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Testing ecological interactions between *Gnomoniopsis castaneae* and *Dryocosmus kuriphilus* 

Guglielmo Lione\textsuperscript{a}, Luana Giordano\textsuperscript{a,\textit{b}}, Chiara Ferracini\textsuperscript{a}, Alberto Alma\textsuperscript{a} and Paolo Gonthier\textsuperscript{a}

\textsuperscript{a} Department of Agricultural, Forest and Food Sciences, University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

\textsuperscript{b} Centre of Competence for the Innovation in the Agro-Environmental Field (AGROINNOVA), University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

Corresponding author: Paolo Gonthier (paolo.gonthier@unito.it)

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**Role of Authors**

GL and LG conducted samplings and fungal isolations. GL performed statistical analyses and LG molecular diagnostics assays. GL and PG designed the experiments and wrote the manuscript. CF helped with the manipulation of insects and with the interpretation of results. AA supervised the work on insects and revised the manuscript.
Abstract

An emerging nut rot of chestnut caused by the fungus *Gnomoniopsis castaneae* was reported soon after the invasion of the exotic gall wasp *Dryocosmus kuriphilus* in Italy. The goal of this work was to assess the association between the spread of the fungal pathogen and the infestation of the pest by testing if:

I) viable inoculum of *G. castaneae* can be carried by adults of *D. kuriphilus*;

II) the fungal colonization is related to the number of adults inhabiting the galls;

III) the fungal colonization of chestnut buds and the oviposition are associated.

Fungal isolations and PCR-based molecular assays were performed on 323 chestnut galls and on their emerging *D. kuriphilus* adults, whose number was compared between galls colonized and not colonized by *G. castaneae*. To test the association between fungal colonization and oviposition, Monte Carlo simulations assuming different scenarios of ecological interactions were carried out and validated through isolation trials performed on 597 and 688 chestnut buds collected before and after oviposition, respectively.

Although DNA of *G. castaneae* was detected in a sample of 40% of the adults developed in colonized galls, the fungus could never be isolated from insects, suggesting that the pest is an unlikely vector of viable inoculum.

On average, the emerging adults were significantly more abundant from galls colonized by *G. castaneae* than from not colonized ones (3.76 vs. 2.54, P<0.05), indicating a possible fungus/pest synergy.
The simulations implying no interaction between *G. castaneae* and *D. kuriphilus* after fungal colonization were confirmed as the most likely. In fact, *G. castaneae* was present in 33.8% of the buds before oviposition, while no association was detected between fungal colonization and oviposition (odds ratio 0.98, 0.71-1.33 95% CI). This finding suggests that the fungus/pest synergy is asymmetrically favorable to the pest and occurs after oviposition.

**Keywords:** *Dryocosmus kuriphilus*, fungi, *Gnomoniopsis castaneae*, *Gnomoniopsis smithogilvyi*, insects, Monte Carlo

1. **Introduction**

Interspecific interactions stand among the major forces driving the ecosystem dynamics and resulting in effects which are perceptible at both structural and functional levels (Jones et al., 1994; Tilman, 1999). Interactions between plant pathogens and insects have been well-documented in agricultural and forest ecosystems as pivotal factors enhancing the occurrence, the transmission and the spread of plant diseases. Pathogenic fungi (Paine et al., 1997; Carlile et al., 2001; Harrington, 2013), bacteria (Harrison et al., 1980; Redak et al., 2004), phytoplasmas (Griffiths, 2013) and viruses (Spence, 2001) can be carried and released by insects through mechanical, metabolic, physiological, or trophic processes, depending on the case.
For some infectious diseases of trees, the ecological association relating fungal pathogens to insects has been identified as one of the main underlying features of the epidemiological processes. For instance, many fungal plant pathogens release volatile organic compounds that are highly attractive to some mycophagous insects. During the feeding process, the spores of the pathogen come into contact with the insects and can adhere on the surface of their exoskeleton, be collected in specialized anatomical structures or transported within the insect body. Subsequently, the contaminated insects may allow the fungal dispersal and transmit the pathogen from tree to tree, often through preexisting fresh wounds (Happ et al., 1971; Webber, 2004; Kirisits, 2013; Harrington, 2013).

The role played by the insects in the ecological association with fungal pathogens may extend beyond the transportation and the release of the spores. In fact, the vectors are often pests attacking the same host of the pathogen, leading to complex network of pest-pathogen-host interactions. For instance, pathogenic fungi requiring fresh wounds on the host tissues to start the infection process can be directly inoculated by their vectors during the excavation of galleries generated for feeding or breeding. While the fungal mycelium colonizes the host, the pests can take advantage of its presence either through the direct consumption of the fungus (i.e. mycophagia), or by feeding on an altered substrate with improved nutritional qualities (Paine et al., 1997). The synergy between the pathogen and the vector explains their mutual escalation, whose natural limitation relies in the increased death rate and in the alteration of the age structure occurring in the host trees population (Webber, 2004; Danti et al., 2013; Eckhardt, 2013; Kirisits, 2013).
Despite rarely documented, pests can also activate latent pathogens, whose switch from the endophytic to the pathogenic phase may be associated with the physiological reaction of the host to the pest attack (Sieber and Hugentobler, 1987).

The fungal-insect ecological interactions may explain the association between the spread of emerging pathogens and the biological invasion of exotic pests (Kirisits, 2013). Since 2005, a severe epidemic of nut rot has spread throughout the populations of the European chestnut (*Castanea sativa* Miller) in Italy, and its causal agent was described for the first time in 2012 as *Gnomoniopsis castaneae* G. Tamietti (syn: *G. smithogilvyi* L.A. Shuttlew., E.C.Y. Liew & D.I. Guest), an ascomycete included in the family of Gnomoniaceae (Visentin et al., 2012; Tamietti, 2016). *G. castaneae* is currently regarded as a major pathogen of chestnut in Italy, France and Switzerland (Visentin et al., 2012; Maresi et al., 2013; Dennert et al., 2015; Lione et al., 2015; Lione and Gonthier, 2016). Despite the pathogen lives as a parasite inside the kernel of the nut, it can also be isolated from the buds, the leaves, the bark of juvenile sprouts and from other green tissues of the tree where it does not induce any symptom. While fruiting bodies of the sexual form of *G. castaneae* can be observed on the burr surrounding the nut, the asexual stage of the fungus produces its multiplicative structures on the galls of *Dryocosmus kuriphilus* Yasumatsu (Visentin et al., 2012; Maresi et al., 2013). *D. kuriphilus*, commonly known as the Asian chestnut gall wasp, is a pest belonging to Hymenoptera Cynipidae that was accidentally introduced to Europe in the early 2000s (Quacchia et al., 2008). In the summer, *D. kuriphilus* lays eggs into the buds of chestnut, where its larvae overwinter. During the following vegetative season, the larvae develop within cells located in the inner tissues of the galls inducing
the formation of greenish-red galls, suppressing shoot elongation and causing twig
dieback (Ôtake, 1980). New adults emerge from the galls in the summer. A massive
presence of galls results in a dramatic reduction of the photosynthetic area, inhibits the
growth of the tree, decreases the chestnut vigor and determines substantial yield losses
(Kato and Hijii, 1997; EFSA, 2010; Sartor et al., 2015). Severe reduction of fruiting
estimated between 65% and 85% was observed in northern Italy, but recently the
presence of the pest has significantly decreased thanks to the biological control
programs performed with the Hymenoptera Torymidae Torymus sinensis Kamijo
(Ferracini et al., 2015).

Based on the first confirmed reports of the presence of G. castaneae and D. kuriphilus in the European chestnut populations, the invasion of the pathogen occurred
a few years after the invasion of the pest and started in the same areas located in the
Cuneo Province, in north-western Italy (Brussino et al., 2002; Visentin et al., 2012),
suggesting that possible interactions between the pathogen and the pest may occur.
Although galls necrosis along with mortality of D. kuriphilus were observed in
association with G. castaneae (Magro et al., 2010), the ecological interaction between
the fungal pathogen and the exotic pest still remains widely unexplored.

The main goal of this work was to test some among the possible ecological
interactions between G. castaneae and D. kuriphilus by combining theoretical and
empirical approaches. In detail, three specific hypotheses of ecological interactions
were tested:

I) whether viable inoculum of G. castaneae can be carried by adults of D. kuriphilus;
II) whether the colonization of the gall tissues by *G. castaneae* is related to the number of inhabiting adults of *D. kuriphilus*;

III) whether the fungal colonization of plant buds and the oviposition by the insect are associated.

2. Methods

2.1. Experimental design

In each of the three sites located in north-western Italy (Table 1), five chestnuts were randomly selected. Branches with a maximum diameter of 1 cm harbored most *D. kuriphilus* galls and chestnut buds (see below), and thus were deemed to be representative for sampling purposes. From the crown of each tree, 10 branches were excised during two distinct samplings, the first one performed on 20th June 2013, before the oviposition timeframe of *D. kuriphilus*, while the second one was carried out on 25th September 2013, after the oviposition timeframe. The approximate starting date and the length of the oviposition timeframe were estimated according to EPPO (2005) and Alma et al. (2014).

2.2. Biological analyses

During the first sampling, 323 galls of *D. kuriphilus* were collected (up to two galls per branch, if present) and incubated separately into aerated plastic cages stored at
room temperature (\(~25\pm2 \degree C\)) and normal daylight exposure. The cages were visually
inspected twice a day, for 3 weeks, and the 35\% of the emerging adults of *D. kuriphilus*
was plated into 6 cm diameter Petri dishes to isolate *G. castaneae* according to the
protocol described in Giordano et al. (2013). The remaining adults were individually
transferred using sterile tweezers into sterile 1.5 mL microcentrifuge tubes and stored at
-20 °C. After 5 days without new emerging adults, the galls were removed from their
incubator. In order to isolate *G. castaneae*, 5 fragments of approximately 5 × 5 × 1 mm
were randomly excised from both the inner and the outer tissues of each gall and plated
into 9 cm diameter Petri dishes filled with malt extract agar (MEA) (Visentin et al., 2012).

The isolation trials described above for the galls were performed on 597 and 688
buds (up to 5 buds per branch) after the first and the second sampling, respectively. A
subset of 60 buds collected from the former sampling and all the buds obtained from the
latter were sectioned under a dissecting microscope (20 × magnification) to assess the
presence of *D. kuriphilus* eggs.

For both samplings, the identification of the pathogen was performed based on
the macro and micro-morphological features of growing colonies, as described in
Visentin et al. (2012) and Lione et al. (2015).

### 2.3. Molecular analyses

A subset of 40 insects, half emerged from galls colonized by *G. castaneae* and
half from galls not colonized (see Results), was drawn from the adults of *D. kuriphilus*
previously stored at -20 °C. On this subset, PCR-based molecular analyses were
performed to validate the results of the isolation trials (i.e. molecular validation). Fungal DNA extraction was carried out by using the E.Z.N.A.™ Stool DNA Isolation Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer’s instructions. *G. castaneae* was identified through a taxon-specific molecular diagnostic assay. The two primers Gc1f (5’-AGCGGGCATGCCTGTCGAG-3’) and Gc1r (5’-ACGGCAAGAGCAACGCCAG-3’) were used as described in Lione et al. (2015) to specifically detect *G. castaneae*. All PCRs were carried out by setting the thermocycler parameters as follows: an initial 95°C denaturation step of 5 min, followed by 35 cycles of 95 °C denaturation for 30 s, 62 °C annealing for 45 s and 72 °C extension for 1 min, and a final 72 °C extension step of 10 min. The specific amplicon of *G. castaneae* (168 bp) was visualized in gel containing 1% (w/v) of high resolution MetaPhor (Cambrex) and 1% (w/v) of standard agarose, after electrophoretic migration. In order to assess the efficiency of fungal DNA extraction, an additional PCR amplification of the Internal Transcribed Spacer (ITS) was performed with the universal primers ITS1 and ITS4 (White et al., 1990; Gardes et al., 1991).

Moreover, the above cited taxon-specific molecular assay was carried out on a random subset of 50 putative colonies of *G. castaneae* to confirm the morphological identifications.

### 2.4. Statistical analysis and Monte Carlo simulations

According to the results of the isolation trial, the galls were classified as colonized (GC+) or not colonized (GC−) by *G. castaneae*. Negative binomial generalized
linear regression models (nbGLM), parametrized with the intercept, were run to compare the average number of adults of *D. kuriphilus* emerged from GC\(^+\) (codified as 1) and GC\(^-\) (codified as 0) (Venables and Ripley, 2002; Kéry, 2010). One nbGLM per site and an overall model for the three sites were fitted.

In both GC\(^+\) and GC\(^-\), the proportion (%) of *D. kuriphilus* adults positive to *G. castanea* (A\(^+\)) was calculated separately for the isolation trial and for the molecular validation along with the exact 95% confidence intervals (95% CI) obtained as reported by Blaker (2000). The proportion of A\(^+\) was compared between GC\(^+\) and GC\(^-\) with the Fisher's exact test (Crawley, 2013).

A Monte Carlo (MC) experiment (Carsey and Harden, 2014) was set to test *in silico* the association between the colonization of *G. castanea* and the oviposition by *D. kuriphilus*. An artificial environment was designed to simulate the oviposition and the fungal colonization processes at buds level. In a cubic Cartesian space with edges bounded between 0 and 40 cm, three branches of 35 cm, each one with three twigs of 5 cm were designed. A single branch included 20 buds, while 5 buds were located along each twig, achieving a total of 105 buds. The probability density functions (PDFs) of the distances separating two consecutive buds on the branches and on the twigs were estimated through the fit of the distribution types included in the Pearson system (Pearson, 1895; Lachene, 2013). The fit was performed on the pooled distances measured on four model branches and on their twigs collected in the field during the first sampling. The optimal Pearson type PDF was selected according to the minimum Akaike Information Criteron (AIC) (Akaike, 1973; Crawley, 2013). Two scenarios of asymmetric association (Somers, 1962) between fungal colonization and oviposition,
and between oviposition and fungal colonization were simulated: scenario A) the fungal colonization may influence the insect oviposition; scenario B) the insect oviposition may influence the fungal colonization. Each scenario was simulated under two competing hypotheses. In the scenario A, the hypothesis A₁ assumed that the buds colonized (BC⁺) and not colonized (BC⁻) by *G. castaneae* were equally attractive to *D. kuriphilus*, while under the alternative hypothesis A₂ the BC⁺ were more likely to attract ovipositing insects than BC⁻. Similarly, in the scenario B, the buds oviposited (BO⁺) were as attractive to the fungus as the not oviposited ones (BO⁻), under the hypothesis B₁, while BO⁺ were more attractive to *G. castaneae* than BO⁻, under the competing hypothesis B₂. For the scenario A, the attraction exerted by the buds was modelled as a series spherical buffers (Mitchell, 1999). Each spherical buffer was centered in the associated bud coordinates, while the radius was weighted differently according to the hypothesis being simulated. The radius was set constant at 0.5 cm for all the buds under A₁, while under A₂ it was set to 0.5 cm for BC⁻ and to 1 cm for BC⁺. For the scenario B, the attraction was modeled as a numeric weight assigned to the buds. Under B₁ the weight was set to 0.5 in order to assign to all buds an even probability of extraction regardless of the oviposition status. The probability of extraction was automatically rescaled by the sampling algorithm based on random number generators (Mitchell, 2005; Carsey and Harden, 2014). Under B₂ the weight was tuned to 0.2 for BO⁻ and to 0.8 for BO⁺ in order to simulate a different attraction, depending on the oviposition status, without introducing an asymmetry hyperparameter (i.e. different attractiveness weights were evenly spaced around the equal attractiveness weight) (Mitchell, 2005; Kéry, 2010; Carsey and Harden, 2014). The oviposition process of a single adult of *D. kuriphilus*
was simulated by modelling the insect trajectory as a Lévy flight. The Lévy flight was generated as a three dimensional random walk whose steps were derived from a power-law tailed PDF, with parameter $a=1$ and $\mu=2$, and whose zenith and azimuth were drawn, at each step, from a uniform distribution bounded between 0 and $2\pi$ radians (Reynolds and Frye, 2007; Edwards, 2008; Kéry, 2010). The status $BO^+$ was assigned to the bud whose buffer included for the first time the ending point of a single step of the Lévy flight. The fungal colonization was modelled as a stochastic process, based on a random number generator, drawing buds and assigning to them the $BC^+$ status according to an extraction probability proportional to a weight (Carsey and Harden, 2014). The weight was attributed to the buds depending on the hypothesis being tested (see below). Within each hypothesis the ratio between $BC^+$ and the total buds (i.e. $g$) was set to $g = 20\%, 40\%, 60\%, 80\%$. Similarly, the number of adults was set as a proportion of the maximum number of buds that could be oviposited in percent, selecting for this percent ($d$) the same values attributed to $g$ (i.e. $d = 20\%, 40\%, 60\%, 80\%$). Each combination between $g$ and $d$ and the additional combination $g=50\%$ and $d=60\%$ were set as a couple of fixed parameters to run a block of 5000 Monte Carlo (MC) simulations. Within each hypothesis, 17 blocks of MC simulation were run, for a total of $3.4\cdot10^5$ simulations (2 scenarios $\cdot$ 2 hypotheses $\cdot$ 17 blocks $\cdot$ 5000 simulations).

In the scenario A, each MC simulation consisted in the following steps: 1) drawing the $g$ $BC^+$ after the fungal colonization process was run with a weight set constant for all the 105 buds; 2) running $d$ independent oviposition processes of a single adult of $D. kuriphilus$ to gather $BO^+$, with the radii of the spherical buffers set as described above for each hypothesis; 3) cross-tabulating the $BC^+$, $BC^-$, $BO^+$ and $BO^-$ and calculating the
odds ratio $\theta$ as the measure of association between fungal colonization and oviposition (Agresti, 2001). Every MC simulation in the scenario B was performed through the following steps: 1) retrieving the $BO^+$ from $A_1$, step 2; 2) running the fungal colonization process with the weights assigned to $BO^+$ and $BO^-$, depending on the buds attraction tuned according to hypotheses described above, and drawing the $g BC^+$; 3-4) replicating previous steps 3 and 4. The 5000 $\theta$ values obtained within the blocks were used to estimate the PDFs of the odds ratios, whose average $\bar{\theta}$ was calculated with its 95% CI lower and upper bounds (i.e. $\bar{\theta}_l$ and $\bar{\theta}_u$) (Buckland, 1983; Jones et al., 2009). MC algorithms were run in R 3.2.1. (scripts are available as Supplementary Material Appendix A).

The MC simulations were biologically validated with the results gathered during the isolation trials performed on the buds. For both samplings, the ratio $g_o$ between $BC^+$ and the total buds was calculated with its 95% CI (Blaker, 2000) separately for each site and conjointly for all sites. The $g_o$ values were compared between the two samplings with the Fisher’s exact test. For the second sampling, the ratio $d_o$ between $BO^+$ and the total buds was calculated as described for $g_o$. The fungal colonization and the oviposition status of the buds were cross-tabulated and the odds ratio $\theta_o$ with its 95% CI (i.e. $\theta_{ol}$ and $\theta_{ou}$) were calculated with the Fisher’s method. Subsequently, $\theta_o$, $\theta_{ol}$ and $\theta_{ou}$ were compared to the average odds ratios and their associated 95% CI obtained from the MC simulations (Agresti, 2001; Carsey and Harden, 2014). The comparison was performed by assessing the probability ($L$) of gathering in silico an outcome statistically equivalent to the values obtained through the field samplings and the laboratory analyses, conditional to the occurrence of each hypothesis (i.e. the likelihood of $\theta_o$, $\theta_{ol}$
and θ_{ou}). L was calculated within the hypotheses as the relative frequency of MC simulation blocks whose θ, θ_l and θ_u were statistically equivalent to θ_o, θ_{ol} and θ_{ou} (i.e. L = \frac{\sum I(\theta_l < 1 < \theta_u)}{17} if θ_{ol} < 1 < θ_{ou}, L = \frac{\sum I(\theta_l > 1)}{17} if θ_{ol} > 1, otherwise L = \frac{\sum I(0 < \theta_u < 1)}{17}, where I is the indicator function according to Iverson’s notation) (Gerber et al., 2003; Kéry, 2010; Carsey and Harden, 2014).

A threshold of 0.05 was set as cut-off for the rejection of the null hypotheses of all statistical tests.

3. Results

The taxon-specific molecular assay confirmed the morphological identifications of all the putative colonies of G. castaneae tested. The isolation trial performed on the galls resulted in 120 GC^+ and 203 GC^−, with a ratio between GC^+ and total number of galls ranging from 16.0% to 67.7%, depending on the site. A total of 966 adults of D. kuriphilus emerged from the galls, 451 from GC^+ and 515 from GC^−. On average, the insects from GC^+ were significantly more abundant than the ones emerged from GC^− (2.62-3.90 vs. 0.62-2.89, P<0.05), as shown by the P-values displayed by the β coefficients in all the nbGLM (Table 2).

On the subset of adults scored for the presence of G. castaneae, 180 emerged from GC^+ and 159 from GC^−. The proportion of A^+ assessed through the isolation trial was 0% both for D. kuriphilus adults emerged from GC^+ (0-2.0% 95% CI) and for adults deriving from GC^− (0-2.2% 95% CI), resulting in a not significant Fisher’s exact test (P>0.05). On the contrary, the proportion of A^+ determined through the molecular assay
raised to 40.0% (20.9-63.1% 95% CI) for the adults from GC, remaining stable at 0%
(0-16.0% 95% CI) for the others. The former proportion resulted significantly larger than
the latter (P<0.05).

The optimal fits for the PDFs of the distances separating two consecutive buds
corresponded to Pearson type III curves with shape, location and scale parameters of
1.89, 2.07 and 12.6 for the branches (AIC=435.3), and 2.20, -0.16, 5.72 for the twigs
(AIC=182.1). Depending on the g-d combination, the blocks of MC simulations
displayed mean values $\bar{\theta}$ of odds ratios ranging from 0.127 to 1.394 under the
hypothesis $A_1$, and $\bar{\theta}$ values comprised between 1.07 and 1.34 under the hypothesis
$B_1$. Under $A_1$, the 95% CI associated with $\bar{\theta}$ showed bounds comprised within the
ranges 0-0.46 for $\bar{\theta}_l$ and 1.47-5.46 for $\bar{\theta}_u$, while under $B_1$ the bounds ranged from 0.18
to 0.46 for $\bar{\theta}_l$ and from 2.14 to 5.68 for $\bar{\theta}_u$. No significant association between the
colonization of G. castaneae and the oviposition by D. kuriphilus emerged under both $A_1$
and $B_1$, since the value 1 was comprised between $\bar{\theta}_l$ and $\bar{\theta}_u$ in all blocks. Under the
alternative hypotheses, the corresponding g-d blocks of MC simulations displayed
ranges from 2.24 to 8.50 for $\bar{\theta}_l$, from 4.78 to 35.47 for $\bar{\theta}$ and from 6.25 to 102.5 for $\bar{\theta}_u$
in $A_2$, and from 1.60 to 5.44 for $\bar{\theta}_l$, from 5.74 to 21.86 for $\bar{\theta}$ and from 7.90 to 60.80 for
$\bar{\theta}_u$ in $B_2$. A positive and significant association between fungal colonization and the
oviposition arose, since the condition $1 \leq \bar{\theta}_l$ occurred in all blocks simulated under $A_2$
and $B_2$ (Table 3).

Depending on the site, the ratio $g_0$ was comprised between 1.1% and 52.2%, and
between 30.7% and 48.5% in the first and in the second sampling, respectively,
displaying a significant increase only in the site of Aymavilles (P<0.05). The overall ratio
of g₀ attained 33.8% in the first sampling and 40.7% in the second one, showing a significant raise between samplings (P<0.05) (Fig. 1). The subset of buds inspected for D. kuriphilus eggs showed the absence of oviposition in all buds collected during the first sampling. Instead, in the second sampling, the values displayed by the ratio d₀ were 87.1% (82.0-91.2% 95% CI) in Aymavilles, 96.1% (92.8-98.2% 95% CI) in Nomaglio and 0% (0-1.6% 95% CI) in Robilante, with an overall value of 61.0% (57.3-64.7% 95% CI). The odds ratio θ₀ obtained for the sites of Aymavilles (1.45, 0.59-3.58 95% CI), Nomaglio (0.75, 0.14-3.56 95% CI) as well as the overall value (0.98, 0.71-1.33 95% CI) were not significantly different from 1 (P>0.05). Instead, the corresponding value for Robilante could not be estimated since no oviposited buds were detected in the second sampling. The values of L were calculated according to the fact that the overall and within site 95% CI satisfied the condition θ₀₁ < 1 < θ₀u. In all cases, L attained the value 100% under A₁ and B₁, and 0% under the competing hypotheses.

4. Discussion

The spread of plant pathogens and the invasion of exotic pests may be closely associated, especially if they share the same hosts. Often, pathogen-pest ecological interactions have been proved to drive the spread of both the pathogen and the pest. Pathogens and pests can operate in synergy through a series of processes leading to mutual advantages, to the detriment of the host plant. In the case of fungal plant pathogens, pests can operate as vectors of viable inoculum, as agents responsible for the inoculation process and as constructors of micro-environments favorable to the
fungal colonization and reproduction. In return, the pathogen can alter the physical and chemical qualities of the host tissues, providing the pests with an improved substrate for feeding and breeding. In some cases, the fungus may also represent a trophic resource for mycophagous insects (Paine et al., 1997; Webber, 2004; Danti et al., 2013; Eckhardt, 2013; Harrington, 2013; Kirisits, 2013).

The spatial and temporal overlaps between the outbreak of the nut rot of the European chestnut caused by _G. castaneae_ and the biological invasion of _D. kuriphilus_ occurring in Italy suggest a possible association between the emerging fungal pathogen and the exotic pest. The isolation trials indicate that _D. kuriphilus_ is unlikely to carry viable inoculum of _G. castaneae_. In fact, if the pest was a vector, a certain amount of adults emerged from the colonized galls (GC+) should have resulted positive to _G. castaneae_. Instead, the fungus was never isolated from the adults of _D. kuriphilus_, regardless of the presence or absence of viable inoculum colonizing the gall tissues. On the contrary, DNA of _G. castaneae_ could be detected in a subset of the insects emerged from the colonized galls. These findings may indicate that, when _G. castaneae_ colonizes the gall tissues, the emerging adults of _D. kuriphilus_ are potentially able to carry a certain amount of inoculum. However, this inoculum is no longer viable and, consequently, it cannot contribute to the infection process and to the spread of the pathogen. Despite the separation between the putative inoculum present in the inner tissues of the insect body from the inoculum adhering on the exoskeleton was not performed, the isolation technique was suitable to reveal the presence of both, since the insects were mostly smashed during the plating process because of their structural fragility. However, since _D. kuriphilus_ consumes part of the inner tissues of the galls, the
inoculum of *G. castaneae* in the form of mycelium might have been ingested and
subsequently inactivated during the digestive processes, rather than transported on the
surface of the exoskeleton. It should be noted that no specialized anatomical structures
allowing fungal inoculum transportation are present on the exoskeleton of *D. kuriphilus*.

The significantly larger number of adults of *D. kuriphilus* hosted in the galls
colonized by *G. castaneae* could be the result of an interaction occurring between the
fungus and the pest during oviposition. *G. castaneae* colonizing the buds could exert an
attractive effect towards the pest, yet also the oviposited buds could represent a more
favorable environment for the fungal colonization. Testing these hypotheses *in silico*
through Monte Carlo methods was advantageous, since such methods allowed to
model and simulate iteratively the ecological phenomena (i.e. oviposition/colonization
and attractiveness of oviposited/colonized buds) under different scenarios (i.e. influence
of fungal colonization on oviposition, or influence of oviposition on fungal colonization)
and with various combinations of biologically relevant parameters (i.e. number of adults
and colonized buds). The oviposition by *D. kuriphilus* and the fungal colonization were
modeled as stochastic processes based on Lévy flight and on random sampling from
probability distributions, respectively. Despite the absence of studies specifically
focused on *G. castaneae* and *D. kuriphilus*, these approaches have been proved to be
effective and consistent with in-field observations when simulating the flight trajectory of
insects and the dynamics of fungal colonization in several natural and semi-natural
ecosystems (Reynolds and Frye, 2007; Edwards, 2008; Honkaniemi et al., 2014;
Jarnevich and Young, 2015). Also the assignment of differential weights and the
delimitation of buffers to model the buds attractiveness towards the fungus and the
insect were consistent with the methods proposed in the literature to investigate ecological interactions (Mitchell, 1999; Mitchell, 2005; Gonthier et al., 2012). Regardless of the scenario and of the number of adults and colonized buds, the outcomes of the simulations (i.e. average odds ratios and associated 95% CI) showed that the association between fungal colonization and oviposition by *D. kuriphilus* depends on which hypothesis about the attraction exerted by the oviposited/colonized buds on the fungus/pest is assumed. When modelling all the buds as equally attractive, no ecological association between the two phenomena could be detected, meaning that no interaction between *G. castaneae* and *D. kuriphilus* occurred. Conversely, when the oviposited/colonized buds were modeled as more attractive to the fungus/pest, significant and positive average odds ratios were displayed, indicating the existence of an underlying interaction between *G. castaneae* and *D. kuriphilus*. The biological validation is a pivotal step to carry out when testing hypotheses through MC simulations (Thébaud et al., 2005). Even though the infection biology and epidemiology of *G. castaneae*, with a few exceptions (Lione et al., 2015; Lione and Gonthier, 2016), are still mostly unknown and, thus, no prior information is available to discriminate which scenario is most likely to occur in nature, the option A seems to fit better to the processes of fungal colonization and oviposition. Moreover, the probability of gathering *in silico* outcomes statistically equivalent to the results gathered from the field trials showed that the hypotheses of equal attractiveness exerted by all the buds towards the fungus/insect are the most likely. These conclusions are supported by the fact that the fungus was isolated from approximately 1/3 of the buds sampled prior to the oviposition period, and also from the buds collected from the site where the oviposition was virtually
absent in both samplings. Despite the overall ratio of colonized buds showed a significant increase between the two samplings, the raise was not substantial (approximately +7%) and it was mostly influenced by the data gathered from one out of three sites. Considering that the incidence of G. castaneae has been shown to increase with warmer temperatures during the months preceding its assessment (Lione et al., 2015), and provided that in north-western Italy the warmest months of the year are comprised between the two sampling dates (Biancotti et al., 1998), the observed increase could depend on the climate, rather than on the oviposition by D. kuriphilus. While the hypothesis of an ecological interaction between G. castaneae and D. kuriphilus during the oviposition stage is not supported, the larger number of adults inhabiting the galls colonized by the fungus suggests the possibility of an interaction occurring subsequently, during the insects development. The fungal mycelium could increase the survival rate of the pests by providing an additional nutritive source for direct consumption (i.e. mycophagy), or by improving the quality of the gall tissues through favorable chemical transformations. The mycelium of G. castaneae colonizing the galls might transform plant tissues into a more digestible biomass rich in nutrients. Despite no experimental results can be provided to support these hypotheses, the positive role of fungi on the diet and on the population dynamics of many insects has been largely documented in the literature (Kendrick, 2000; Carlile, 2001). The colonization of galls by G. castaneae may also result in a physical alteration of the plant tissues. Several fungi have been shown to induce changes in the firmness of substrates by softening their texture (Kendrick, 2000), which might result in a reduced mechanical resistance leading to an advantage to the insect. However, as noticed by Magro et al.
(2010), galls alteration might also be detrimental to *D. kuriphilus*. It is worth noting that since no necrosis or observable physicals alterations were observed on the galls colonized by *G. castaneae* in our study, the role of the fungus as inhibitor of *D. kuriphilus* that was suggested by Magro et al. (2010) would deserve further investigations. Moreover, specific studies are needed to clarify whether and how *G. castaneae* can modify the galls tissues.

5. Conclusion

The theoretical approach proposed and used in this study combined the results from different possible ecological interactions between *G. castaneae* and *D. kuriphilus*, while the biological analyses and the validation performed on field data allowed the detection of the most likely hypothesis. All lines of evidence suggest that the ecological interactions between *G. castaneae* and *D. kuriphilus* are asymmetrically favorable to the insect, rather than to the fungus. This is not surprising considering that the two species did not coevolve in their current area of sympatry. It is worth noting that, potentially, the infestations of *D. kuriphilus* could also have increased the spreading ability of the pathogen, since asexual fruiting bodies of *G. castaneae* have been observed on the galls surface (Maresi et al., 2013). However, asexual reproduction has been recently shown to play a minor role compared to sexual reproduction in the spread of the fungal pathogen based on population genetics data (Sillo et al., 2016). Moreover, as documented for other fungi (i.e. latent pathogens), the stress induced on the chestnut by a massive attack of the pest could increase the incidence of the disease (Petrini,
1991). Such observations suggest that the level of complexity in the ecological interaction between *G. castaneae* and *D. kuriphilus* might be influenced by other co-occurring factors. However, no studies on these topics are available yet and, to date, our results suggest that the diffusion of *G. castaneae* and the consequent outbreak of the nut rot of chestnut could have boosted the spread of the exotic pest in the newfound area of invasion in Italy.

**Acknowledgements**

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**References**


Figure captions

Figure 1. Percentage of buds colonized by *Gnomoniopsis castanea* (g₀) in the two samplings. The values of g₀ observed in sampling 1 (white bars) and sampling 2 (gray bars) are reported separately for each site as well as conjointly for all the sites (i.e. Overall). Different letters on the top of the bars indicate a significant difference (P<0.05) between the g₀ values of the two samplings according to the Fisher’s exact test. Error bars refer to the 95% confidence interval of g₀.
Table 1. Main characteristics of the sampling sites for the collection of buds and galls of *Dryocosmus kuriphilus* from the European chestnuts.

<table>
<thead>
<tr>
<th>Site</th>
<th>Coordinates (UTM WGS84 zone 32N)</th>
<th>Elevation (m a.s.l)</th>
<th>Aspect</th>
<th>No. of galls sampled</th>
<th>No. of buds sampled (20/06/2013)</th>
<th>No. of buds sampled (25/09/2013)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aymavilles</td>
<td>361954, 5060322</td>
<td>980</td>
<td>W</td>
<td>175</td>
<td>186</td>
<td>225</td>
</tr>
<tr>
<td></td>
<td>410670,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nomaglio</td>
<td>5043485, 381836</td>
<td>580</td>
<td>WSW</td>
<td>124</td>
<td>161</td>
<td>233</td>
</tr>
<tr>
<td></td>
<td>4906525</td>
<td>730</td>
<td>WNW</td>
<td>24</td>
<td>250</td>
<td>230</td>
</tr>
<tr>
<td>Robilante</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>
Table 2. Negative binomial generalized linear regression models (nbGLM) comparing the average number of *Dryocosmus kuriphilus* adults emerged from galls colonized (GC⁺) and not colonized (GC⁻) by *Gnomoniopsis castaneae*. The intercept, the β coefficient and its related P-value are reported for each site and conjointly for all the sites (i.e. Overall). The β coefficients showing a significant difference (P<0.05) between the averages are marked with the symbol *.

<table>
<thead>
<tr>
<th>site</th>
<th>No. of <em>D. kuriphilus</em> adults emerged from GC⁺ (average)</th>
<th>No. of <em>D. kuriphilus</em> adults emerged from GC⁻ (average)</th>
<th>intercept</th>
<th>β</th>
<th>P-value</th>
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<tr>
<td>Aymavilles</td>
<td>3.64</td>
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<td>1.06</td>
<td>0.23*</td>
<td>3.61·10⁻²</td>
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<td>Nomaglio</td>
<td>3.90</td>
<td>2.00</td>
<td>0.69</td>
<td>0.67*</td>
<td>4.17·10⁻⁷</td>
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<td>Robilante</td>
<td>2.62</td>
<td>0.62</td>
<td>-0.47</td>
<td>1.43*</td>
<td>1.88·10⁻⁴</td>
</tr>
<tr>
<td>Overall</td>
<td>3.76</td>
<td>2.54</td>
<td>0.93</td>
<td>0.39*</td>
<td>5.88·10⁻⁹</td>
</tr>
</tbody>
</table>
Table 3. Blocks of Monte Carlo simulations defined by the combination of *Gnomoniopsis castaneae* colonization (g) and *Dryocosmus kuriphilus* oviposition (d). The average of the odds ratios $\bar{\theta}$, along with its 95% CI lower and upper bounds ($\bar{\theta}_l, \bar{\theta}_u$), are reported for each g-d combination and hypothesis within scenario as measures of association between fungal colonization and oviposition.

<table>
<thead>
<tr>
<th>block</th>
<th>scenario A</th>
<th>scenario B</th>
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<tr>
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<tr>
<td>20</td>
<td>40</td>
<td>0.97 (0.00, 2.60)</td>
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<tr>
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<td>60</td>
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<td>80</td>
<td>0.25 (0.00, 1.82)</td>
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<tr>
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<td>20</td>
<td>1.14 (0.34, 2.75)</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>0.93 (0.00, 2.20)</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>1.08 (0.44, 2.20)</td>
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<tr>
<td>40</td>
<td>80</td>
<td>0.16 (0.00, 1.48)</td>
</tr>
<tr>
<td>50</td>
<td>60</td>
<td>0.15 (0.00, 1.54)</td>
</tr>
<tr>
<td>60</td>
<td>20</td>
<td>1.20 (0.36, 2.97)</td>
</tr>
<tr>
<td>----</td>
<td>-----</td>
<td>------------------</td>
</tr>
<tr>
<td>60</td>
<td>40</td>
<td>0.96 (0.00, 2.28)</td>
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<td>60</td>
<td>1.10 (0.46, 2.25)</td>
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<td>20</td>
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<td>80</td>
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</tr>
<tr>
<td>80</td>
<td>80</td>
<td>0.15 (0.00, 1.54)</td>
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