

Efficient Transmission of an Introduced Pathogen Via an Ancient Insect-Fungus Association

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Abstract In *Cupressus sempervirens* the association between seed insects and tree pathogens has resulted in optimal exploitation of the cones. A fungus-infected cone can be inhabited by the nymphs of a true seed bug (*Orsillus maculatus*), the adults of which may carry a heavy spore load at emergence. Cones are infected when eggs are laid within the cone, most frequently via the emergence holes of a seed wasp (*Megastigmus wachtli*). This symbiotic association evolved with the nonaggressive fungus *Pestalotiopsis funerea* within the natural range of the cypress. When the aggressive cypress canker disease (*Seiridium cardinale*) was introduced into Europe, it was transmitted by *O. maculatus* to cones usually colonized by *Pestalotiopsis funerea*, with disastrous consequences for the regeneration and survival of *C. sempervirens* in the entire Mediterranean area.

Insects feeding on conifer seed cones represent a specialized guild among phytophagous insects (Turgeon et al. 1994). Fungi pathogenic to the cones are often less specialized (Manion 1991) because they can develop on other parts of the tree as well as on the cones (Sutherland et al. 1987). The role of insects as vectors of infectious spores of plant pathogens is widely known (Webber and Gibbs 1989; Martin 1992; Roy 1993), but only few studies concern cone and seed insects (Hoover et al. 1996).

How the agent of the cypress canker disease [*Seiridium cardinale* (Wagener)] is transmitted has long been debated, without an adequate conclusion being reached (Wagener 1928; Graniti 1998). Rain seems to be an important factor in the distribution of spores (conidia) within tree crowns or between ad-

joining trees, but the long-distance dispersal is not well understood. Conidia are unlikely to be carried by the wind, because they are embedded in a sticky substance (Wagener 1928; Panconesi and Ongaro 1982). Insects are often assumed to be important carriers of the fungus, and evidence for this has been obtained for bark beetle (Covassi et al. 1975) and moth (Frankie and Parmeter 1972) transmission. Birds are also possible vectors of the fungus (Wagener 1939). However, the rapid spread of this disease after its first recording in California, as well as where it was introduced in Europe and Africa (Barthelet and Vinot 1944; Grasso 1951), is still unexplained. It is noteworthy that the fungus readily produces infectious spores in laboratory cultures when seeds are added to the substrate (Intini and Panconesi 1974), and that in nature the fruiting bodies are more abundant on cones (Fig. 1a) than on the bark of infected trees (Intini and Panconesi 1974). However, cone and seed insects have been overlooked as possible vectors. In addition, there is little knowledge of the insect species involved, and extensive surveys have been performed only recently (Roques 1983; Guido et al. 1995).

The finding that the ligid bug *Orsillus maculatus* (Fieber) feeds on cypress seeds and inhabits the cone throughout its development is also recent (Battisti et al. 1997). The strict relationship between the seed bug and the cypress was supported by the perfect overlap of their geographic ranges, extended from the native area of the cypress (southeastern Europe and Middle East) to the wide area of introduction (southwestern Europe and northern Africa; Battisti et al. 1997). The cypress seed bug may feed

on the seeds from outside the cone, penetrating through the cone scales with the mouthparts (adult; Fig. 1c), or from inside (nymph). Feeding can be detected either by the presence of the salivary sheath (Miles 1972; Fig. 1d) or by radiography of the seeds (Fig. 1e). However, the ovipositor cannot penetrate the cone scales, and therefore an incidental opening must be available for egg-laying to be possible. Such openings are provided when scales shrink and separate after colonization of the cone by a fungus [*Seiridium cardinale* and *Pestalotiopsis funerea* (Desm.) Stey.] or a caterpillar (*Pseudococcyx tessulatana* (Staudinger), or an emergence hole of the adult of the seed wasp *Megastigmus wachtlei* Seitner (Roques and Raimbault 1986; Fig. 1b).

In an experiment performed in a cypress stand in northern Italy three cone-bearing branches on each of 25 trees were monitored from flower initiation to cone maturation. Insects were excluded from one branch per tree with a sleeve, allowing light, water, and air to pass through. In the unprotected branches total insect and fungus damage amounted to 13.81% and 32.04%, respectively, of the initial cone number ($n=373$). By contrast, no insect or fungus damage was observed in the branches from which insects were excluded (initial cones $n=169$). Thus the fungus attack of the cones was related to insect feeding, and the seed bug appeared to be a major agent as traces of its feeding or oviposition were found in nearly all the cones killed by the fungi. The frequency of oviposition between shrunken scales (0.53) was not significantly different from that observed in *Megastigmus* holes (0.67; likelihood ratio $G=0.401$, 1 df, $P=0.526$, with a sample size greater for the second type openings, i.e., 102 cones bearing emergence holes vs. 30 cones with shrunken scales). Similar or even higher rates of exploitation of cones infested by *Megastigmus* have been observed at other sites in France, Greece, and Italy (unpublished). During nymphal development of the seed bug all cones eventually become partially or completely colonized by one or both fungi, which produce abundant fruiting bodies on seeds and scales. Both nymphs and adults of the seed bug then become covered with infectious spores (Fig. 1f).

A survey was conducted in natural (Crete) and artificial (Crete, southern France, central Italy) stands of cypress to evaluate the spore load of seed bugs. Two of the artificial stands (France, Fréjus; Italy, Roselle) were made of clones recently selected for resistance to the canker fungus, whereas in each region at least one artificial stand severely infested with the canker fungus was considered. Fungal spores were detected in 67.4% of the insects examined ($n=271$), with a distinct pattern in the propor-

tion of each fungus among the stands of each region. The endemic, nonaggressive *Pestalotiopsis* (Guba 1961) was found with a similar frequency on insects collected both in natural and artificial stands, whereas the introduced and aggressive *Seiridium* was scarcely detected in natural stands or in stands selected for resistance to this fungus (Fig. 2). The estimated spore load varied consistently, reaching its heaviest in the stands severely infested with *Seiridium*. The spore load of *Pestalotiopsis* showed a lower variation and did not differ significantly among stands (Table 1).

The possibility that the adults of *O. maculatus* may actually transmit the fungi to cypress cones was verified in a field experiment using insects artificially contaminated with conidia of *S. cardinale* and *P. funerea* (Table 2). Fungus transmission rarely occurred when insects fed only on the cones, but it was frequent when eggs were laid in the cones. Transmission was consistent with the fungus species that was applied to the insects. The five cases of fungus

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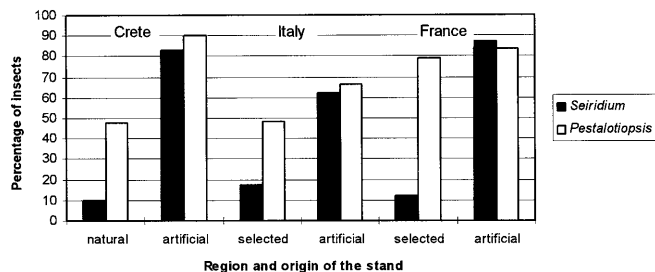


Fig. 2. Percentage of seed bugs having at least the labeled fungus in various type of stands and regions. Legend and sample size are given in Table 1. The proportion of insects carrying the fungus did not vary significantly either between stages (adults vs. nymphs) or between sexes (2×2 contingency table, G test with Yates correction for continuity applied to original data). The frequency of insects carrying fungi did not vary significantly among natural+selected stands for both *Seiridium* ($\chi^2_{(2)}=0.687$, $P>0.05$) and *Pestalotiopsis* ($\chi^2_{(2)}=3.129$, $P>0.05$); the same was observed among artificial stands (*Seiridium*: $\chi^2_{(2)}=3.097$, $P>0.05$; *Pestalotiopsis*: $\chi^2_{(2)}=2.343$, $P>0.05$). The frequency of insects carrying *Seiridium* spores was significantly higher in artificial than in natural+selected stands ($\chi^2_{(1)}=26.62$, $P<0.05$), but this was not the case for *Pestalotiopsis* ($\chi^2_{(1)}=2.800$, $P>0.05$). Probability value ($\alpha=0.05$) was adjusted for the number of simultaneous tests ($k=6$) according to the sequential Bonferroni technique (Rice 1989)

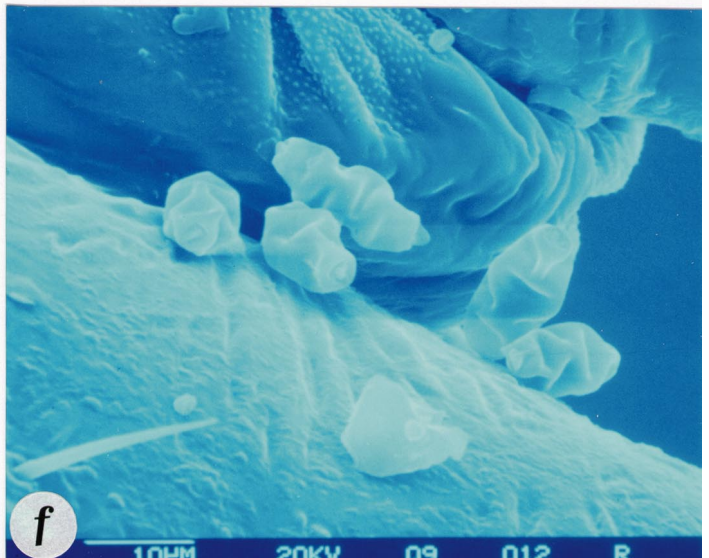
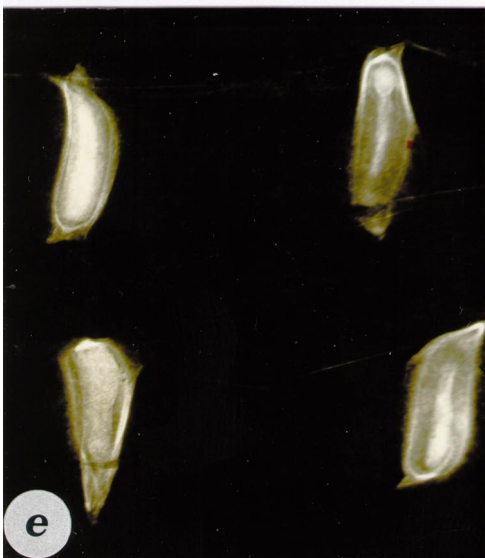
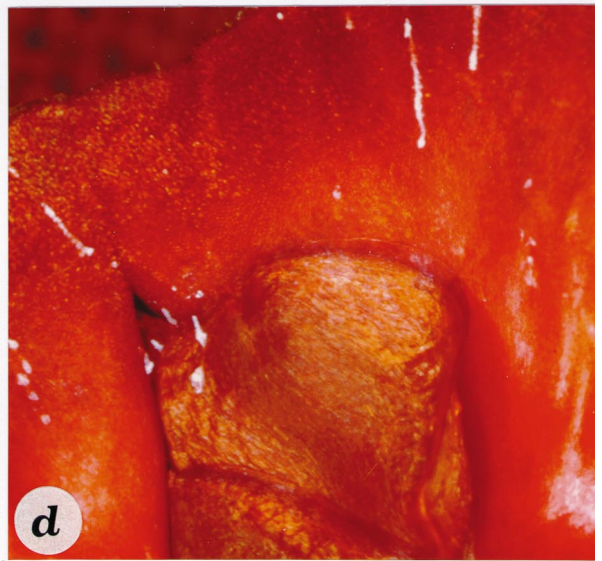


Table 1. Average load of conidia of *S. cardinale* and *Pestalotia funerea* on adults and nymphs of *O. maculatus* collected in different stands and regions

Region, site	Type of stand	<i>n</i>	Conidia of <i>Seiridium</i> /ins.	Conidia of <i>Pestalotiopsis</i> /ins.
Crete, Chania	Artificial	30	125.01 ± 27.13 ^a	275.12 ± 100.62 ^a
Crete, Lefka ori	Natural	40	7.50 ± 3.82 ^b	43.75 ± 15.93 ^a
S France, Montpellier	Artificial	51	311.94 ± 63.78 ^a	169.68 ± 27.48 ^a
S France, Fréjus	Selected	34	5.03 ± 2.76 ^b	58.33 ± 14.03 ^a
C Italy, Antella	Artificial	83	355.49 ± 67.85 ^a	135.93 ± 19.57 ^a
C Italy, Roselle	Selected	39	7.93 ± 3.31 ^b	144.48 ± 70.05 ^a

Different letters within a fungus denote significant differences between means by the Tukey's test for unequal *n*, performed on Log (*x* + 1) transformed data to homogenize variance. The probability value ($\alpha=0.05$) was adjusted for the number of simultaneous tests (*k* = 6) according to the sequential Bonferroni technique (Rice 1989). Each living insect had been washed in 400 μ l sterile water with 1% deter-

gent (Tween80), shaking for 1 min at 40 Hz. The number of conidia in the suspension was estimated with a Thoma cell, with ten replicates for each insect. The volume of suspension examined in each replicate was 1 μ l. The method removes about 90% of the conidia from an insect's body, while keeping insects alive and available for rearing

Table 2. Effect of insect feeding and egg-laying on transmission of fungi (*S. cardinale* and *P. funerea*) to the cypress cones

Insects loaded with	<i>n</i>	Cages with insect feeding only		Cages with insect feeding and egg laying			
		<i>n</i>	<i>Seiridium</i> on cones	<i>Pestalotiopsis</i> on cones	<i>n</i>	<i>Seiridium</i> on cones	<i>Pestalotiopsis</i> on cones
<i>Pestalotiopsis</i>	15	5	0	2	10	3	9
<i>Seiridium</i>	14	8	1	0	6	5	1
Control	14	2	0	0	12	2	3
Likelihood ratio <i>G</i>			0.26	4.66		24.71	29.56
<i>P</i>			>0.05	>0.05		<0.05	<0.05

The frequency of each fungus at each treatment was tested against the null hypothesis (H_0 : equal number of cages with infested cones at each treatment). The probability value ($\alpha=0.05$) was adjusted for the number of simultaneous tests (*k* = 4) according to the sequential Bonferroni technique (Rice 1989). Laboratory-reared adults, each carrying fewer than 100 conidia of both fungi, were artificially loaded with one of the fungi by immersion and shaking for 30 s in a suspension of conidia (2×10^6 conidia/ml). Conidia were obtained from infested cones collected in April 1996 in a cypress stand in Barbarano, northern Italy. The germination rate after 48 h incubation at 20° C was 91% for *S. cardinale* and 95% for *P. funerea*. The final spore load of the adults was $1.451 \pm 0.387 \times 10^3$ conidia/insect (*n* = 5). Fifteen trees with-

out symptoms of canker disease were selected in the same stand and all the three treatments (pairs of adults loaded with *Seiridium*, *Pestalotiopsis*, and control) were applied as randomized complete block design. Adults were caged at the beginning of May within a sleeve (30 × 50 cm) containing the branch apex with at least two cones entering the third year. The sleeve excluded other insects while allowing light, water and air to pass through. In November 1996 the branches were removed and the sleeve searched for the presence of insects. Two replicates each of the *Seiridium* treatment and control disappeared during the summer. When no eggs or living insects were found, only feeding on the cones was presumed to have occurred. In all cases the cones were examined for the detection of the fungi

transmission (two for *S. cardinale*, three for *P. funerea*) obtained with untreated adults may be explained by the presence of residual conidia on the washed insects or with an infection occurring in the field from spores penetrating the rearing cage. Conidia may either be introduced with the ovipositor or deposited near the hole during egg laying, because the female remains the entire time with the lower abdomen in contact with the cone surface. Thus the probability of transmission should be related to the load of conidia at the time of egg laying.

The relationship between the seed bug *O. maculatus* and the fungi pathogenic to the cypress depends ultimately upon the availability of holes suitable for

egg laying in cones entering their third year. Other cone and seed insects, especially the seed chalcid *M. wachtlii*, produce such holes. In all likelihood the relationship evolved over a long period of coexistence between insects and a nonaggressive fungus such as *P. funerea*. However, a change in the pathogenicity of the fungus or the introduction of another more aggressive species with similar niche requirements may lead to dramatic consequences for the survival of the tree and the perpetuation of the whole system. *O. maculatus* would seem to be acting as a long range, hardly detectable, but swift and efficient vector of *S. cardinale*, making attacked cones an important source of inoculum for the tree.

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- Barthelet J, Vinot M (1944) Notes sur les maladies des cultures méridionales. *Ann Epiphyties* 10:11–23
- Battisti A, Colombari F, Frigimelica G, Guido M (1997) Life history of *Orsillus maculatus*, a true bug damaging seeds of *Cupressus sempervirens*. In: Battisti A, Turgeon JJ (eds) Proceedings of the 5th IUFRO WP Cone and Seed Insects, University of Padua, Italy, pp 215–220
- Covassi M, Intini M, Panconesi A (1975) Preliminary notes on the relationship between *Coryneum cardinale* Wag. and *Phloeosinus aubei* Perr. in Tuscany. *Redia* 56:159–166
- Frankie GW, Parmeter JR (1972) A preliminary study of the relationship between *Coryneum cardinale* (Fungi Imperfecti) and *Laspeyresia cupressana* (Lepidoptera: Tortricidae). *Plant Dis Reporter* 56:992–994
- Graniti A (1998) Cypress canker: a pandemic in progress. *Ann Rev Phytopathol* 36:91–114
- Grasso V (1951) A new pathogen of *Cupressus macrocarpa* Hartw. in Italy. *Italia Forestale Montana* 6:62–65
- Guba EF (1961) Monograph of *Monochaetia* and *Pestalotia*. Harvard University Press, Cambridge
- Guido M, Battisti A, Roques A (1995) A contribution to the study of cone and seed pests of the evergreen cypress (*Cupressus sempervirens* L.) in Italy. *Redia* 78:211–227
- Hoover K, Wood DL, Storer AJ, Fox JW, Bros WE (1996) Transmission of the pitch canker fungus, *Fusarium subglutinans* f. sp. *pini*, to Monterey pine, *Pinus radiata*, by cone- and twig-infesting beetles. *Can Entomologist* 128:981–994
- Intini M, Panconesi A (1974) Studies on the in vitro fructification of *Coryneum cardinale* Wag. *Riv Patol Vegetale* 10:337–342
- Manion PD (1991) Tree disease concepts. Prentice-Hall, Englewood Cliffs
- Martin MM (1992) The evolution of insect-fungus associations: from contact to stable symbiosis. *Am Zoologist* 32:593–605
- Miles PW (1972) The saliva of Hemiptera. *Adv Insect Physiol* 9:183–255
- Panconesi A, Ongaro L (1982) *Seiridium (Coryneum) cardinale* (Wag.) Sutton & Gibson in cypress stands of Monte Morello (Firenze). *Riv Patol Vegetale* 18:109–121
- Rice WR (1989) Analyzing tables of statistical tests. *Evolution* 43:223–225
- Roques A (1983) Les insectes ravageurs des cônes et graines de conifères en France. INRA, Paris
- Roques A, Raimbault JP (1986) Distribution and biological cycle of the Cypress seed chalcid, *Megastigmus wachtli* (Seitn.) (Hymenoptera, Torymidae), in the Mediterranean. *J Appl Entomol* 101:370–381
- Roy BA (1993) Floral mimicry by a plant pathogen. *Nature* 362:56–58
- Sutherland JR, Miller T, Quinard RS (1987) Cone and seed diseases of North American conifers. North America Forestry Commission, Victoria
- Turgeon JJ, Roques A, De Groot P (1994) Insect fauna of coniferous seed cones: diversity, host plants interaction, and management. *Ann Rev Entomol* 39:179–212
- Wagener WW (1928) *Coryneum* canker of Cypress. *Science* 67:584
- Wagener WW (1939) The canker of *Cupressus* induced by *Coryneum cardinale* n. sp. *J Agricultural Res* 58:1–46
- Webber JF, Gibbs JN (1989) Insect dissemination of fungal pathogens of trees. In: Wilding N, Collins NM, Hammond PM, Webber JF (eds) Insect-fungus interactions. Academic, London, pp 161–189