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DIPARTIMENTO PER LA INNOVAZIONE NEI SISTEMI BIOLOGICI AGROALIMENTARI E FORESTALI

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TO: Fungal Biology Editorial Board

Object: MS FUNBIO-D-16-00165R1

Dear Editors,

I resubmit the MS FUNBIO-D-16-00165R1 taking into account all the suggestions and comments risen by the reviewers.

I'm looking forward your feedback,

Sincerely yours

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Response to reviewer's and editor:

All the editorial changes suggested were approved in the text.

Double spaces were all eliminated

A space was left above and below each section heading

The dates format was changes as suggested

Gnomoniopsis gall necrosis

Does Gnomoniopsis castanea contribute to the natural biological control of chestnut gall wasp?

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Abstract

Gnomoniopsis castanea has been reported as the causal agent of necrosis of chestnut wasp (*Dryocosmus kuriphilus*) galls. The fungus is frequently observed on galls in chestnut stands infested by the insect in Italy. In the present study the impact of gall necrosis and the dynamic of its development have been studied in mature and young *Castanea sativa* stands in Central Italy during spring and early summer, before the *Dryocosmus kuriphilus* adult flies. Results suggest that gall necrosis develops from resident endophytic inoculum of *Gnomoniopsis castanea*. During the 2 years of monitoring, no differences were found in incidence and severity of the disease. Gall necrosis increased exponentially during the season, reaching the maximum of severity at the end of August. Gall necrosis was shown to have a severe impact on *Dryocosmus kuriphilus* vitality, mostly impacting the adults inside the galls. Gall necrosis by *Gnomoniopsis castanea* appears to efficiently control gall wasp in chestnut stands, although the high virulence of the fungus to chestnut fruits precludes its use as biocontrol agent in biological control strategies.

Keywords: *Gnomoniopsis castanea; gall wasp; biological control; endophyte; Dryocosmus kuriphilus*

Introduction

Occurrence of *Gnomoniopsis* spp. associated with chestnut tissues and organs has been widely reported in the past few years in Europe and Australasia on *Castanea sativa*, *C. crenata* and European X Japanese hybrids (Shuttleworth et al., 2013). Two species were described independently in 2012, *Gnomoniopsis smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew & D.I. Guest in Australia associated with brown rot of kernels of *Castanea* spp. and hybrids (Shuttleworth et al., 2012), and *Gnomoniopsis castanea* G. Tamietti, also associated with brown rot of *C. sativa* kernels (Visentin et al., 2012). However ,using ITS and tef1-α in combination with a morphological analysis provided evidence that *G. castanea* and *G. castanea* must be considered synonyms (Shuttleworth, et al., 2015) and co-specific with *Gnomonia pascoe* prov. nom. previously described in New Zealand associated with diseased kernels (Smith and Ogilvy 2008). Most recently, phylogeny data, while confirming the co-specificity of these taxa, highlighted some differences in sequence of neutral markers suggesting the existence of lineages (Pasche et al., 2016). A debate is ongoing on the legitimate name of the species (Shuttleworth, et al., 2015; Linaldeddu et al., 2016; Tamietti, 2016). In this study we will adopt the name *Gnomoniopsis castanea* G. Tamietti until a definitive agreement on the legitimate name of this fungus is reached.

Gnomoniopsis castanea is recognized as the causal agent of brown rot of chestnut kernels in Europe and Australasia (Smith and Ogilvy 2008; Visentin et al., 2012; Shuttleworth et al., 2013; Dennert et al., 2015). Although it has been associated with more than one causal agent, brown rot of chestnut has been recorded for many years in several chestnut areas worldwide. It may represent the most threatening disease of chestnut kernels, being responsible of damages affecting up to 50% and 60-70% of the production in pre- and post-harvest yields, respectively, with obvious economic consequences (Maresi et al., 2013). Furthermore *G. castanea* causes leaf and shoot blight in chestnut and most recently has been reported as the causal agent of twigs canker in India and Europe (Dar and Rai, 2015; Pasche et al., 2016). *Gnomonipsis castanea* is a cryptic species whose origin has not been determined yet. It has been recorded in Australasia and India on introduced chestnut accessions from Japan (*C. crenata*) and Europe (*C. sativa*). It lives

asymptomatically as an endophyte in most chestnut organs and tissues only expressing symptoms following the occurrence of favourable environmental factors. For instance, chestnut brown rot has been reported to be increased by rainfall during flowering (Ogilvy 1998) most likely caused through a floral infection by overwintered ascospores released from dead burrs on the orchard floor (Smith and Ogilvy 2008; Shuttleworth et al. 2012). In Italy and Switzerland, a noticeable increase of brown rot incidence was recorded following chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu) invasion beginning in the mid-2000s. The first official European record of gall wasp was in 2002 (Brussino et al., 2002) in Italy although the insect was probably introduced some time before in the mid-1980s through importation of propagation material from China (Aebi et al., 2006). Since then, the gall wasp has become widespread in Italy and has been recorded in most of the chestnut range in Europe. Since then, the gall wasp has become widespread in Italy and has been recorded in most of the chestnut range in Europe. *Dryocosmus kuriphilus* is a member of the oak gall wasp tribe Cynipini, and is one of only two species in this tribe to induce galls on *Castanea* (Felt, 1940; Stone *et al*., 2002). Attack by *D. kuriphilus* on chestnut commonly reduces wood production (Kato & Hijii, 1997) plant vitality (Moriya *et al* ., 2003) and fruit yield by 50–75% (Payne *et al*., 1983). Biological control of gall wasp can be efficiently achieved with the exotic parasitoid *Torymus sinensis*, although several native parasitoids seem to contribute naturally to the containment of the infestation (Aebi et al., 2007; Speranza et al., 2009). Magro et al. (2010), observed the presence of necrotized *D. kuriphilus* galls and demonstrated the galls and associated leaves were killed by *G. castanea* before the adult flight period. This observation led to the question as to whether, beside its negative impact on nuts quality, *G. castanea* could act as natural bio-control agent of chestnut gall wasp.

The aim of the present study is to determine the role of *G. castanea* as a biocontrol agent of *D. kuriphilus* in nature. For this study, the natural occurrence of gall necrosis caused by *G. castanea* was investigated in different sites to define the patterns of distribution, the dynamic of disease development, and its impact on the developmental stages of *D. kuriphilus* within the galls.

Materials and Methods

79

80 This work was carried out during the growing seasons of 2010-2012 in one of the largest chestnut districts
81 in Central Italy, the Monti Cimini area (province of Viterbo), characterized by highly productive managed
82 orchards and coppices. *Dryocosmus kuriphilus* was first recorded in the area in 2006.
83 Meteorological data related to the investigated sites were obtained by the databases of the Latium Region
84 Agriculture development Agency (ARSIAL) for the year 2011 and 2012.

85

86 *Endophytic occurrence of* Gnomoniopsis *castanea*

87

88 In order to provide a baseline on the endophytic occurrence of *G. castanea* in chestnut stands in the
89 investigated areas, a survey was conducted in October 2010, and samples were collected from 15 *C. sativa*
90 trees in two sites located at the southwest (site A, 42°17'31.4"N 12°09'01.9"E, gall wasp infestation first
91 recorded in 2006) and east (site B, 42°18'38.3"N 12°13'29.5"E, gall wasp infestation first recorded in 2010)
92 of the volcanic cone of Vico Lake, including trees from orchards (O trees) and coppices (C trees) (Table 1).
93 Three terminal shoots (5 cm), including buds, leaves, and 3 kernels were collected from each tree, stored in
94 separate “snap-lock” plastic bags, placed at 4°C and processed within 2 days. Isolation of the pathogen was
95 attempted from buds, bark, and leaves. Isolation from kernels was done by separating the fruit from the
96 shell, and distinguishing perisperm, endosperm, and embryo. Each sample was surface sterilized according
97 to Luchi *et al*., 2006 (60 s in 75% ethanol, 3 min in 3% NaClO and 30 s in 75% ethanol), rinsed in sterile
98 distilled water and aseptically cut into 5 fragments not exceeding 5 × 5 mm. Fragments were plated onto
99 Petri dishes containing potato dextrose agar (PDA, Oxoid, 39g/l) amended with streptomycin sulfate (0,06
100 g/l), and incubated at 20 ± 2°C. After 7 days of incubation, plates were observed and the presence *of G.*
101 *castanea* colonies was recorded. Identification was based on morphological (Shuttleworth et al., 2012) and
102 molecular traits (see below). Additional fruits (up to 10) were collected from each tagged tree in separate
103 “snap-lock” plastic bags, brought to the laboratory, and incubated in a damp chamber at room temperature

for 10 days before visual analysis for presence/absence of *G. castanea* asexual reproductive structures on the fruit surface, and isolation in pure culture from asexual structures (Shuttleworth et al., 2012).

Natural impact of gall necrosis in orchard trees

The impact of *G. castanea* gall necrosis was assessed on 32 *C. sativa* adult trees randomly chosen in site A (Table 1). The survey was conducted in the 2012 growing season on July 12. Four branches from each tree were identified and tagged. On each branch, the number of healthy and symptomatic galls was determined according to symptoms description by Magro et al. (2010). Galls were classified according to a visual scale of 4 grades: healthy galls, 0; slightly necrotic (0- 30%), 1; partially necrotic (30- 60%), 2; fully necrotic (60-100%), 3. A total of 4.307 galls were inspected for necrotic symptoms. In October 2012, a total of 480 kernels were collected from the tagged branches. Fruits were incubated in the damp chamber at room temperature for 10 days before visual analysis of internal symptoms according to the same scale used for galls (listed above). Necrosis and brown rot incidence and severity were recorded for galls and kernels respectively. Incidence ($I = n/N$) was calculated as the number of symptomatic units (n) among the total number of evaluated units (N). Severity (S) was calculated using the equation $S = \Sigma(x_i n_i) / n$ where x_i represents the visual scale grade, n_i the number of symptomatic units in grade x_i, and n the total number of symptomatic units. The variance-to-mean ratio (VM) was used to estimate the type of distribution patterns (regular, random or clustered) of the data (Campbell and Madden, 1990). This index of dispersion is calculated by dividing the sample variance by the sample mean: VM=s2/ where s2 is the sample variance and the sample mean. VM is expected to be <1 for regular spatial patterns, =1 for random patterns and >1 for aggregated patterns. The expected value of 1 for a random pattern is related to the idea that the Poisson distribution (by definition population mean = population variance) is appropriate for describing frequency count data for a random pattern. In general, the value VM increases as aggregation increases. Isolation from a sub-set of 40 galls (10 per each necrotic class), collected on July 12 from 10 trees randomly chosen among the 38 inspected was performed as described in the previous paragraph by collecting the 5

fragments from asymptomatic tissues in galls in class 0, from the advancing edge of the lesions of galls in classes 1 and 2, and from necrotic tissues of galls in class 3.

Gall necrosis development

Gall necrosis development during the growing season was studied in 2011 and 2012 in a young (5 years old in 2011) sweet chestnut plantation heavily infested by gall wasp (site C, 42°17'42.0"N 12°08'23.3"E). The plantation extends for 10 hectares and consists of 1500 wild rootstocks of *C. sativa* grafted with scions of Marrone cultivar 'Fiorentino' planted at 8 x 8 meters. The choice of the young plantation was determined by the ease of monitoring and inspecting the crown of young trees compared to mature orchard or coppice trees. In the 2011 experiment, 30 trees were randomly selected in the plantation. For each tree, 3 branches were tagged in April 2011. All the galls present on each tagged branch were monitored for development of necrotic symptoms; on 29th April at the stage of first leaves separating (FLS); 5th May at the stage of first leaves unfolded (FLU); 19th May, 10th and 20th June at the stage of fully expanded leaves (FEL) (Mejer et al., 1994), before the gall wasp adult emerged and flew away. In 2012 the experiment was replicated with the same design on trees different from those monitored in 2011. Again all galls on each tagged branch were monitored at FEL stage on 24th May; 6th, 14th, 28th June; 13th July. Assessment of necrotic galls was performed using the same visual scale and disease descriptors charcterized in the previous paragraph. On 7th July 2011, and 13th July2012, and at the end of the monitoring period, 4 galls from each tagged tree were collected, 1 from each of the 4 grades of the visual scale, for a total of 120 galls. Galls were taken to the laboratory and processed for isolation of *G. castanea* as described above.

Impact of gall necrosis on Dryocosmus kuriphilus *vitality*

In 2011 and 2012, 100 healthy galls were randomly collected every week from trees in site C, starting at the first appearance of gall in May and ending at adults emergence in July. Galls were taken to a laboratory and

dissected in order to assess the developmental stage (i.e., larvae, white pupae, white pupae with red eyes, black pupae, and adults) and life cycle of *D. kuriphilus*. *Dryocosmus kuriphilus* mortality was assessed on 28th June 2011, and 13th July 2012, corresponding to the peaks of adult emergence. Two hundred and four hundred galls were randomly collected from tagged branches in site C, in 2011 and 2012, respectively. After necrosis grade-scale assessment, galls were dissected to score the number of live and dead insects in each nutrition cell, and development stage (larvae, white pupae, red eye pupae, black pupae, adults). Mortality was then calculated for each necrotic class and development stage.

The entomopathogenic potential of *Gnomoniopsis castanea* was tested on *Galleria mellonella* (Lepidoptera: Pyralidae) larvae due the inability to test it directly on *D. kuriphilus* . This lepidopteran is normally used for assessing the virulence of microorganisms and is considered highly susceptible to many fungal pathogens (Reeves et al., 2004).

Three different concentrations of *G. castanea* conidial suspensions were prepared: 2 x 10^7, 1 x $10^{7,}$ and 5 x 10^6 conidia/ml. Eight late-instar larvae of *G. mellonella*, reared individually in Petri dishes, were dampened with 200 µl of each conidial suspension. Eight larvae were dampened with sterile water as the control treatment. Assays were repeated three times.

Treated and control larvae were then maintained in Petri dishes at 24 °C ± 1 under dark conditions and mortality was assessed every 48 hours until the emergence of the adults. Finally, all dead specimens were placed in moist-chambers for fungal isolation.

Gnomoniopsis *castanea molecular identification*

For all the isolates, species identity was confirmed by sequencing fragments of the β-tubulin, the elongation factor 1-α (EF 1-α), the RNA polymerase II (rpbII) genes as well as the internal transcribed spacer (ITS). DNA was extracted from fresh mycelium grown on PDB (potato dextrose broth) with the NucleoSpin Plant II mini kit (Mackery Nagel, Germany) following the manufacturers' instructions. DNA concentration was assessed by gel electrophoresis and DNA was diluted 1:10 to perform PCR. Amplification of EF 1-α, RPB

II, ITS and β-tubulin was done using the primers designed in other studies (Table S1, Supplementary material). For each PCR reaction, the master mix consisted of 2X MyTaq MIX (Bioline, UK), 0.50 µM of each primer and approximately 5–20 ng DNA in a final reaction volume of 25 µL. Cycling conditions consisted of an initial denaturation of 3 min at 95°C, followed by 35 cycles consisting of 15 sec at 94°C, 15 sec at the respective annealing temperature and 10 sec at 72°C, followed by a final elongation of 5–10 min at 72°C. Amplicons were purified with NucleoSpin Gel and PCR Clean-up (Mackery Nagel). Sequencing reactions were performed by Macrogen Europe Laboratory (Amsterdam, The Netherlands) and forward and reverse sequences were assembled and edited using BioEdit (Ibis Bioscience) and compared to NCBI database (https://blast.ncbi.nlm.nih.gov/ Blast.cgi?PAGE_TYPE=BlastSearch).

Statistical analysis

One-way ANOVA, non-parametric Kruskal-Wallis, multiple comparison parametric and non-parametric post-test (Tukey's Multiple Comparison Test and Dunn's post-test) were carried out with Graphpad Prism version 5.00 (GraphPad Software, San Diego California USA). Non-linear data fit and comparison of independent fits with Akaike's Information Criteria (AICc) were carried out with Graphpad Prism version 5.00. Principal Component Analysis was carried out with PAST version 2.14 (Hammer et al., 2001). Meteorological data for the investigated areas are reported in supplementary material (Table S2, Supplementary material).

Results

Endophytic presence of Gnomoniopsis *castanea in chestnut tissues*

Endophytic presence of *G. castanea* in healthy chestnut tissues and organs in the two surveyed sites is presented in Figure 1. There were significant differences among groups (Kruskal-Wallis test , P=0,010,

Gaussian approximation). Differences were found with Dunn's Multiple Comparison Test between fruits and the other tissues (bark, leaves and buds). Figure 2 shows the Principal Component Analysis (PCA) based on isolation of *G. castanea* from different chestnut tissues: the first and second components accounted for 86.7 and 7.9% of the variance, respectively. Isolation frequency of *G. castanea* was generally lower in site B than site A and, relative to site B, there appeared to be a weak effect associated with forest type. Of 146 healthy kernels inspected, 120 (84,5%) showed the presence of a variable number of conidiomata of *G. castanea* on the epicarp after incubation in the damp chamber; however they did not show any internal brown rot symptoms. *Gnomoniopsis castanea* was recovered from 50% of the attempted isolations from 33 randomly chosen spiral mucilaginous cirri erupting from asexual fruit bodies. All the *G. castanea* isolates were confirmed by morphological and molecular analyses.

Natural impact of gall necrosis in orchard trees

According to the VM ratio values (Table 2) both incidence and severity of gall necrosis showed regular spatial patterns in the investigated sites (underdispersed distribution). Incidence of gall necrosis was 14.3±1.9 with a high severity value (2.1±0.06 SE over a maximum of 3) at the time of the survey corresponding to the period of gall wasp adult flights (June/July 2012). Both incidence and severity of brown rot of kernels followed regular spatial patterns as well (Table 2). Incidence of brown rot assessed in October 2012 on kernels after placing them in the damp chamber was very high (74.5±1.9), while average severity was 1.6±0.04 SE (over a max value of 3). *Gnomoniopsis castanea* was consistently found associated with gall necrosis and rotted kernels. Figure 3c presents the percent of isolation from gall fragments (5 for each gall, 200 in total) in site A in 2012 as related to the graded necrosis class scale. No significant differences were found among classes (ANOVA $P>0.05$). Results suggest a consistent isolation of the fungus from all graded necrotic classes including healthy galls. Five additional fungi were obtained from galls in the different necrotic classes but were not further investigated (Figure 3c) .

Gnomoniopsis gall necrosis

Galls necrosis development

Development of gall necrosis during the period April 29-June 20 2011 is reported in Figure 4 and expressed as severity of symptoms. Comparison of independent fits of the 2011 and 2012 data with Akaike's Information Criteria (AICc) revealed that one exponential growth curve was representative of the two datasets. In mid-July the severity value was 2.3 corresponding to 75.4% of galls totally necrotized (class 3). The distribution of symptoms in the four disease classes at the last monitoring date (June 20, 2011 and 28, 2012), before the *D. kuriphilus* adult flight period, is reported in Figure 5. Incidence of gall necrosis was 23.4±1,8 and 34.5±1,7(SE) in 2011 and 2012 respectively. Isolation of *G. castanea* from the subset of galls collected on July 7, 2011 and July 13, 2012, was equally distributed in the four disease classes and is reported in Figure 3a and b. Sixteen additional fungi were isolated from necrotic galls in the 4 classes, among which were two isolates of the chestnut blight fungus *Cryphonectria parasitica* from two fragments of one totally necrotized gall, and seven isolates of *Fusarium* sp from four and three fragments of one totally and one partially necrotized gall.

Impact of gall necrosis on Dryocosmus kuriphilus *vitality*

Assessment of the *D. kuriphilus* life cycle in the two year study revealed an approximate 10-15 days delay in peak of development stages in 2012 compared to 2011 (Figure 7, Supplementary material). The impact of gall necrosis to *D. kuriphilus* vitality in 2011 and 2012 is presented in Figure 6. Galls in Class 3 (totally necrotized) contributed to insect mortality. Highest total mortality was reached in the 2012 in Class 3 with 64.2%. dead individuals *Dryocosmus kuriphilus* larvae mortality was very low in all necrosis classes and it never exceeded 3.2%. Most of the mortality occurred in the adult stage (33.9 and 32.6 in 2011 and 2012 respectively), while high mortality of red eye pupae was recorded in 2012. Overall mortality recorded in 2011 and 2012 was 16.5 and 21.7%, respectively.

In the entomopathogenic test, no significant differences in terms of mortality were observed among treatments of larvae of *G. mellonella* (ANOVA $P > 0.05$). No *G. castanea* outgrowth from treated specimens was observed in the damp chamber.

Discussion

Gnomoniopsis castanea has been recognized as endophyte found in healthy chestnut tissues and organs in the investigated sites in Italy since 2010. The fungus was prevalent in bark, leaves, and buds and rarely found in fruits. Endophytic behaviour of *G. castanea* is not new and has been reported by Shuttleworth et al. (2012) in Australia and by Visentin et al. (2012) in Italy. In the present study, an effect of site on the isolation of *G. castanea* from asymptomatic tissues was observed by PCA analysis. That is, isolation of *G. castanea* was more successful from the site that has been heavily infested by gall wasp since 2006, suggesting a possible interaction between gall wasp infestation and presence of *G. castanea* in asymptomatic tissues. However, the comparison of only two sites cannot be considered proof of an existing interaction, although this possibility cannot be ruled out. Meyer et al. (2015) proposed an interaction between the gall wasp infestation and *Cryphonectria parasitica* isolated from abandoned galls. Turchetti et al. (2012) and Maresi et al. (2013) reported an increase in *Gnomoniopsis* brown rot following the gall wasp infestation in Italy, and speculated an existing interaction between the two organisms. Related to the aims of this study, Magro et al. (2010) demonstrated the interaction between gall wasp and necrotrophic activity of *G. castanea* to galls which appear to be an optimal substrate for colonization by the fungus, and eventually for the production of new inoculum (Maresi et al., 2013). Whether or not gall colonization by *G. castanea* occurs via external inoculum or endophytic colonization is still unclear. Abundant sources of asexual inoculum are produced on nuts in moist conditions, as demonstrated in the present study. Shuttleworth et al. (2012) also suggested that overwintered ascospores released from dead burrs on the floor could account for infection on the plant, specifically for floral infection. The same pattern of infection has been hypothesized for *Sclerotinia pseudotuberosa* , causal agent of black root of chestnut and oak

kernels. An endophytic behaviour has also been demonstrated for this fungus in kernels and other tissues of the plant (Vettraino at al., 2005). Other weakly pathogenic fungi found on chestnut, such as *Amphiporte castanea*, *Diplodina castaneae,* and *Pezicula cinnamomea*, have been found to be endophyte in chestnut tissue (Bissigger and Sieber 1994). Another pathogen causing fruit rot of *C. sativa*, *Phomopsis castanea*, was reported to occur as an endophyte in different organs and tissues, including flowers, leaves, shoots, and fruits, and has been shown to be seed transmitted (Washington, Hood and Stewart-Wade 1999, Wadia et al. 2000). Gall necrosis (and kernel brown rot) found in the sites investigated in this study follow a regular distribution pattern (VM value approaching zero), that is compatible with gall pathogenic colonization from *G. castanea* endophytic inoculum. An external source of inoculum cannot be excluded; however, taking into account the pattern of distribution, it is unlikely that the inoculum source is ascospores produced on kernels and burrs on the ground, since a random distribution would be expected. Furthermore, in orchards especially, very few kernels and burrs are left on the ground after the harvest season. A possible source of homogeneous external inoculum is represented by old abandoned galls still on the tree. These were found to produce abundant asexual inoculum (Maresi et al., 2013). However, the high frequency of *G. castanea* isolations from totally asymptomatic galls (class 0), following a stringent external sterilization procedure, supports the hypothesis that pathogenic colonization of galls comes from endophytic inoculum. Furthermore, data on gall necrosis development in 2011 and 2012 expressed as both incidence and severity, fit unique exponential growth curves in spite of substantial differences in frequency and distribution of precipitation between the two year (427 vs 197 mm in the period March –July 2011 and 2012, respectively) This can be considered an additional indirect demonstration of gall pathogenic colonization from endophytic inoculum. In fact, it is well known that in coelomycetes the abundance of external inoculum, in terms of conidiomatal formation and conidial dispersal, is strictly associated with water availability during the growing season (Nag Raj, 1981). *Gnomoniopsis castanea* is the most abundant taxon isolated from galls either in the endophytic or pathogenic stages on partially to totally necrotized galls. Magro et al. (2010), clearly demonstrated the pathogenicity of this fungus to galls and successfully satisfied Koch's Postulates. However, a cohort of additional fungal species were present in galls examined

in this study which might contribute to the desiccation of the tissues. the present study, a total of 16 different taxa were randomly isolated from asymptomatic and necrotized galls from the study sites, including common saprotrophs such as *Penicillum* spp. and *Aspergillus* spp.; potential pathogenic taxa such as *Fusarium* spp.; and recognized taxa pathogenic to chestnut such as *Cryphonectria parasitica*. Most of these taxa have been previously reported by Meyer et al. (2015) as colonizers of abandoned galls in Switzerland and by Addario and Turchetti (2011), who found some necrotrophic activity by *Fusarium* spp. The presence of *C. parasitica* in necrotized galls is not new, as it was reported by Prospero and Forster (2011) and Meyer et al. (2015) in Switzerland, hypothesizing an interaction between the two alien invasive organisms. The presence of *Fusarium* spp. has been associated with entomopathogenicity to gall wasp (Addario and Turchetti, 2011; Tosi et al., 2015). *Fusarium* spp. isolated from inside galls were reported by Addario and Turchetti (2011) and Tosi et al. (2015), where *Fusarium* spp. was isolated from the bodies of the insect at different development stages. Tosi et al. (2015) demonstrated the entomopathogenicity of *F. proliferatum* to *D. kuriphilus* in laboratory tests on intact or sectioned galls. However, based on results of the present study, the mortality of gall wasp in galls was probably not caused by direct parasitism by *Fusarium* spp., since its presence in galls was negligible (less than 1%). A positive correlation was found between severity of gall necrosis and mortality of *D. kuriphilus* inside the galls before the adult emergence/flying period. A baseline of adult mortality, never exceeding 4%, was recorded in healthy galls. Such mortality can be considered physiological and associated with different causes (Cooper and Rieske, 2010). Larvae were only slightly impacted by gall necrosis. Looking at the life cycle of *D. kuriphilus*, the peak of larval development has been reported to be in the second and third week of May in 2011 and 2012, respectively, corresponding to a nearly null incidence of gall necrosis. Differently, adults are strongly impacted by gall necrosis. The peak of adults present in galls was recorded in late June/early July, corresponding to exponential growth of both incidence and severity of gall necrosis. Excluding a direct entomopathogenic impact of *G. castanea* on *D. kuriphilus*, mortality of adults appear to be related to other factors. Cooper and Rieske (2010), reported that one of the primary mortality factors for gall wasp was the failure of adult gall wasp to emerge. Necrotic gall tissues are dry and hard which could probably make it

difficult for the adult to emerge. The large number of dead red eye pupae, recorded in 2012 in partially and totally necrotized galls, could be due to the delay of about 10 days in the maturation of development stages compared to 2011. The delay may be attributed to the colder temperatures recorded in 2012 compared to 2011, during the period April-May corresponding to bud burst and galls development. As previously reported, differences in temperature and precipitation did not change the gall necrosis development calendar between 2011 and 2012. As a consequence, the delay in life cycle completion probably caused the peak of development to the red eye pupal stage to be late in June during the presence of high severity values of gall necrosis. Their mortality could have been caused by an unsuitable environment within necrotized galls.

In conclusion, gall wasp mortality was associated with gall necrosis before the adult flight period. Gall necrosis could be largely attributed to the pathogenic activity of *G. castanea* on galls. Mortality in totally necrotized galls was relevant, even exceeding 60% . Overall mortality was high as well, in the range of 15-20%, placing gall necrosis by *G. castanea* as one of the most efficient sources of natural biological control of *D. kuriphilus* in Europe. The endophytic behaviour of the fungus guarantees its presence in chestnut tissues independently from dynamics of external inoculum sources. Furthermore, *G. castanea* is massively and regularly present in chestnut in the investigated areas. Unfortunately, this fungus is also recognized as the main cause of brown rot of kernels 'in planta' and in post-harvest (Shuttleworth et al., 2012; Visentin et al., 2012). As a consequence, the increase in its population possibly associated to gall wasp infestation might represent a serious threat for chestnut fruit quality and market.

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Figure captions

Figure 1. Cumulative isolation frequency of *Gnomoniopsis castanea* isolations from asymptomatic chestnut organs and tissues in 2010. Bars represent SE (n=15). Kruskal-Wallis test P<0.0002. Different letters indicate significant differences at the Dunn's Multiple Comparison Test

Figure 2. Principal Component Analysis (PCA) based on isolation of *Gnomoniopsis castanea* from different chestnut tissues: the first and second components accounted for 86.7 and 7.9% of the total variance respectively.

Figure 3. Results of isolation from galls belonging to the four necrosis classes in site C (July 7, 2011 and July 13, 2012) and site A (July 12, 2012): (black bars) negative isolation; (grey bars) *Gnomoniopsis castanea* isolation; (light grey bars) other fungal taxa isolation. Bars represent SE (n=10). Healthy galls, 0; slightly necrotic (0- 30%), 1; partially necrotic (30- 60%), 2; fully necrotic (60-100%), 3. Different letters within groups evidence significant difference at Dunn's Multiple Comparison Test.

Figure 4. Pattern of development of gall necrosis expressed as incidence (A) and severity (B). Comparison of independent fits of the 2011 and 2012 with Akaike's Information Criteria (AICc) revealed that one curve was representative of the two datasets for both incidence and severity.

Figure 5. Mean value (%) of galls in the four necrosis classes for each sample tree in site C on June 20, 2011, and June 28, 2012, at the beginning of gall wasp adult flies. Bars represent SE. Different letters indicate significant differences at the Tukey's Multiple Comparison Test. Incidence (%) of gall necrosis was

Gnomoniopsis gall necrosis

489 23.4±1,8(SE) and 34.5±1,7 in 2011 and 2012, respectively . Healthy galls, 0; slightly necrotic (0- 30%), 1;
490 partially necrotic (30- 60%), 2; fully necrotic (60-100%), 3.
491
492 Figure 6. Mortality of *D. kuriphilus* development stages in galls belonging to the four necrosis classes
493 recorded on June 28 and July 13 in 2011 and 2012, respectively.
494
495 Figure 7 (Supplementary material 3) Peaks of *Dryocosmus kuriphilus* developmental stages, in 2011 and
496 2012.

Table1

TABLE 1. Description of the sweet chestnut sites investigated in the present study

Site	Geographic coordinates	Forest types	Gall wasp first record in the area
A	42°17'31.4"N 12°09'01.9"E	orchards & coppice	2006
B	42°18'38.3"N 12°13'29.5"E	orchard & coppice	2010
C	42°17'42.0"N 12°08'23.3"E	young plantation	2006

Table2

TABLE 2. Incidence (I) severity (S) and Variance-to-mean ratio assessed on July 12 and October zz 2012 for gall necrosis and kernels brown rot respectively . SE indicates the standard error.

Descriptor	Average ± (SE)	Variance to-mean ratio [a]
Gall necrosis		
I (%)	14.3 (1.4)	0.04
S	2.1 (0.06)	0.07
Kernel brown rot [b]		
I (%)	74.5 (1.9)	0.016
S	1.6 (0.04)	0.033

[a] Variation to mean ratio = s^2/x, where s^2 = sample variance and x = sample mean

[b] Assessed for kernels after 10 days dump chamber incubation

Figure1

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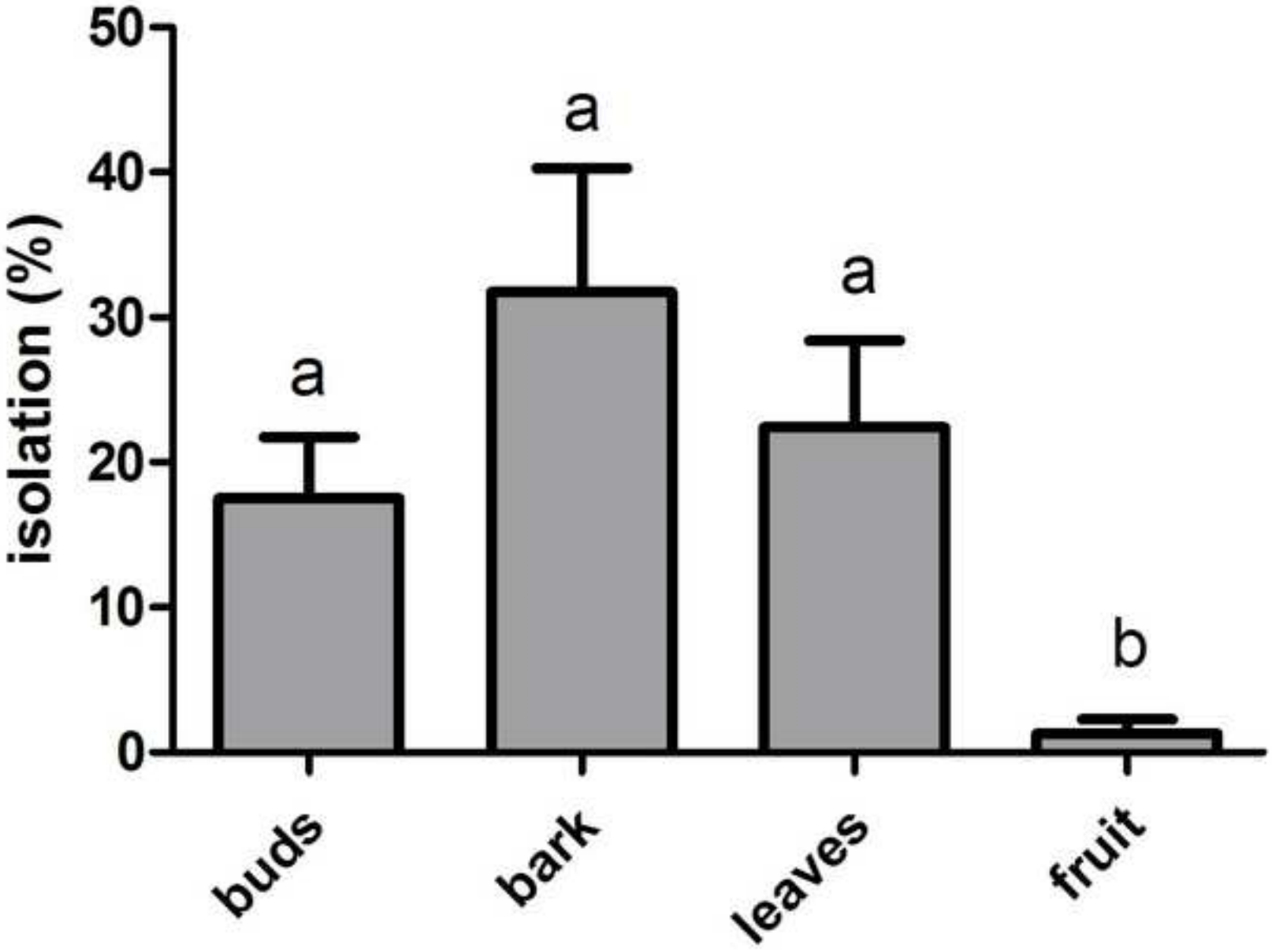

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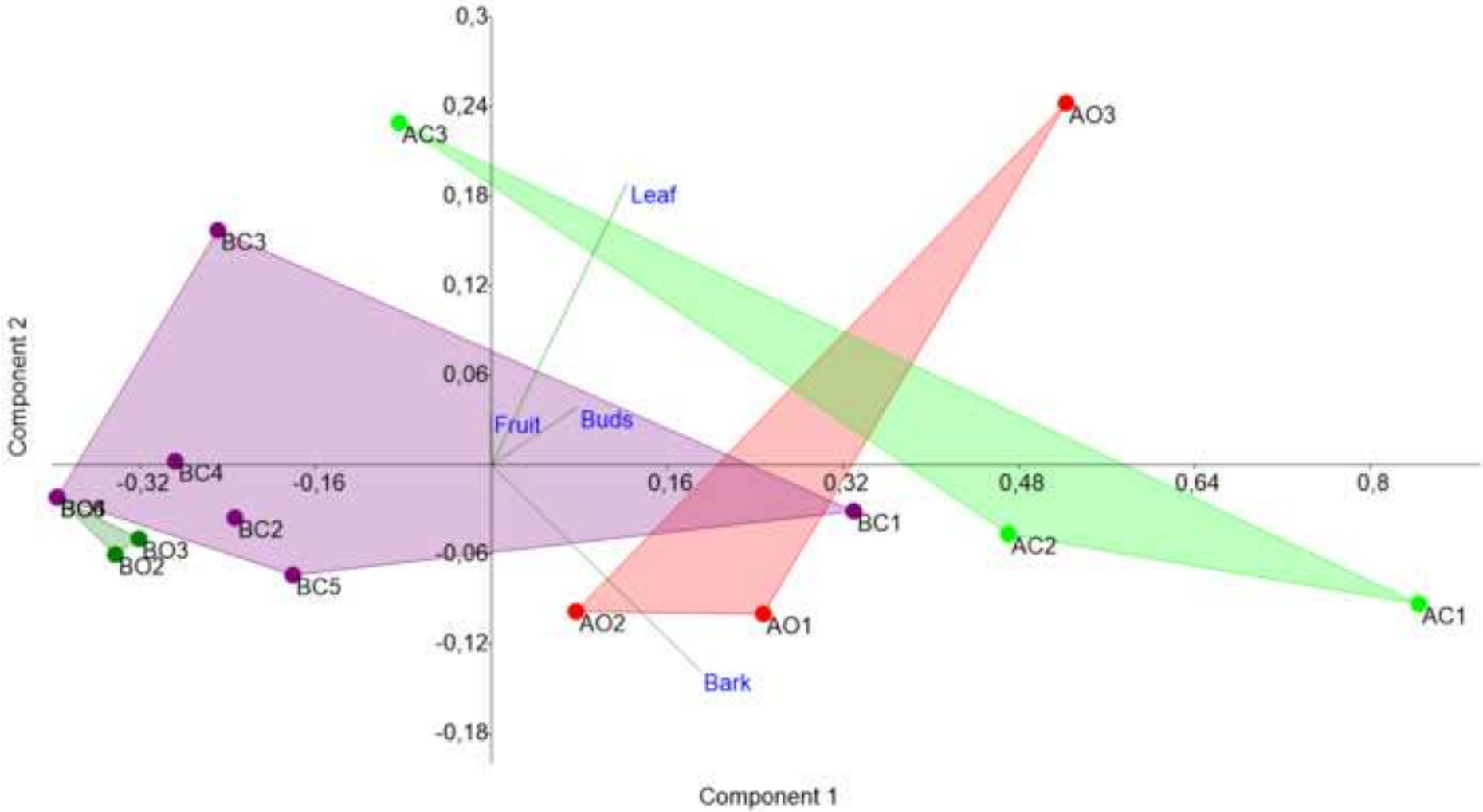

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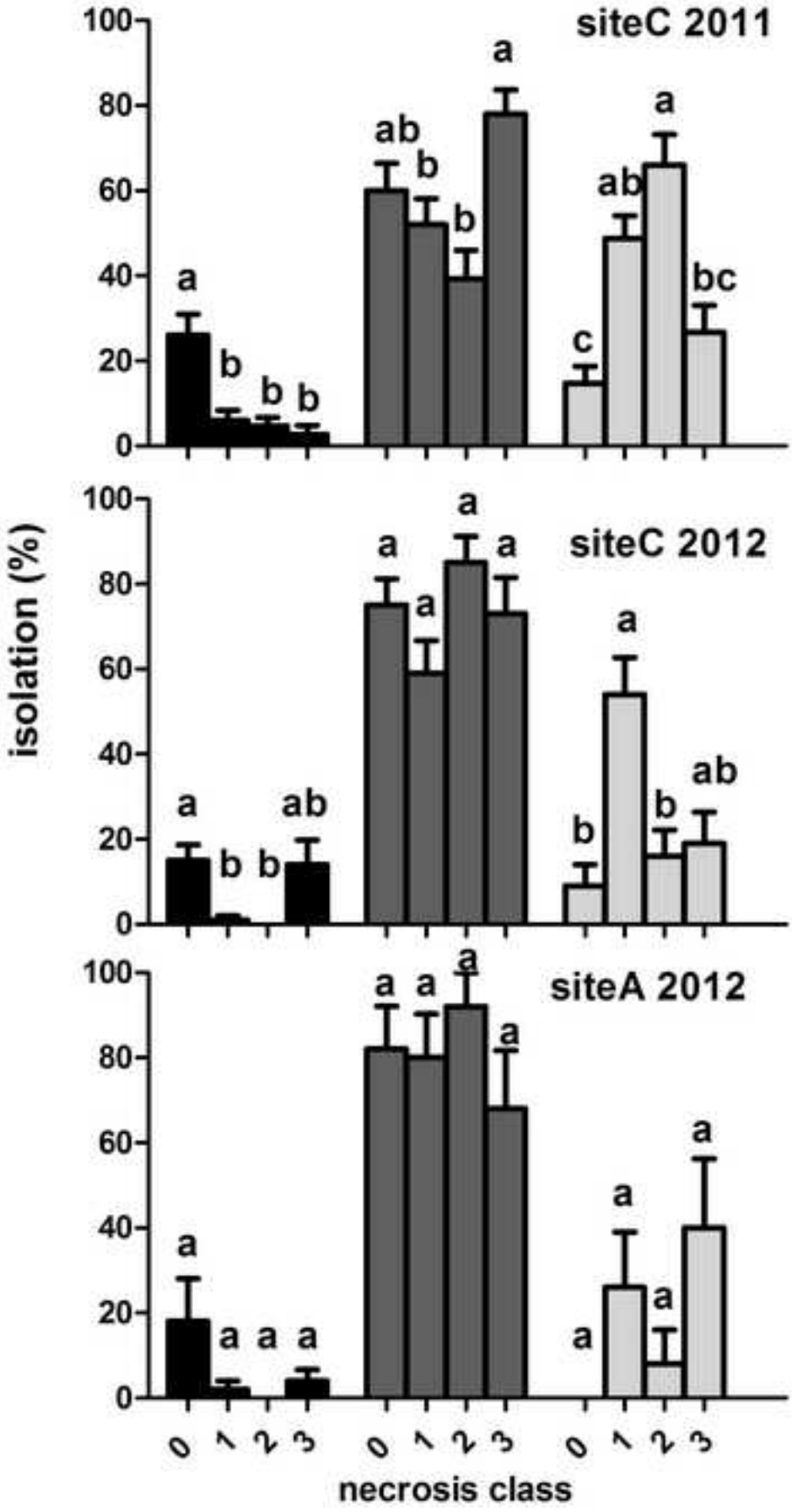

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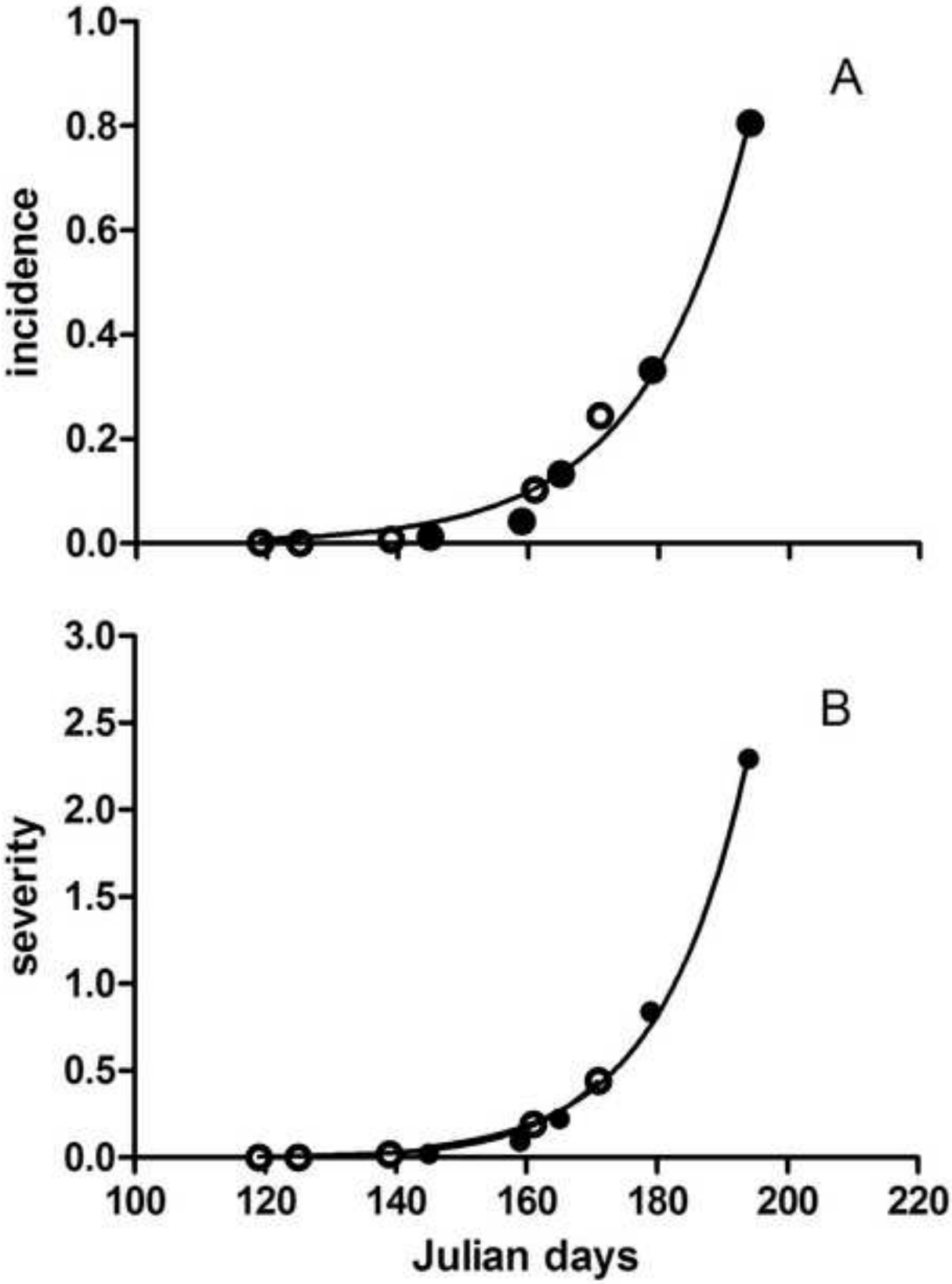

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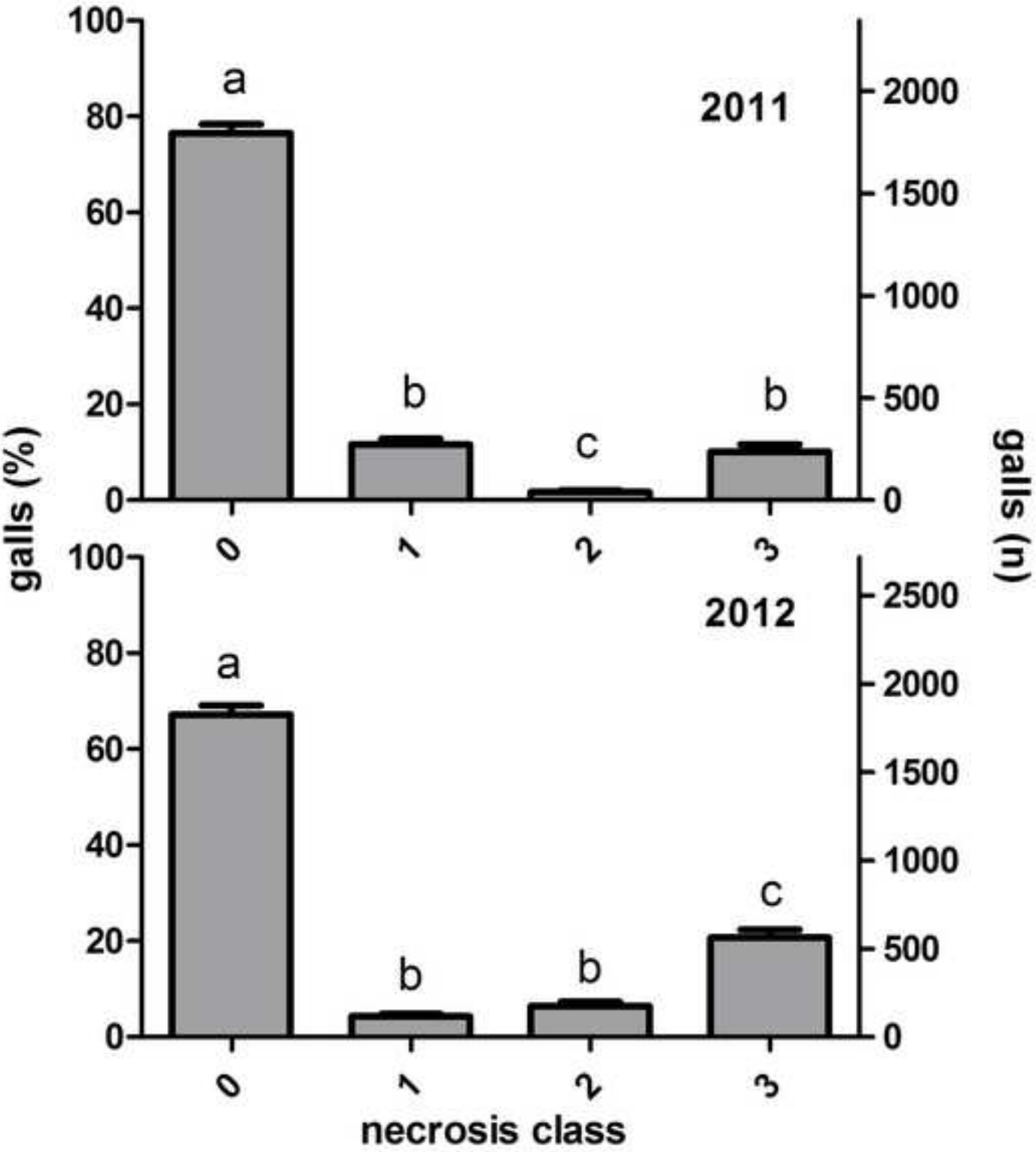

Figure6

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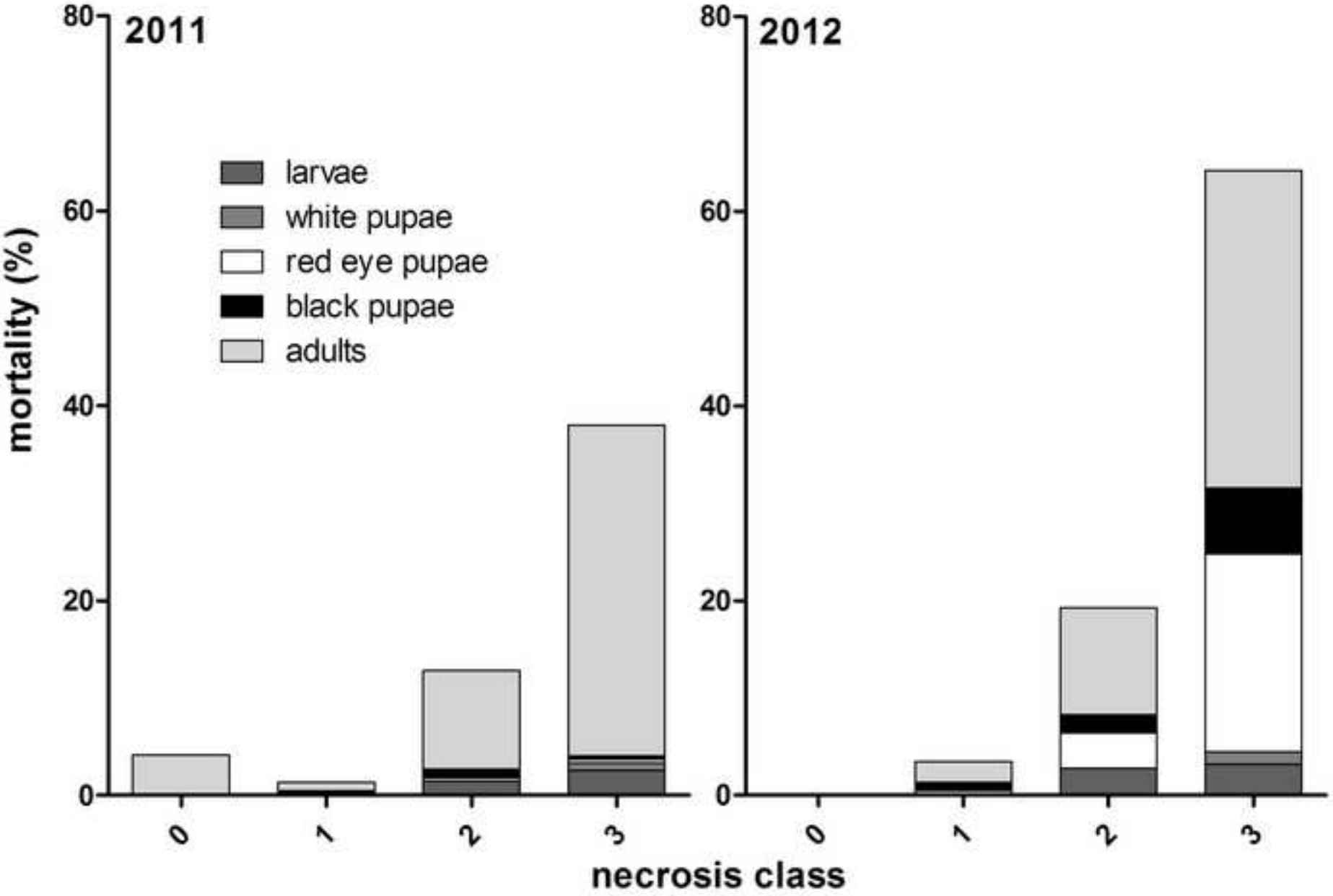

Supplementary File 1

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