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Occurrence and distribution of *Armillaria gallica* genets in a declining oak stand of southern Italy

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Summary. Outbreaks of *Armillaria* root rot in conifer plantations and declining oak stands are frequently due to the spread of the fungus in the soil over long periods. This paper examines the occurrence and distribution of *Armillaria* genets in a declining mixed oak stand of southern Italy. Samples of rhizomorphs, mycelial mats and fruit bodies of *Armillaria* were collected from the soil, stumps, and living and dead trees. A total of 111 *Armillaria* isolates were collected, all belonging to the species *A. gallica*. They were grouped in 28 genets by somatic incompatibility. The largest genet covered an area of about 2.6 ha with a linear extent of 300 m. On the basis of an estimated 0.5 m annual growth in the soil, its age was assumed to be about 3 centuries. The results confirm the ability of *A. gallica* to remain alive and stable in a large area over a long time.

Key words: somatic incompatibility, population dynamics, epidemiology.

Introduction

Basidiomycetes of the genus *Armillaria* include serious root-rot pathogens of mainly woody plants, with a worldwide distribution in both natural and planted forests (Shaw and Kile, 1991). In natural forests, *Armillaria* species generally behave as wood-rotting saprophytes which can develop on dead stumps without necessarily involving a pathogenic stage (Kile *et al*., 1991). These fungi can become pathogenic in natural stands weakened by diverse causes; in fact, they are indicated as coresponsible for oak decline both in Europe and in North America (Bruhn *et al*., 2000; Ragazzi *et al*., 2000; McLaughlin, 2001; Thomas *et al*., 2002). Of the six species occurring in Italy (Guillaumin *et al*., 1993), *A. gallica* Marxm. & Romagn. is frequently encountered as a weak pathogen of oak trees both in Italy and other countries (Guillaumin *et al.,* 1985; Wargo, 1993; Luisi *et al.,* 1996).

A number of investigations into the population dynamics of *Armillaria* species in forest sites have been carried out by studying genotype distribution. Different methods of study have been employed: somatic (or vegetative) incompatibility (SI) (Korhonen, 1978; Kile, 1983), comparison of mating-type alleles (Korhonen, 1978; Kile, 1983), isozyme markers (Rizzo and Harrington, 1993), mitochondrial and nuclear DNA analysis by means of both restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) (Smith *et al.,* 1990; 1992). More recently, a comparison of those different methods (Guillaumin *et*

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al., 1996) indicated that somatic incompatibility was a reliable tool for epidemiological studies on *Armillaria*.

In this study, SI was used to assess genets of *Armillaria* in terms of the number, size and shape of the areas covered, and to estimate their development over time in a declining oak stand of southern Italy, for a better understanding of the spreading potential of *Armillaria*.

Materials and methods

Study site

The study was carried out in the "Difesa Grande" oak stand, in the district of Gravina in Puglia, the largest forest site in the province of Bari, southern Italy. This stand is considered to be a spontaneous residual mesophyte forest, and today has an area of about 1,800 ha, mostly consisting of *Quercus cerris* L., *Q. frainetto* Ten. and *Q. pubescens* Willd. The stand was previously managed as a coppice with 15–16-year rotations in order to produce firewood, railway sleepers and acorns for animals. Silvicultural practices stopped at least 40 years ago, hence the stand, now about 45–50 years old, is almost unmanaged. Consequently it has undergone a gradual deterioration of its vegetative conditions, and is one of the forests affected with the oak decline syndrome (Sicoli *et al.*, 1998). Studies carried out in this stand indicated that a multiplicity of factors concur to induce plant decline (Luisi *et al.*, 1993; Manicone *et al.*, 1993; Sicoli *et al.*, 1998). Moreover, a frequent association of fungal species, in particular *Armillaria* spp., with declining oak trees was observed (Luisi *et al.*, 1996; Sicoli *et al.*, 1998).

Sampling

Armillaria genets in the stand were mapped on the basis of a systematic sampling. The area to be investigated, about 6.25 ha, was divided according to a grid pattern into twenty-five square plots 50 m to a side. In the autumn of 1997 rhizomorphs and mycelial mats of *Armillaria* were collected near each corner of these plots. Any basidiomes of the fungus detected in the entire plot area were also sampled. A rough map was initially drawn up showing all the places where samples had been found. When the squares required further investigation, more detailed sampling was undertaken (Kile, 1986; Legrand *et al.,* 1996), dividing plots into 10-m sided square sub-plots (100 m^2) and more samples were collected from each corner of these sub-plots. In a few sub-plots systematic sampling of rhizomorphs was impossible due to disturbance of the soil or the dominance of a thick shrubby vegetation.

Isolation and culture conditions

A selective medium for basidiomycetes was used for isolations from infected wood or rhizomorphs (Kuhlman and Hendrix, 1962). Rhizomorphs were sterilised for 2–3 minutes in bleach, then rinsed in sterile water, cut into pieces, split lengthwise, and the two halves put in Petri dishes. Fragments of inner tissues of the pilei or the stipes of the basidiomes were transferred to water-agar or to the selective medium. All *Armillaria* isolates were lastly cultured on 2% malt extract agar (MEA) in Petri dishes at 23±1°C in the dark.

Identification of somatic incompatibility groups (SIGs)

Clones were differentiated on the basis of SI, i.e. by means of diploid-diploid matings. Pairings were performed by placing 3-mm-diameter plugs of each isolate 3–5 mm apart in Petri dishes containing 3% MEA. The plugs consisted of undifferentiated mycelium (i.e. without crusts and rhizomorphs) cut from the edge of actively growing colonies. Each Petri dish contained two different pairings (Smith *et al.,* 1994; Falk and Parbery, 1995) and each pairing was replicated at least once. Pairing was scored after incubation for 12, 20 and 30 days at 23±1°C in the dark. Isolates growing side by side without either producing a pigmented zone line or being clearly separated from the other were considered to belong to the same genet or somatic incompatibility group (SIG).

In order to reduce the number of the pairings necessary for assessing the number of SIGs [a complete set of mycelial interactions would have required n(n-1)/2 different tests], each isolate was paired with its three nearest neighbours according to the rough map, then one isolate from each resulting homogeneous group was tested against one isolate from the nearest neighbouring group. After the SIG of each isolate was determined, the distribution of different SIGs was marked on the map.

Species identification

Isolates representing each SIG were specifically identified by means of compatibility tests, pairing every haploid or diploid isolate with known haploid tester strains of the Italian *Armillaria* species (Korhonen, 1978). Pairings were performed by placing 3-mm-diameter plugs of each isolate 1–2 mm apart in Petri dishes containing 2% MEA. Plugs were taken from the edge of actively growing colonies and consisted of undifferentiated mycelium. Four–six weeks of incubation at 23±1°C in the dark was sufficient for observation of the mating reaction, i.e. the change in colony morphology. In fact, haploid colonies are generally white and fluffy, but when fusion of compatible mating types occurs, the coalesced colonies become dark brown, appressed and crustose. If the isolates are from different species, the colonies will not grow together and will maintain their morphology (Anderson and Ullrich, 1982).

Results

The sampling yielded 111 *Armillaria* isolates: 36 from rhizomorphs, 20 from wood and 55 from basidiomes. Ten out of 25 plots required to be divided into sub-plots.

With few exceptions, the reaction between paired diploid isolates was either clearly compatible or clearly incompatible, so that only a few pairings had to be replicated.

The SI tests discriminated 28 genets of *Armillaria*, of which 9 were represented by one isolate, and 8 by two (Table 1). All these genets belonged to *A. gallica* according to the interfertility matings.

Table 1. Main parameters of the 28 genets of *A. gallica* detected in the 6.25 ha test site in the Difesa Grande oak wood, Gravina in Puglia, southern Italy.

Genet	Number of isolates	Area (m^2)	Maximum extension (m)
1	34	26,169	300.0
2	5	4,266	88.3
3	5	2,482	70.0
4	9	1,886	70.0
5	7	1,505	79.1
6	4	1,141	50.0
7	5	744	39.2
8	7	641	48.3
9	2	410	30.0
10	2	340	28.3
11	2	282	23.3
12	3	254	24.2
13	2	240	22.5
14	2	230	23.3
15	2	212	21.6
16	4	204	26.6
17	2	196	18.3
18	2	189	23.3
19	3	132	15.8
20	1	95	14.3
21	1	95	14.3
22	1	95	14.3
23	1	95	14.3
24	1	95	14.3
25	1	95	14.3
26	1	95	14.3
27	1	95	14.3
28	1	95	14.3

The area and maximum extension of each genet are also given in Table 1 and their shape is drawn on the map in Fig. 1. On this map the lines group isolates of identical SIGs without rigorously delimiting the area in which the mycelium of each genet occurred.

The largest genet covered an area of 26,169 m^2 with a maximum extension of 300 m. The smallest genet had an area of 95 m^2 and a maximum extension of 14.3 m. The total area covered with *A. gallica* genets was about 42,378 m^2, 67.8% of the investigated area. The average area of all genets was 1,513.5 m^2 and the average maximum extension 40.4 m.

Discussion

The study found that *A. gallica* spread, as a single species, over a relatively large area in a South Italian oak forest in a state of decline, and that the test site was colonised by 28 genets of this fungus. In this study somatic incompatibility worked well as a surveying tool; the choice of method was validated by a number of experiments which found, with few exceptions, that genets identified in this way coincided in area with genets demarcated by nucleic acid analysis (Kile, 1983; Guillaumin and Berthelay, 1990; Smith *et al*., 1990; 1994; Guillaumin *et al*., 1996), even if molecular technique was more rapid.

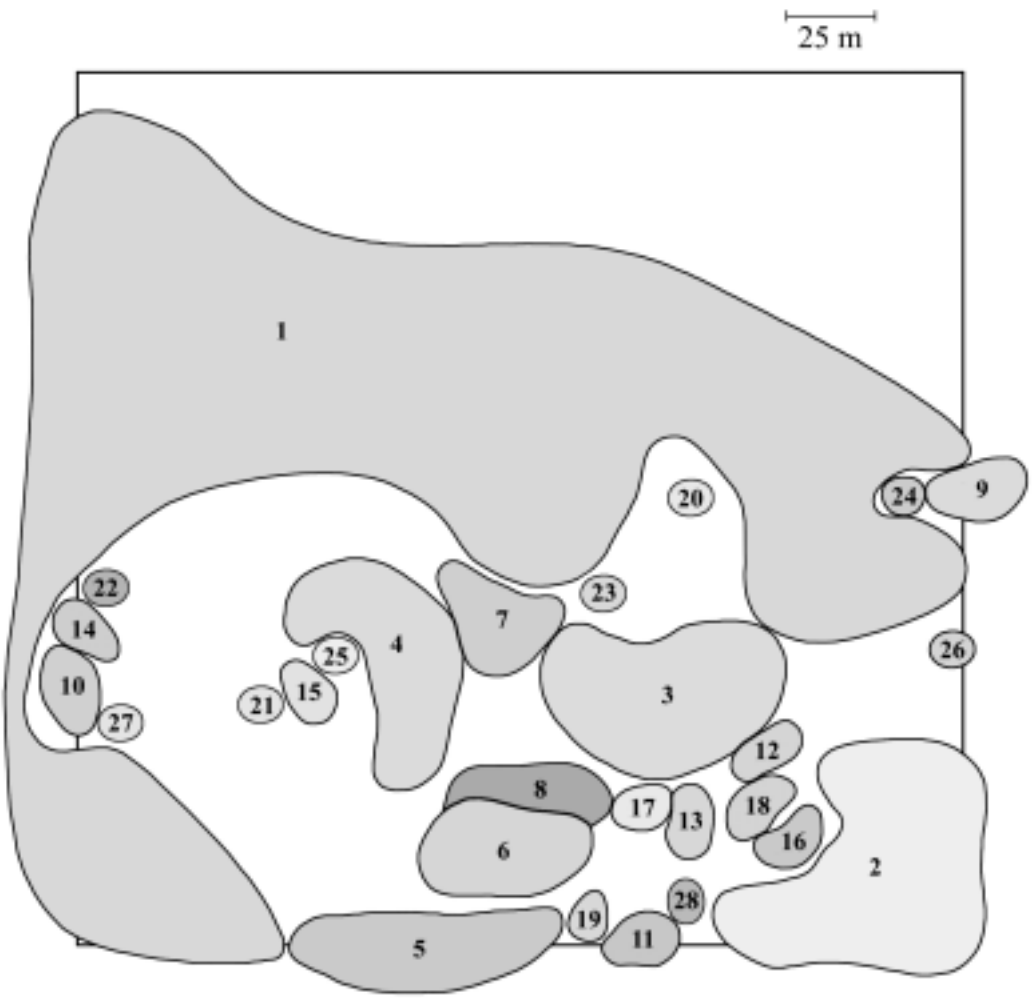

Fig. 1. Distribution of the 28 genets of *A. gallica* detected in the 6.25 ha test site at the Difesa Grande oak wood, Gravina in Puglia, southern Italy (lines do not precisely demarcate the spatial limits of mycelium of each genet).

The 50-m distance between samples here adopted for studying the epidemiology of this rhizomorph-producing species was already used for assessing the size and distribution of *A. gallica* infection foci in England (Rishbeth, 1991). However, such a long distance is not likely to give completely certain results because small genets may escape detection, and it is in any case difficult to determine what a reasonable minimal distance between samples is (Legrand *et al.*, 1996). In this work, samples of mycelial fans, rhizomorphs and basidiomes were even collected at 10-m intervals. Moreover, in order to detect new and small genets, sampling from wood or basidiomes is preferable to that from rhizomorphs since the latter often belong to the same isolate over long distances.

If it is assumed that at the altitude of the sampled area the annual growth of *A. gallica* is up to 0.5 m (Smith *et al.*, 1992; Legrand *et al.*, 1996), the largest genet detected would be about 3 centuries old and its size as large as that of the genets reported by Legrand *et al*. (1996). Moreover, since genet 1 may well have extended beyond the area sampled, it may by even older.

As regards the size and topographical shape of the various genets of *A. gallica* in the test area (Fig. 1), there was one large continuous genet partially encircling a number of smaller genets that were mostly represented by only one or two isolates. Since inbred sibling clones are relatively rare in *Armillaria* (Korhonen, 1978) and mutation is not a significant source of new genotypes (Kile, 1983), the occurrence of 27 small genets, differing from each others by their SI, could mean they were new air-borne originated individuals that had lately begun colonising stumps and declining trees. On the other hand, they can also represent remnants of a larger, older clone, as reported by Kile (1983). Within an old genet, the oldest central parts probably disappear first because of the decomposition of the substrate. These are then replaced by different genets colonising new stumps. Conversely, the most recent, peripheral parts remain alive longest (Guillaumin *et al*., 1994; Legrand *et al*., 1996).

The data reported so far clearly show that in declining oak stands *A. gallica* spreads in the soil through rhizomorphs and has the capacity to spread and remained genetically stable for long periods (Rayner and Todd, 1982; Rayner, 1991; Smith *et al*., 1992). *A. gallica* has for centuries colonised wood debris without causing major damage to the trees, living side by side with oaks as long as site conditions were presumably more favourable to them, confirming that in this oak wood this fungus plays only a secondary role in any outbreaks of oak decline (Sicoli *et al*., 1998).

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