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Parasitic fungi on *Dryocosmus kuriphilus* in *Castanea sativa* necrotic galls

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Abstract

The spread of Asian chestnut gall wasp (ACGW) *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera Cynipidae) is an emergent constraint factor for Italian chestnut stands and orchards, considering its potential to reduce plants vigour and chestnut production. Biological control with the introduced parasitoid *Torymus sinensis* Kamijo (Hymenoptera Torymidae) is being performed in Italy, but search for autochthonous antagonists appears as an urgent topic to improve strategies for the protection of chestnut stands. This study reports the first finding and the parasitic action of fungi detected in necrotic wasp galls in some stands of *Castanea sativa* Mill. in Tuscany and Piedmont regions: *Fusarium incarnatum-equiseti* species complex (FIESC) 25-b and 3-b, *Alternaria alternata* (Fr.) Keissl., *Botrytis* sp. were identified. After fungi were isolated, artificial inoculations were carried out in field: six selected strains were tested on 10 healthy galls each (two strains of FIESC 25-b, one of FIESC 3-b, two of *Botrytis* spp., and one of *A. alternata*). Dead adults of wasp were found inside 47% of the 60 treated galls, and the highest lethal effect was produced by the *Fusarium* strains (60-70%) and by one strain of *Botrytis* spp. (60%). Moreover mycelial linear growth in plate of the six strains was tested on gall extract agar medium (GEA), and compared to that on PDAmb and water-agar: on GEA the significantly ($p < 0.001$) highest linear growth was recorded for three strains after 4 days and four strains after 8 days. Isolation of *Fusarium* mycelia from bodies of dead adults, inside galls both naturally and artificially necrotized, emphasises their infective ability. In conclusion the two *Fusarium* species show interesting perspectives and verifiable opportunities for ACGW biocontrol.

Key words: *Dryocosmus kuriphilus*, *Fusarium incarnatum-equiseti* species complex, *Castanea sativa*, biocontrol.

Introduction

The invasion of the Asian chestnut gall wasp (ACGW) *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera Cynipidae) is affecting many chestnut ecosystems in the world (Bosio *et al.*, 2010). Since its first report in Piedmont region of Italy (Brussino *et al.*, 2002) it represents a new threat for chestnut cultivation, which had already been affected in the past by the impact of pathologies such as blight and ink disease. In Tuscany region the first report of this parasite dates back to 2008 (Graziosi and Santi, 2008) and its rapid spread is currently taking place across the region.

The knowledge of gall wasp biology and ecology is an essential requirement for the development of suitable strategies for the protection of chestnut stands: the complex interactions among the new insect, its host, the abiotic factors and its natural enemies need to be investigated. Biological control is the main management action currently adopted in Italy: the introduction of the parasitoid *Torymus sinensis* Kamijo (Hymenoptera Torymidae) is being undertaken and indigenous insects are studied as parasitoids (Speranza *et al.*, 2009).

However also fungi may act as natural parasites of gall wasp. The presence of *Gnomoniopsis* sp. associated with ACGW gall necrosis and gall wasp mortality was reported in the area of Monti Cimini in Central Italy (Magro *et al.*, 2010). Necrotic galls containing dead insects and invaded by unidentified fungi were observed in the United States (Cooper and Rieske, 2007; 2010).

This study aims to investigate the necrosis production of fungi, found in some Italian stands of *Castanea sativa* Mill., both in natural and artificial infections of ACGW galls, to evaluate their action against the parasite insects, and to carry out their isolation and identification.

Materials and methods

Collection of gall samples

Surveys were carried out in chestnut stands near Vaie and Condove (province of Turin, Piedmont) and near Quarrata and San Mommè (province of Pistoia, Tuscany) in June 2010. Necrotic galls, covered by white or greyish mycelia, were frequently observed on groups of 3-10 chestnut trees. In each of the four study sites 3 trees were selected and 10 gall samples per tree were collected. Most galls (60%) contained dead ACGW individuals.

Potato dextrose agar with biotin (1 mg/l) and methionine (100 mg/l) (PDAmb) was used for isolations in Petri dishes from infected gall tissues and from bodies of some mycelium-enveloped dead cynipids (figure 1). Isolations were incubated for 7-8 days (25 °C) in the dark. Six strains were selected in relation to their ability to sporulate on the surface of galls and were subcultured on PDAmb. Single-spores cultures were obtained by plating dilution series on water-agar and transferring germinated conidia on PDAmb after 24 hours of incubation.

Identification of fungal strains

The six strains were identified on the basis of morphological characters, according to Leslie and Summerell (2006) for *Fusarium* spp., Ellis (1971) for *Botrytis* sp., Simmons (1967) for *Alternaria alternata* (Fr.) Keissl. Molecular investigations for *Fusarium* spp. were then performed at the Department of Agroenvironmental Sciences and Technologies (University of Bologna), where colonies were previously grown on carnation leaf agar (CLA) to produce homogeneous macroconidia. After DNA extraction using CTAB method, the transla-

Figure 1. White mycelium of FIESC 3-b enveloping dead individuals of *D. kuriphilus* inside their gall chambers.

Figure 2. A cynipid gall colonized by mycelium of FIESC 25-b. Also FIESC 3-b produced identical necrosis.

tion elongation factor 1-α (TEF) gene region was amplified with primers EF1−EF2 (O'Donnell *et al*., 1998), cloned in *Escherichia coli* using plasmid vector pGEM-T Easy, and sequenced by Eurofins MWG Operon Company. Sequences were finally compared with those collected in the Fusarium-ID v.1.0 database (http://fusarium.cbio.psu.edu) (Geiser *et al*., 2004). Identity of *A. alternata* was confirmed at Plant Biology Department (University of Turin) through culturing on malt extract agar (MEA) to improve sporulation, sequencing of ITS1−ITS2 regions of ribosomal DNA, and comparison of sequences in the NCBI database (http://www.ncbi.nlm.nih.gov).

Artificial inoculation tests

Artificial inoculations were performed in a chestnut experimental plot in Montesenario near Florence (Tuscany, Italy) at the beginning of July 2010. On two different trees, 5 galls were inoculated with each of the six fungal strains. A total of 60 galls was treated. This number of galls was limited by the necessity to select galls the more homogeneous as possible for dimensions and still containing individuals of wasp, therefore only galls without emergence holes were chosen. Mycelial plugs taken from 6-days-old PDAmb cultures were placed in microlesions on galls made with a sterilized scalpel, protected with masking tape. Controls were ten galls (five for each tree) microlesioned and not inoculated. After 7 days both treated galls and controls were examined and collected. Reisolations from artificially inoculated galls on PDAmb plates were incubated in the dark (25 °C) for 7 days.

Growth of fungal strains on different media

In laboratory tests the strains were cultured on gall extract agar (GEA) to compare their growth on PDAmb and water-agar (WA). Gall extract was prepared by grinding and homogenizing 60 g of green galls in 250 ml of distilled water; a pre-filtration was made with sterile gauze and filter paper. This mixture was then filtered for sterilisation using 0.45 μ pore-sized cellulose filters, measured for pH value (4.5) and added in aseptic conditions to a water agar medium (10 g agar/250 ml distilled water) which had been previously sterilised in autoclave (120 °C, 1 Atm, for 20 minutes).

The six strains were cultured on the three different media (three replications each) placing a fragment of mycelium (0.5 cm) in the centre of each plate. A total of 54 Petri dishes was prepared and incubated at 25 °C in the dark. Two periodic controls (after 4 and 8 days) were carried out to verify the growth of the colonies: two linear measurements per plate were taken, from the edge of the mycelium fragment to the distal edge of the colony. Averages in linear growth of each strain comparing different media and of the six strains compared on GEA were statistically analysed (ANOVA and post-hoc Tukey test) using the package Statistica 7.0 (StatSoft, Tulsa, OK, USA).

Results

Isolation and identification of fungal strains

Three of the six strains belong to the *Fusarium incarnatum-equiseti* species complex (FIESC): two of them are included in the clade Incarnatum, haplotype 25-b (F25-1, F25-2) and one in the clade Equiseti, haplotype 3-b (F3-1). F25-2 and F3-1 were isolated from dead cynipid bodies, whereas F25-1 from gall tissues (figure 2). This classification refers to the multilocus haplotype nomenclature recently described by O'Donnell *et al.* (2009): the authors assert that no traditional Latin binomial should correspond with total confidence to these two FIESC species, so any morphological and ecological information in previous literature about *F. equiseti* and *F. incarnatum* (in the past synonymous of *F. semitectum* or *F. pallidoroseum*), although useful, can be applied to them with some caution.

One *A. alternata* and two *Botrytis* sp. strains were obtained from gall tissues and not from cynipid cadavers. *A. alternata* had obclavate conidia with a short beak and formed in long, often branched chains; conidiophores

were simple or branched and short (40-70 × 3-4 µm thick). The sequence obtained in molecular determination showed a high omology (> 99%) with those of *A. alternata*, *A. tenuissima* and *A. arborescens* available in the NCBI database, but the last two species were excluded for evident differences in morphological characters. *Botrytis* sp. had typical morphological traits: cultures were greyish-coloured; conidiophores had pale apex, a stipe and a head of branches; single-celled conidia were ellipsoidal or subspherical (mean = 10.6 × 8.0 µm, n = 100) and black sclerotia were observed.

Artificial inoculation tests

Evident necrotic processes were observed on all the 60 artificially infected galls 7 days after the inoculations; white, greyish and dark mycelial covers, often formed by conidial masses, were easily detectable. These symptoms were equivalent to those previously observed in natural infections. Control galls were asymptomatic. Reisolations were carried out and confirmed the presence and the necrotic ability of the selected strains.

The variable dimensions of the necrotic areas emphasise a different ability of the six strains to colonise wasp galls (table 1). Necrosis were present on the whole surface of all the 20 galls infected by *Botrytis* sp., while *A. alternata* produced necrosis only on limited surfaces; both *Fusarium* strains F25-1 and F25-2 caused necrosis on the whole surface of 90% of infected galls, whereas the strain F3-1 produced the same effect on the 60% of the inoculated galls.

Dead adults of wasp were found in the 47% of treated galls. The strain F25-1 produced the highest lethal effect to ACGW (in 70% of necrotic galls), followed by F3-1 (60%) and B2 (60%), whereas A1 showed the mildest effect. Withering, but not resembling necrosis, was observed on limited areas of leaves around infected galls.

Growth of fungal strains on different media

Data reported in table 2 show that gall extract agar (GEA) is the medium on which the significantly

Table 1. Results of artificial inoculations (after seven days) of six fungal strains on *D. kuriphilus* galls. WS - with fungal necrosis produced on the whole surface; PS - with fungal necrosis produced on partial surface; DC - containing dead individuals of cynipid.

Strains	Strain abbreviation	Place of origin	No. of galls				
			treated	necrotic	WS	PS	DC
FIESC 25-b	F25-1	Vaie	10	10	9	1	7
FIESC 25-b	F25-2	Vaie	10	10	9	1	5
FIESC 3-b	F3-1	Condove	10	10	6	4	6
Botrytis sp.	B1	Quarrata	10	10	10	0	3
Botrytis sp.	B2	S. Mommè	10	10	10	0	6
Alternaria alternata	A1	Vaie	10	10	0	10	1
Controls			10	0	0	0	0

Table 2. Average linear growth (mm) and standard error of each fungal strain in Petri dishes comparing three different media: water-agar (WA), gall extract agar (GEA) and PDAmb (PDA). Measurements of 4-days-old and for 8-days-old cultures are reported. Means followed by different letters are significantly ($p < 0.001$) different according to ANOVA and post-hoc Tukey test.

Fungal strain	Medium	Growth (mm) after 4 days			Growth (mm) after 8 days		
		mean		standard error	mean		standard error
FIESC 25-b (F25-1)	WA	22.58	a	0.55	38.67	a	0.60
	PDA	26.00	b	0.85	42.00	b	0.00
	GEA	27.83	b	0.86	42.00	b	0.00
FIESC 25-b (F25-2)	PDA	17.00	a	0.47	28.42	a	1.02
	WA	22.67	b	0.90	37.67	b	0.77
	GEA	26.67	c	0.25	42.00	c	0.00
FIESC 3-b (F3-1)	PDA	10.50	a	0.45	16.67	a	0.53
	WA	17.67	b	0.70	35.75	b	0.75
	GEA	22.42	c	0.30	41.33	c	0.31
Botrytis sp. (B1)	WA	19.25	a	0.92	31.17	a	1.33
	PDA	27.42	b	0.57	35.50	ab	2.14
	GEA	31.50	c	0.29	40.83	b	0.83
Botrytis sp. (B2)	WA	12.33	a	0.48	28.08	b	0.45
	PDA	14.50	ab	2.03	23.25	a	1.21
	GEA	17.17	b	0.84	37.58	c	1.38
A. alternata (A1)	PDA	18.17	a	0.59	26.58	a	0.37
	WA	21.67	b	0.88	32.58	b	1.55
	GEA	22.92	b	0.30	38.83	c	0.42

(p < 0.001) highest mycelial linear growth was recorded, compared to water-agar (WA) and PDAmb, for three strains after 4 days and four strains after 8 days. For strains F25-2 and F3-1 the growth on GEA was the highest both after 4 and 8 days. In the remaining cases growth values on GEA were statistically equivalent to those on PDA, but never lower. Moreover, the highest conidial production was observed in the cultures developed on GEA.

Comparing cultures of the different strains on GEA (figure 3), the significantly (p < 0.001) higher linear growth after 4 days was that of strain *Botrytis* B1, followed by the two strains of FIESC 25-b; after 8 days all the three *Fusarium* strains and B1 showed the statistically highest (p < 0.001) linear growth values.

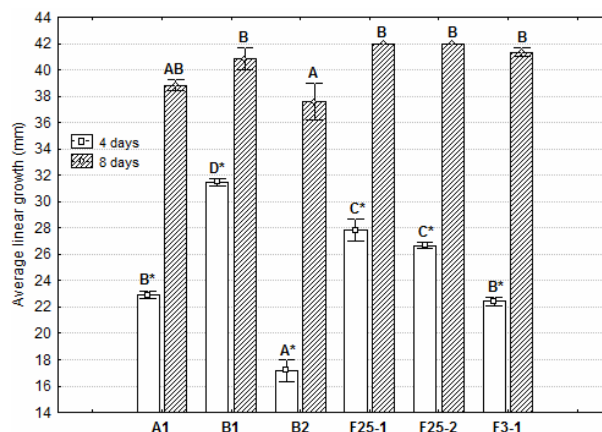

Figure 3. Average linear growth (mm) of the six fungal strains on gall extract agar (GEA). Data are illustrated both for 4-days-old and for 8-days-old cultures. Means followed by different letters are significantly (p < 0.001) different according to ANOVA and post-hoc Tukey test. Letters marked by an asterisk refers to statistical comparison of strains after 4 days; letters without asterisk refer to comparison after 8 days. Bars symbolise standard error of the mean.

Discussion and conclusions

The invasive ACGW poses a severe threat to chestnut stands in Italy, considering its capacity to reduce plants vigour and chestnut production (Kato and Hijii, 1997; Turchetti *et al.*, 2010): the safeguard of chestnut cultivation in many Italian areas hardly infested is a current management emergence. The introduction of the parasitoid *T. sinensis* may help control ACGW in chestnut stands. However, the time between parasitoid introduction and establishment of self-sustaining populations, which effectively reduce gall wasp populations under an acceptable threshold of damage, could not be short and accentuates the need of search for integrative biocontrol agents. An important role among constraint factors could be performed both by autochthonous parasitoids and other biocontrol organisms. To this concern the present study proves the existence in *C. sativa* orchards of naturally spreading fungi, present in the phylloplane and phyllosphere region, associated with necrosis of galls and wasp mortality, which were isolated and identified.

The species FIESC 25-b and FIESC 3-b might particularly offer interesting but verifiable opportunities for use in biocontrol: their efficacy in causing the death of ACGW individuals, their rapidity of action (only seven days) and abundant sporulation are elements that arouse particular interest. An entomopathogenic capacity may be supposed for their ability to growth enveloping cynipid bodies; in support to this hypothesis it is possible to see that in literature *F. incarnatum* was sometimes reported as an entomopathogen, for example by Hareendrath *et al.* (1987) who found similarly dead mummified insects of *Aphis craccivora* Koch. The production of micotoxins by these fungi might be supposed as another mortality factor for the gall maker: it is remarkable that both *F. incarnatum* and *F. equiseti* were cited for their ability to produce insecticidal toxins (Logrieco *et al.*, 1998; Blaney *et al.*, 1985).

In laboratory tests was produced and reported for the first time an extract of ACGW galls for employment as a nutrient medium for mycelial culturing in plate. The growth of cultures further indicated that gall tissues are a favourable substrate for the investigated fungi, perhaps in relation to the content of particularly nutrient elements. This result was attested above all for the two *Fusarium* species, and also for the B1 strain of *Botrytis* sp. This species (strain B2) showed also a good ability in necrotizing wasp galls, but did not envelope wasp bodies as FIESC species did. However, the constraining consideration is that *Botrytis* sp. is a well-known widespread saprobe and opportunistic phytopathogen, so the verification of its hypothetic employment in biocontrol against ACGW is hardly proposable. The same consideration can be made for *A. alternata*, together with the fact that this strain (A1) produced smaller necrosis compare to the other fungi and lower effect on wasp vitality, so its contribute in damaging and limiting chestnut cynipid appears to be unimportant. In relation to the previous classifications of *Fusarium* genus, *F. incarnatum* and *F. equiseti* are not reported significantly as plant pathogens (Leslie and Summerell, 2006), however the role of the two detected FIESC species in the environment must be verified.

After the preliminary indications provided by these tests, subsequent investigations should especially confirm parasitic efficacy of these *Fusarium* species, with specific attention to verify their capacity of direct penetration into the tissues of galls, and to deepen the aspect of any collateral effects on leaves and twigs. Also ascertaining their natural diffusion in chestnut stands is an important topic, because such phenomenon could be more widespread than so far observed in the visited stands. Further investigations on the probable interactions between the selected strains and other gall inhabitants such as the native and introduced parasitoids are desirable, considering also possible relationships with phytopathogen fungi as *Cryphonectria parasitica* (Murrill) Barr.

The susceptibility of galls and the ecological role of the fungi are not clearly known at the moment: fungi invading insect galls can act as pathogens, saprophytes colonizing already dead galls, or inquilines which in-

vade and feed upon healthy gall tissues indirectly causing the death of gall maker, even if it is not always easy to detect a clear difference (Wilson, 1995).

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