside the desiccator. The spores were then wrapped in aluminium foil and stored under vacuum at 5°.

Prior to use in the tests, requisite amounts of aeciospores were rehydrated for 24 h at ambient temperature in a germinator with a water-saturated atmosphere.. Spore viability was tested on potato-dextrose-agar (PDA) and water-agar.

Culture media. A total of 10 different growth media were tested for production of axenic rust cultures. These were based on the medium of Schenk & Hildebrandt (1972) and that of Harvey & Grasham (1974), as modified by Pei & Pawsey (1990) and Pei & Gibbs (1992) respectively, and further modified for the present tests.

The composition of the basic modified Schenk & Hildebrandt medium was as follows (l^{-1} of double distilled water): $NH_4H_2PO_4$, 300 mg; H_3BO_3, 5 mg; $CaCl_2$, 151 mg; $CoCl_2.6H_2O$, 0·1 mg; $CuSO_4.5H_2O$, 0·2 mg; $Na_2.EDTA.2H_2O$, 20 mg; $FeSO_4.7H_2O$, 15 mg; $MgSO_4$, 195·4 mg; $MnSO_4.H_2O$, 10 mg; KI, 1 mg; KNO_3, 2500 mg; $Na_2MoO_4.2H_2O$, 0·1 mg; $ZnSO_4.7H_2O$, 1 mg; Difco Bacto-agar, 8 g.

The composition of the basic modified Harvey & Grasham medium (per litre of double-distilled water) was as follows: $CaNO_3.4H_2O$, 500 mg; $MgSO_4.7H_2O$, 281, 73 mg; $(NH_4)_2SO_4$, 25 mg; $Fe_2(SO_4)_3.7H_2$ 250 mg; KH_2PO_4, 140 mg; $MnSO_4.3H_2O$, 4, 14 mg; Difco Bacto-agar, 8 g.

The composition of these two media was varied by replacing some components (growth regulators, sucrose *v.* glucose) or by increasing the concentration of an existing ingredient (sucrose, Oxoid broth), to produce a total of 10 different growth media, as shown in Table 1.

The pH of all media was adjusted to 5·7–5·8 with 1 N HCl and 1 N NaOH before autoclaving at 121° for 20 min.

Each spore sample was inoculated onto the solidified media using a sterilized hair brush or a small sterile spatula.

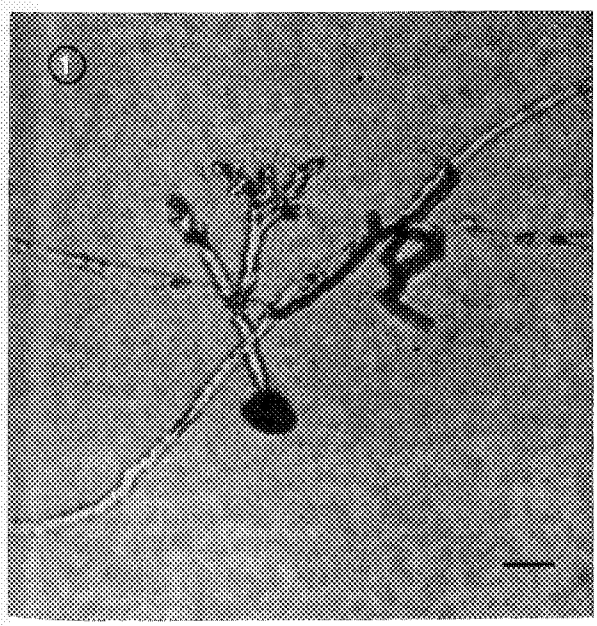


Fig. 1. First mycelium ramifications developing from a short germ-tube produced by a germinated *Cronartium flaccidum* aeciospore on SH2 medium 10 d after inoculation (bar, 20 μm).

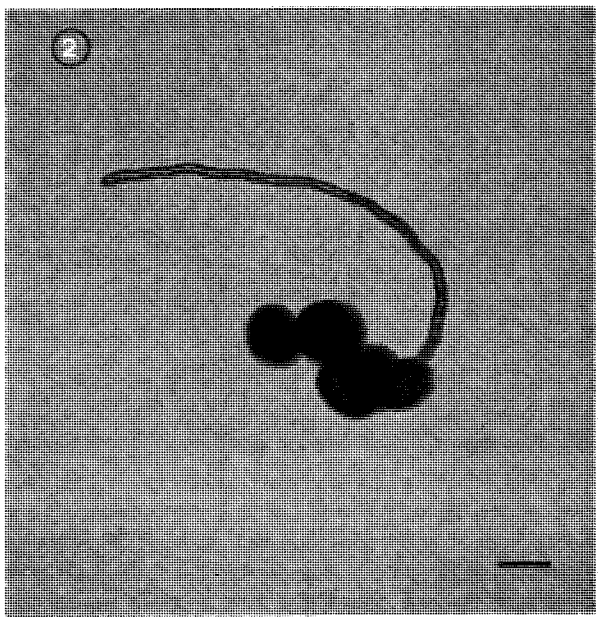
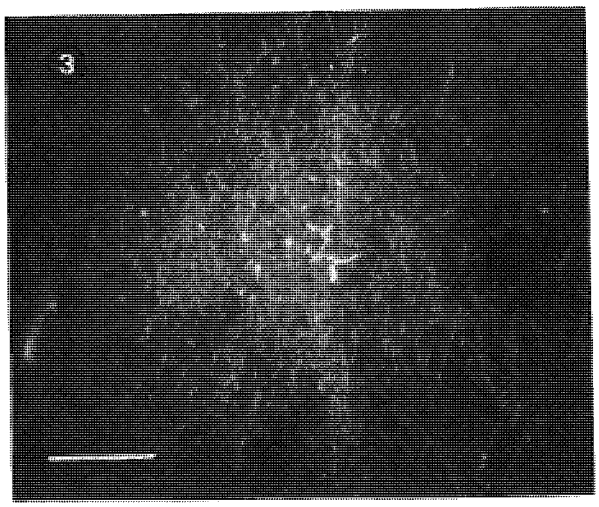
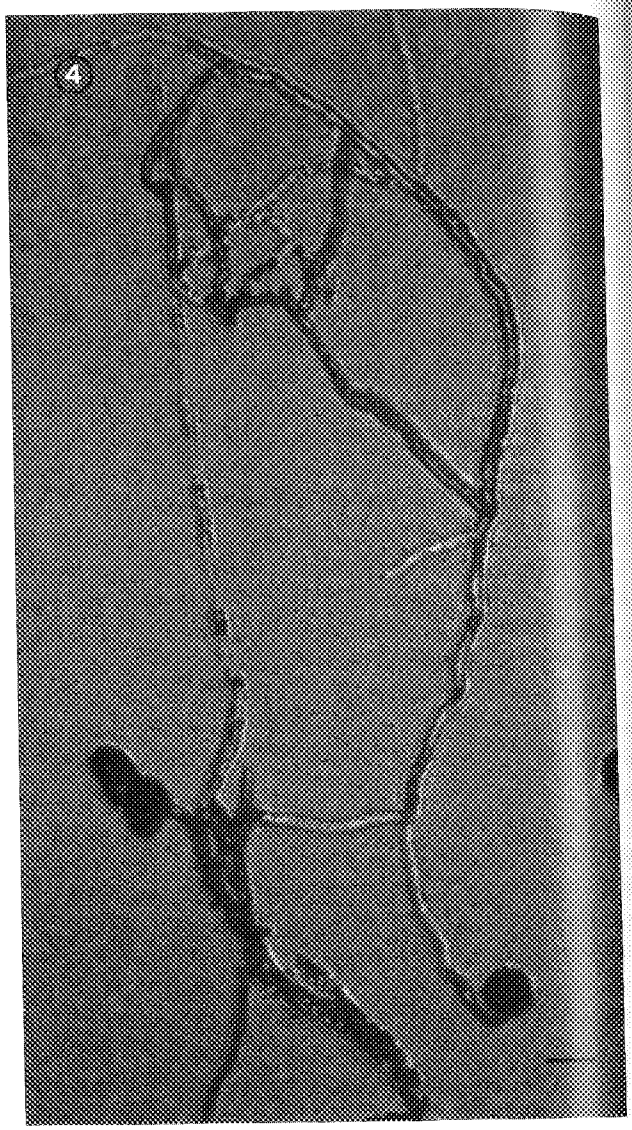
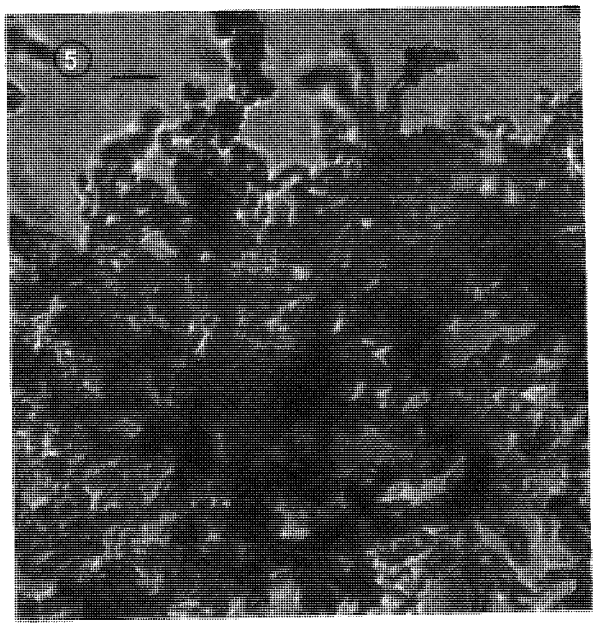
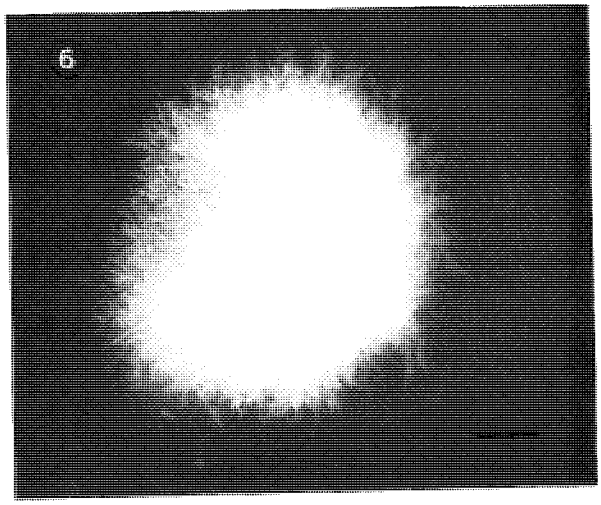


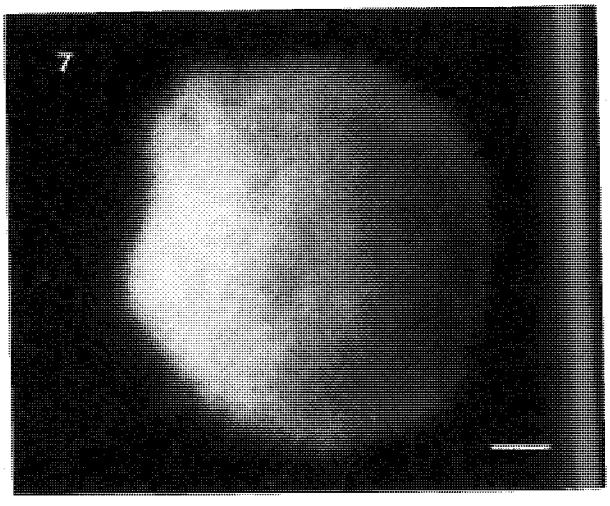
Fig. 2. Spore with germ-tube 173 μm in length after 48 h of incubation on distilled water agar (bar, 20 μm).











Figs 3–7. Fig. 3. Colony visible to the naked eye on HG1 medium 3 wk after inoculation (bar, 1 mm). **Fig. 4.** Long, straight and scarcely branched hyphae rapidly developing from sporelings 5 d after spore seeding on HG1 medium (bar, 20 μm). **Fig. 5.** Twisted hyphae, the most frequent type in culture (bar, 20 μm). **Fig. 6.** A colony with a fluffy and aerial surface texture (bar, 1 mm). **Fig. 7.** A colony with short thin aerial hyphae densely covering the surface of culture (bar, 1 mm).

Table 3. Growth rates of *C. flaccidum* in mm on media SH1–5 and HG1–5 at 24° in the dark over a 5-month period (mean of 5 colonies for each of the 4 spore sources on each of the 10 media)

	1 month				2 months				3 months				4 months				5 months			
	MD	SG	MQ	MV	MD	SG	MQ	MV	MD	SG	MQ	MV	MD	SG	MQ	MV	MD	SG	MQ	MV
SH1	6	4	3	4	11	10	8	8	16	15	11	12	25	24	19	18	38	36	29	28
SH2	6	3	3	3	10	9	7	8	16	11	11	10	29	28	17	17	36	35	27	25
SH3	3	2	2	2	6	5	4	4	11	8	8	9	19	16	12	14	23	20	16	18
SH4	6	4	2	3	10	10	8	7	17	14	10	12	25	15	18	17	37	36	28	27
SH5	5	5	2	3	10	9	7	7	15	14	9	11	25	22	17	16	37	34	27	26
HG1	5	4	3	3	11	9	6	7	17	13	10	11	27	27	17	16	40	39	26	27
HG2	5	4	2	2	10	8	5	7	17	12	9	10	26	27	16	17	39	37	24	27
HG3	4	4	2	2	9	7	5	5	15	12	8	9	26	26	15	17	37	36	25	27
HG4	3	3	2	1	6	5	4	4	10	9	7	6	14	14	10	9	24	22	15	13
HG5	2	3	2	2	5	5	5	4	9	9	7	7	15	14	12	11	23	23	16	16

All Petri dishes (6 cm diam.) were sealed with 'Parafilm M®' (American National) and incubated at 23–25°.

During the first 15 d the cultures were examined daily with a stereomicroscope and any contaminant micro-organisms – easy to detect because their growth rates are much higher than that of the rust itself – removed with a sterile scalpel. Up to 40% of the dishes became contaminated, and of these about half had to be discarded despite repeated removal of contaminant material. Because of the high number of dishes in the sample (1000 dishes, 100 per medium), this did not affect the outcome of the experiment.

RESULTS AND DISCUSSION

Twenty-four h after inoculation, spores from all four locations had germinated well (Table 2). High variation was observed in the length and morphology of germ-tubes (Figs 1, 2), consistent with the observations of Gibbs, England & Wolstenholme (1988) for *Peridermium pini*.

When assessing rust growth, it is vital to determine the precise point at which the spore stops relying on endogenous reserves and is able to meet its nutritional needs entirely from the growth medium (i.e. the point at which it becomes saprotrophic (Maclean, 1982)). To determine this, sporeling development was scored in accordance with the developmental growth scale of Kuhl *et al.* (1971), which uses hyphal growth to define five developmental growth stages. The third stage on this scale, 'extensive secondary and some tertiary branching', certainly represents saprotrophic growth. This stage was reached after about 2 wk. The first appearance of colonies under the microscope was asynchronous, as was their later development. Twenty days after inoculation, vegetative colonies became visible to the naked eye (Fig. 3).

Preliminary trials revealed that where colony growth occurred, the largest colonies always developed in localized areas of high spore density. A high seeding density was therefore essential for good colony formation. These findings are consistent with Hartley & Williams (1971*a*, *b*), Kuhl *et al.* (1971), Green (1976) and Maclean (1982) for specialized forms of *Puccinia*; and with Pei & Pawsey (1990) for *P. pini*. For *C. flaccidum*, the optimal seeding rate was found to be 400–1200 aeciospores mm^{-2}, which yielded 90% colony production with Mercadante (MD) and Serra delle Giumente (SG), and 50% with Monte Quoio (MQ) and Monte Vosgi (MV).

Saprotrophic growth of *C. flaccidum* was observed in all 10 media, although with wide variations among media (Table 3). The SH1 and HG1 media gave the best results. Increasing the carbon source (sucrose) by 10 g (in SH2) did not improve colony growth, and the use of an alternative carbon source (glucose) in SH3 reduced growth somewhat. Adding 1 g of 'Lab-Lemco' broth [a mix of peptone (Oxoid L 37) and 'Lab Lemco' Powder (Oxoid L 29) in the ratio of 5 to 3] to increase organic nitrogen (SH5) also failed to improve growth.

Good growth was also achieved with the SH4 medium, in which the growth-regulating substances BAP (6-benzyl-aminopurine) and NAA (1-naphthaleneacetic acid) replaced K (kinetin) and 2·4 D (2,4-dichlorophenoxyacetic acid) used in the other SH modifications. Clearly these growth-regulating substances are not highly specific. The excellent growth rates observed on HG1, which contained no growth-regulating substances at all, indicates that these substances were not necessary for *C. flaccidum* growth: when either pair of growth regulators was added to this medium (HG2, HG3), growth was not increased. Colony production was lower in HG4 and HG5, in which glucose was used instead of sucrose, with either K/2,4-D or NAA/BAP as growth regulators.

SH1 and HG1, which produced the best growth of *C. flaccidum*, are very similar to the media that produced optimal growth of *Peridermium pini* (Pei & Pawsey, 1990; Pei & Gibbs, 1992). Another point of resemblance between *P. pini* (as described by Pei & Gibbs, 1991) and *C. flaccidum* was the wide cultural variations among the various strains. These variations related to hyphal length and morphology (Figs 4, 5) and to colony margins and morphology (Figs 6, 7).

A clearer understanding of cultural variation and nutritional requirements may help to shed light on the phylogenetic closeness of *P. pini* and *C. flaccidum*.

The establishment of *C. flaccidum* in axenic culture can be expected to have a number of interesting consequences: it will make possible rapid axenic *in vitro* assays to test for hypersensitivity in *Pinus* spp. to different strains of *C. flaccidum*; it will help evaluate the effects of metabolites from susceptible and resistant host tissues on *in vitro* fungal growth and, vice versa, study the production of phytotoxic metabolites

by the rust. Such information will clarify some aspects of the biology of the pathogen and of the factors responsible for pine resistance and susceptibility.

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