Brown rot on nuts of *Castanea sativa* Mill: an emerging disease and its causal agent

Giorgio Maresi (1), Claudia Maria Oliveira Longa (2), Tullio Turchetti (3)

The quality and quantity of nut production are fundamental to the economic viability of chestnut cultivation, yet recent reports indicate that severe damage due to moulds represents a significant problem for growers. We carried out an investigation of the agents of chestnut rot and internal fruit damage in three orchards in Italy. Black and brown rot, as well as insect damage, were found in all the areas examined. Brown rot appeared to be the main cause of damage, affecting 8% to 49% and 2% to 24% of nuts collected from the ground and from burrs, respectively. With respect to morphology and DNA sequencing analyses, fungal isolates obtained from brown rot were homologous with *Gnomoniopsis* sp. obtained from *Dryocosmus kuruhiphus* (Yasumatsu) galls and with *Gnomoniopsis castanea* and *Gnomoniopsis smithogilvyi* described on chestnut in Italy and Australia, respectively. The same fungus was also isolated from the bark of one- and two-years-old healthy shoots at each site, supporting the endophytic behaviour of this rot agent. Brown rot symptoms on nuts associated with *Gnomoniopsis* sp. corresponded with those previously described by several authors and referred to as *Phoma* or *Phomopsis endogena*, suggesting a relationship between these fungi and *Gnomoniopsis* sp. It is to notice that the escalation of brown rot damage in Italy followed several periods of drought and probably the recent invasion of *D. kuruhiphus*, both stress factors for chestnut trees.

**Keywords:** Nut mould, *Gnomoniopsis* sp., *Phoma endogena*, *Phomopsis castanea*, endophytism

**Introduction**

Chesnut cultivation has progressed in Italy and other European countries as a result of a favourable market and the recovery of trees following natural establishment of hypovirulence in chestnut blight (*Turchetti et al. 2008*). Chestnut orchards and stands are still vital to the economy of many mountain communities as elements of the landscape, sources of timber and, in particular, for the production of nuts. The quality of chestnuts is determined by size, shape, color, flavor and texture, features which are very important for both fresh consumption and processing (*Korel & Balaban 2008*) and which are essential to ensuring an advantageous income for growers. These fruits contain sugars, especially monosaccharides and disaccharides such as sucrose, glucose, fructose and raffinose, as well as starch (*Bernardez et al. 2004*). As a consequence, they are highly attractive to insects and fungi, which can cause severe damage and hence pose a considerable problem for chestnut cultivation. Attacks by insect larvae (*e.g.* *Cydia splendana* Hb., *Cydia faggiglandana* Zell. and *Curculio elephas* Gyll.) are well known to be detrimental to the harvesting of healthy chestnut fruits (*Pedrazzoli et al. 2012* ). Several fungi are also capable of colonizing nuts and causing damage: *Phoma endogena* Speg. and *Phomopsis endogena* Speg. Cif. have been described as agents of brown rot, *Sclerotinia pseudotuberosa* Rehm (syn. *Ciboria batschiana* Zopf) can cause black rot, while *Penicillium* spp. and *Penicillium crustaceum* L. Fr. produce greenish moulds. Environmental factors can also influence the quality of fruits (*Ferreira-Cardoso & De Vasconcelos 2009*): low soil water availability and high temperatures are destabilizing factors for normal chestnut growth as they are responsible for decreased plant vigor and standard fruit production and also exacerbate the effect of pest and fungus damage (*Gomes-Laranjo et al. 2004*, *Conedera et al. 2005*).

The recent appearance of the invasive Asian chestnut gall wasp (*Dryocosmus kuruhiphus* Yasumatsu) in Italian chestnut stands and orchards is causing new concern for the sustainability of chestnut cultivation (*Bosio et al. 2010*). An unusual level of fruit damage was reported in Piedmont, more or less in correspondence with the wasp invasion, (*Gentile et al. 2010*) and subsequently in chestnut-producing areas of Italy, such as Trentino, Emilia Romagna, Tuscany, Lazio and Campania (*Maresi, Poli & Sigillo, personal comm.*). The damage in Piedmont was initially attributed to *Gnomonia pseudocoe* (*Gentile et al. 2010*) and recently related to *Gnomoniopsis castanea*, a proposed new species (*Visentin et al. 2012*). Attacks of brown rot have also occurred in chestnut cultivations in Australia, where the agent was identified as *Gnomoniopsis smithogilvyi* (*Shuttleworth et al. 2012*). *Gnomoniopsis sp.* has been found to be associated with necrosis of *D. kuruhiphus* gall galls (*Magro et al. 2010*), raising interest in its use as a biocontrol agent against this hymenopteran (*Vannini et al. 2012*).

In this study, we investigated brown rot damage reported in three different Italian chestnut-growing areas in Piedmont, Trentino and Tuscany with the aim of isolating and identifying the causal agents, verifying their homology, comparing their morphology, testing their pathogenicity and formulating hypotheses regarding the factors responsible for their spread.

**Material and methods**

**Study sites and their environmental features**

Chestnut orchards were selected in October 2011 in three Italian regions: Boarda (Val di Susa, Turin, Piedmont), Montesenario (Mugello, Florence, Tuscany) and Crosano (Val d’Adige, Trento, Trentino-South Tyrol). The general environmental features of each stand are reported in Tab. 1.

Monthly mean temperatures and accumulated precipitation for 2011 were obtained from meteorological stations closest to each of the study sites: Castel Borello (630

© SISEF http://www.sisef.it/iforest/
m a.s.l. - Società Meteorologica Italiana network), Borgo San Lorenzo (193 m a.s.l. - LaMMA network), and Besagno (382 m a.s.l. - FEM agro meteorological network). Only raw, non-validated, non-gap-filled meteorological data series were used. For Besagno, monthly mean temperatures and accumulated precipitation recorded during the vegetative period (April-September) were extracted from data for the whole period 2001-2011. These monthly values were compared with the average of the 10 years data series.

**Sample collection and laboratory testing**

Three chestnut trees were randomly selected in each of the orchards investigated. Forty nuts were collected from burs still attached to the branches of each tree and sixty nuts were collected from the ground under the same trees. Burs were examined for perithecia presence. Fruit samples were packaged separately in paper bags and immediately transferred to the laboratory for assays. Each nut was dissected and examined: fruits showing brown rot were separated from nuts affected by black rot and selected for isolation. A further 10 nuts were collected from ground in each area, stored in field conditions and evaluated for the presence of fruiting bodies.

Ten shoots were randomly collected from the crown of each tree, placed in plastic bags and stored at 4 °C for laboratory assay.

**Fungal isolation and identification**

(A) From nuts

Tissue from the initial decaying endosperm and from mummified fruits were surface-sterilized for 1 min in 70% ethanol, 5 min in 1.25% sodium hypochlorite and 30 sec in 70% ethanol. They were then rinsed twice in sterile, distilled water and blotted on dry sterile filter paper. Using a sterile scalpel, fragments of infected tissue were plated onto Potato Dextrose Agar (Difco, USA) containing 5% sucrose and 2 ml of mycelia scraped from the surface of a 7-10 day culture growing on PDamb. DNA was also extracted directly from initial decaying, brown tissue and chalky tissues in symptomatic nuts from Crosano. A total of seven strains were so assayed.

DNA was extracted using the Nucleospin1 Plant Kit according to the manufacturer’s instructions (Macherey-Nagel, Düren, Germany). Internal transcribed spacer regions 1 and 2, including the 5.8S rDNA, were amplified and sequenced using the primers ITS5 and ITS4 (White et al. 1990). Gene fragments were amplified in 25 µl reactions on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) under the following reaction conditions: 12.5 µl of GoTag Green Master Mix (supplied in 2× Green GoTaq® Reaction Buffer pH 8.5, 400µM of each dNTPs and 3µM MgCl2, - Promega, USA), 0.40 µM of each primer, 10 ng of DNA template and 9.5 µl of nuclease free water. The thermal cycler programme was: 2 min at 95° C followed by 30 cycles of 30 sec at 95° C, 30 sec at 55 °C, and 1 min at 72 °C, with a final extension period of 10 min at 72 °C. Amplification products were separated by electrophoresis on 1% agarose gel at 100V for 30 minutes in TBE buffer (Tris-borate EDTA). The gel was stained with SyberSafe (Applied Biosystems, California, USA) and visualized on a UV transilluminator (Biorad) to assess PCR amplification.

PCR products were purified using Exo-SAP (Euroclone S.p.A., Italy) following the manufacturer’s instructions. Amplified products were sequenced with the Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3130xl Genetic Analyzer. BLASTN comparison of the amplicon sequences was carried out using the NCBI database to confirm the identity of the strains. Raw sequences were edited using Sequencer version 4.5 for Windows (Gene Codes Corporation, Ann Arbor, MI, USA) and deposited in the GenBank.

Nucleotide sequences were aligned with other fungal sequences (Gnomoniopsis spp., Phomopsis spp. and Phoma spp.) from NCBI using the Mega 4.1 software (http://www.megasoftware.net). A distance matrix was generated and an evolutionary tree for the datasets was inferred by the neighbor-joining method. The stability of relationships was assessed by bootstrap analysis of the neighbor-joining data based on 1000 replicates.

(B) From shoots

An 8-10 cm section was cut from each shoot of the current year's and the two-year growth. Each sample was surface disinfected for 10 sec in 95% ethanol followed by 4 min in 2% NaOCl solution with 2 drops of Tween 80® (Stanosz et al. 2001). Ten fragments were cut from various positions on the bark tissue of each sample and placed on potato dextrose agar (PDA, Oxoid) in Petri dishes, five in each dish.

Isolations were incubated at 25 ± 1 °C for 7 days in the dark, after which the growing fungi were identified by their cultural characteristics. The number of fragments colonized by each type of culture was linked with the original sample and plot.

**DNA analysis**

DNA assays were carried out using cultures from individual monoclonal strains randomly selected from each area, two from Crosano and one from each of the other sites. Genomic DNA was extracted from approximately 50 mg of mycelia scraped from the surface of a 7-10 day culture growing

<table>
<thead>
<tr>
<th>Site</th>
<th>Boorda</th>
<th>Montesenario</th>
<th>Crosano</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevation</td>
<td>565</td>
<td>650</td>
<td>550</td>
</tr>
<tr>
<td>Exposure</td>
<td>North</td>
<td>North West</td>
<td>North East</td>
</tr>
<tr>
<td>Morphology</td>
<td>Terrace</td>
<td>Terrace</td>
<td>Slope</td>
</tr>
<tr>
<td>Slope</td>
<td>0%</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>Substrate</td>
<td>Alluvial</td>
<td>Sandstone</td>
<td>Turbidite</td>
</tr>
<tr>
<td>Management</td>
<td>cultivated</td>
<td>Semi-cultivated</td>
<td>Semi-cultivated</td>
</tr>
<tr>
<td>Irrigation</td>
<td>Twice (July, August)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geographic</td>
<td>45.120369° N</td>
<td>43.891551° N</td>
<td>45.827764° N</td>
</tr>
<tr>
<td>Coordinates</td>
<td>7.187719 E</td>
<td>11.330627 E</td>
<td>10.975599 E</td>
</tr>
</tbody>
</table>

**Main environmental features of the study sites.**
70% ethanol, 5 min in 1.25% sodium hypochlorite, 30 sec in 70% ethanol and rinsed in sterilized tap water. After this treatment, the fruits were artificially infected with each of the mononodial strains selected for the DNA assay. An artificial wound 0.5 cm deep was made in the pericarp at the apical part of the fruit using a sterilized scalpel, as reported by Voglino & Bongini (1917). A fragment of mycelium collected from a ten-day-old culture was placed in the wound, which was then covered with masking tape; the nuts were then stored in Petri dishes (25 cm in diameter). Fifteen replicates were run for each strain, another fifteen were wounded but not inoculated to serve as controls. The remaining fifteen unsterilized nuts were wounded as additional controls. All the treated and untreated nuts were stored at 25 °C in the dark. After twenty days all replicates were examined for disease and re-isolations were carried out from the symptomatic nuts on plates containing PDAmb.

**Statistical analysis**

The statistical analysis concerned the symptoms presence and isolations from nuts and shoots. For each symptom detected on nuts, considering the mean value of the three sampled trees, the three sites were compared using the Chi-square ($\chi^2$) test for 2-way tables (coupling the sites two by two). The same analysis was used for fungal isolation from bark tissue of shoots. $\chi^2$ values were compared with critical $\chi^2$ value at significance level $\alpha=0.05$ and 1 degree of freedom.

**Results**

**Study sites and environmental features**

The three sites, located at similar altitudes, vary in exposure and morphology (Tab. 1). They are characterized by three different, but mainly acidic, geological substrates (Alluvial, Sandstone and Turbidite). Different management systems are in operation in the three orchards: Boarda is regularly cultivated (pruned and mowed) and irrigated twice during the summer, while Montesenario and Crosano are partially cultivated: mowing and harvesting are the only agricultural practices employed.

During 2011, high temperatures (over 20 °C) were recorded for August and September, while in the same months rainfall was less than 100 millimeters at all three stations (Fig. 1). In Besagno, August and September 2011 monthly mean temperatures were higher (1.3 and 2.7 °C, respectively) than the average of 10 years series for the same months (data not shown), while precipitations were lower of about 20 mm with respect to the same months and about 50 mm less for April and May. June and July were wetter with precipitations higher by 50 mm more than the 10 years series average for the same months (data not shown).

*D. kuriphilus* galls were observed in abundance at all the study sites.

**Sample collection and description of symptoms**

Significant differences were found between healthy fruits collected from the ground at Crosano and those collected at the other two sites (Tab. 2). The percentages of nuts displaying insect damage collected from the trees and from the ground was recorded for each of the study sites (range 15-23%). *C. splendana* and *C. fagiglandana* larvae were detected in the affected nuts and fungal rot associated with larval galleries was also observed.

The main symptoms recorded in the remaining rotten nuts of the samples were of black rot (Fig. 2a) and brown rot (Fig. 2b).

Initial and final symptoms coexisted in most of the nuts and, in a few cases, black and brown rot were observed together. Both rots were present in the nuts collected from the ground and in those still on the trees, although the extent of damage was greater in the nuts on the ground: black rot affected 8% of nuts gathered from the ground and 2% of nuts on the trees in the Boarda area, while the figures for Montesenario were 7% and

### Tab. 1

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Boarda</th>
<th>Montesenario</th>
<th>Crosano</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Symptoms (%)</td>
<td>62</td>
<td>65</td>
<td>32</td>
</tr>
<tr>
<td>Black rot (%)</td>
<td>8</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Brown rot (%)</td>
<td>11</td>
<td>8</td>
<td>49*</td>
</tr>
<tr>
<td>Insect damage (%)</td>
<td>19</td>
<td>20</td>
<td>16</td>
</tr>
</tbody>
</table>

### Tab. 2

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Nuts from the ground</th>
<th>Nuts from burrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Boarda</td>
<td>Montesenario</td>
</tr>
<tr>
<td>No Symptoms (%)</td>
<td>62</td>
<td>65</td>
</tr>
<tr>
<td>Black rot (%)</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Brown rot (%)</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Insect damage (%)</td>
<td>19</td>
<td>20</td>
</tr>
</tbody>
</table>

**Fig. 1** - Monthly precipitation and mean temperatures recorded during 2011 at the three meteorological stations closest to the study sites.

**Fig. 2** - Percentages of damaged nuts found in the samples collected from the three different chestnut orchards. Chi-squared test 2-way tables was performed. Critical $\chi^2$ value was at $\alpha=0.05$ and df = 1. (*): values which differ in a significant way.
1%, respectively, and for Crosano 3% and 0%, without significant differences among sites (Tab. 2).

Brown rot was widespread in the Crosano area, where 49% of the nuts from the ground and 24% of the nuts from the trees showed symptoms. In this stand, the disease was significantly more present than in the other two sites either in nuts taken from the ground (vs. Boarda: $\chi^2$=19.21, p<0.05; vs. Montesenario: $\chi^2$=23.64, p<0.05) or from the burrs ($\chi^2$=6.27, p<0.05). The brown rot

Fig. 2 - (a) Nut completely destroyed by black rot due to *Sclerotinia pseudotuberosa*: it generally starts in the torch, producing grey coloration on the endosperm that rapidly turns blackish with evidence of black mycelium in the rotten tissue. (b) Nut affected by brown rot: the fungus initially causes light brown coloration on the margin of the endosperm, followed by loss of tissue consistency as it whitens and hardens, resulting in a chalky appearance. (c) Black pycnidia from brown rot tissue erupting below the episperm. (d) Culture of *Gnomoniopsis* sp.: colony on PDAm. (e) Conidia obtained from stroma or pycnidia in cultures and from pycnidia on nuts. (f) Pycnidia of *Gnomoniopsis* sp. on Asian chestnut wasp gall.

Tab. 3 - Comparison of conidiomata, conidia and cultural characteristics of fungi associated with brown rot obtained in this study compared with other findings reported in the literature. All studies cited reported the fungus on *Castanea sativa*. (n.r.): not reported.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Perithecia (μm)</th>
<th>Conidiomata (μm)</th>
<th>Conidia (μm)</th>
<th>Cultural morphology</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gnomoniopsis</em> sp. (this study)</td>
<td>-</td>
<td>200 x 158</td>
<td>5.1-8.9 x</td>
<td>Light brown mycelium. Orange masses of conidiomata in concentric rings</td>
<td>Brown rot, endophytic on shoots Brown rot</td>
</tr>
<tr>
<td><em>Phoma endoidea</em> (Spegazzini 1879)</td>
<td>n.r.</td>
<td>n.r.</td>
<td>1.9-3.9</td>
<td>Not reported</td>
<td>Brown rot</td>
</tr>
<tr>
<td><em>Phoma endoidea</em> (Voglino &amp; Bongini 1917)</td>
<td>-</td>
<td>200 x 160</td>
<td>6-8 x 2-3</td>
<td>Light brown mycelium. Orange masses of conidiomata in concentric rings</td>
<td>Brown rot</td>
</tr>
<tr>
<td><em>Phoma endoidea</em> (Ride &amp; Gudin 1960)</td>
<td>-</td>
<td>n.r.</td>
<td>6-7.5 x 2.4</td>
<td>Light brown mycelium. Orange masses of conidiomata in concentric rings</td>
<td>Brown rot</td>
</tr>
<tr>
<td><em>Gnomoniopsis</em> sp. (Magro et al. 2010)</td>
<td>-</td>
<td>n.r.</td>
<td>8.1 x 2.4</td>
<td>White mycelium in concentric rings</td>
<td>White mycelium in concentric rings Brown rot, necrosis on shoots and leaves Colonisation of D. kuriphilus galls</td>
</tr>
<tr>
<td><em>Gnomoniopsis castanea</em> (Visenti et al. 2012)</td>
<td>244-268 x 146-190</td>
<td>5.5-8.0 x</td>
<td>2.0-3.0</td>
<td>Light brown mycelium. Orange conidiomata in concentric rings</td>
<td>Colnisation of D. kuriphilus galls Brown rot, endophytic on shoots</td>
</tr>
<tr>
<td><em>Gnomoniopsis smithogilvyi</em> (Shuttleworth et al. 2012)</td>
<td>238-242</td>
<td>6.5-9.5 x</td>
<td>2.0-4.0</td>
<td>Greyyish brown mycelium with concentric rings of conidal stroma</td>
<td>Brown rot, endophytic on shoots</td>
</tr>
</tbody>
</table>
was also present in the other areas without significant differences: in Boarda 11% of
the nuts from the ground and 2% of the nuts from the trees were affected, while in
Montesenario the figures were 8% and 4%, respectively. In neither of these areas the dis-
case was observed in previous years. No dif-
fences between the three sites were ob-
served for insect damage.
Storage of the nuts, whether controls or
producing fructifying bodies in natural condi-
tions, exacerbated the brown rot as all the
fruits displayed chalky tissue after three
months. An abundance of pycnidia, first
brownish-grey then blackish, appeared on
the surface of the endosperm below the epi-
sperm (Fig. 2c). These fructifications were
also observed in the invaginations and inside
the endosperm where holes and cavities in
the rotten tissue were detected. Conidia
collected from these pycnidia were analogous to
those obtained from the cultures (Tab. 3). No
perithecia were observed on collected burrs
and on infected nuts.

Fungal isolation and identification

Cultures of greyish-black mycelia were
isolated from black rot tissue and identified
as the species Sclerotinia pseudotuberosa
(syn. Ciboria batschiana, anamorph Rhaco-
diella castaneae) according to the morpholo-
gical diagnostic criteria described by Ellis &
Ellis (1997).

Colonies with light brown mycelium were
isolated from initial brown tissue and white,
chalky tissue. Orange masses of conidial
growth appeared in concentric rings on the
surface of the cultures (Fig. 2d). On older
colonies, blackish, ellipsoid pycnidia (158-
200 μm) also produced conidia, which were
generally ovoid, oval or oblong (5.15-8.91
μm x 1.90-3.96 μm - Fig. 2e). Based on the
above characteristics the fungus was identi-
fied as Gnomoniopsis sp. (Tab. 3). Homo-
geneous morphological characteristics were
found in all the mononconidial strains from
all the study sites. Observed differences in
size between conidia produced on chestnut
fruits and those produced on nutritive media
were non-significant.

High percentages of positive isolation were
obtained from the bark tissue of both one-
year and two-years-old shoots at each site
(Tab. 4). Most of the colonies displayed the
same characteristics as the fungus obtained
from isolations from brown rot nuts. As for
1-year-old shoots, Crosano recorded the
highest presence of Gnomoniopsis sp with
respect to the Boarda and Montesenario (χ²=
21.9 and 27.4, respectively; p<0.05). Colo-
nies of the brown rot agent were found on
70% of the fragments, on average. Boarda
33%) and Montesenario (48%) showed sim-
ilar values as for one-year-old shoots, while
they differed for 2-years-old shoots
(χ²=4.6; p<0.05). Sparodic isolates of

Cryphonectria parasitica were obtained,
though no differences in frequencies were
observed among sites. Crosano recorded also
a presence of other fungi higher than
Montesenario in one-year and two-years-old
shoots (χ²=13.21 and 8.99, respectively;
p<0.05). Tab. 3 reports a comparison of the
morphological characteristics of these iso-
lates of Gnomoniopsis sp. with those of fungi
associated with the same symptoms reported
by other authors (Spegazzini 1879, Voglino
& Borgini 1917, Ridé & Gudin 1960,
Magro et al. 2010, Visentin et al. 2012,
Shuttleworth et al. 2012).

DNA analysis

Morphological identification of fungal iso-
lates was confirmed with molecular data ob-
tained from DNA amplification. Four ITS
sequences obtained from the DNA of fungal

Tab. 4 - Mean percentage of fungi isolations from bark tissue of healthy shoots (one- and
two-years old) of chestnut plants. The Chi-squared test for 2 way table was performed (pres-
ence/no presence for each isolate and coupled sites). Critical χ² value at α=0.05 and df=1.
(⁎): values which differ in a significant way.

<table>
<thead>
<tr>
<th>Shoots</th>
<th>Species</th>
<th>Crosano</th>
<th>Montesenario</th>
<th>Boarda</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 year old</td>
<td>Gnomoniopsis sp.</td>
<td>70⁎</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Cryphonectria parasitica</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>other fungi</td>
<td>21⁎</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Sterile fragments</td>
<td></td>
<td>9</td>
<td>61</td>
<td>55</td>
</tr>
<tr>
<td>2 years old</td>
<td>Gnomoniopsis sp.</td>
<td>71⁎</td>
<td>48⁎</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Cryphonectria parasitica</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>other fungi</td>
<td>17⁎</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Sterile fragments</td>
<td></td>
<td>9</td>
<td>47</td>
<td>55</td>
</tr>
</tbody>
</table>
cultures and three sequences obtained directly from nut tissue were analyzed. A Megablast search in NCBI revealed the above fungal specimens having the highest similarity with the *Gnomoniopsis* sp. strain M6EmB (JN793536.1), with identity rates in the range of 99-100% (Tab. 5).

Phylogenetic analysis of *Gnomoniopsis* spp., *Phomopsis* spp. and *Phoma* spp. revealed their classification in three distinct groups (Fig. 3), and a very close relationship between the *Gnomoniopsis* sp. strains from this study and those found in GenBank derived from *Dryocosmus* galls (Viterbo) and chestnut rot in Italy (*Torino* - *Gnomoniopsis castanea*), Australia (*Gnomoniopsis smithogilvyi*) and New Zealand, as well as with strains from India whose host was not specified in the database (Fig. 3). The chestnut *Gnomoniopsis* sequences seem quite different from other *Gnomoniopsis* strains detected in the USA on other plant species. The *Phomopsis castanea* sequence is completely different from *Gnomoniopsis* ones; this strain derived from *Castanea mollissima* in China, but no other information are recorded.

Artificial inoculation of nuts

All fifteen nuts assayed to test their healthiness were found to be uncontaminated by moulds.

The *Gnomoniopsis* isolates tested engendered symptoms of brown rot and were detected in most (61 to 71%) of the artificially infected nuts. No differences between the strains were evident (Tab. 6). The disinfectants and wounded controls did not show any symptoms, while only two (13%) of the wounded but non-disinfected controls showed initial symptoms of colonization by *Penicillium* sp.

### Discussion and conclusions

A high degree of damage was found in all three orchards sampled and both black rot and brown rot fungi were common, especially in the Crosano orchard where more than half of the crop appeared to be affected by mould fungi. Insect damage was also severe, confirming its destructiveness in nut production.

*Sclerotinia pseudotuberosa* is known to be a ubiquitous endophyte capable of direct nuts colonization, starting with infection of the flowers (Vetraino et al. 2005). Warm, dry weather during the harvest period, as recorded in 2011, may have restricted the presence of the fungus in the nuts sampled. Indeed, several authors consider these conditions to be the main factors constraining the spread of black rot (Sieber et al. 2007, Delaur & Morelet 1979, Ridé & Gudin 1960). Nonetheless, it is worth noting that black rot was present in all the study areas and could pose a serious problem for nut storage in the absence of water-curing treatments (Migliorini et al. 2010).

The unusual spread of brown rot, the high incidence of damage caused by this disease and the extent of the damage found in Crosano justifies growers’ worries about this disease, which has been highly destructive in other parts of Piedmont and Tuscany (Visentin et al. 2012, Poli, personal comm.). The presence of brown rot in fruits still in the burr exacerbates the already harmful impact of this disease and reduces the quality and quantity of chestnuts with consequences for the economic viability of cultivation.

*Gnomoniopsis* sp. was found to be the causal agent of brown rot in all three areas. In addition to genetic analyses, culture morphology and the shapes and sizes of conidia also confirm homology of the isolated strains with those recently found in association with *D. kuriphilus* galls in the Viterbo area (Monti Cimini - Magro et al. 2010). We were also able to confirm a complete homology with the newly described *Gnomoniopsis castanea* (Visentin et al. 2012) and *Gnomoniopsis smithogilvyi* (Shuttleworth et al. 2012, 2013). As suggested by Vannini et al. (2012) and Visentin et al. (2012), evidence by isolation from bark support a massive presence of this fungus as endophytic in one- and two-year-old shoots. This behavior could explain its ability to colonize gall tissue and to infect nuts still in burrs, maybe in bud burst. It could also explain its sudden appearance in various chestnut-growing areas of Italy, because probably already present on trees. Nevertheless, this fungus, like *S. pseudotuberosa*, was not reported in previous investigations on endophytism in chestnut shoots carried out in Switzerland (Bissegger & Sieber 1994). Other fungi belonging to the *Gnomoniopsis* genus were also described as endophytic on *Fagaceae* (Walker et al. 2010). It is worth noting that isolation of *Gnomoniopsis* was not related to bark alteration (cankers or necrosis) and that no fructification was observed on bark tissue in the present study, while pycnidia were found on galls instead. The common presence of this fungus on chestnut tissue suggests a possible role in checking gall wasp populations by colonizing the gall tissue and perhaps the insect inside chambers (Vannini et al. 2012), but its pathogenicity on fruits is a formidable constraint for its potential use as a bio-control agent.

According to the owner of the Crosano orchard, brown rot has been present since 2003, although its presence has increased in the last three years. Its emergence does not appear to be related to the appearance of *D. kuriphilus* infestations in Trentino, which was first recorded in the province in 2007 and far away from the stand examined (Salvadori et al. 2007). The recent finding (January 2013) of *Gnomoniopsis* pycnidia on galls which remained overwintering on trees (Fig. 2f - Maresi G, personal comm.) calls for a more detailed investigation on the possible role of *D. kuriphilus* galls in the spread of the brown rot fungus.

Meteorological records report a warm, dry period in all the study areas during the 2011 vegetative season, but in the area least affected (Boarda) irrigation helped the trees. Stress and drought periods have been common in the Crosano area since 2003. Plant stress brought about by drought and gall
wasp attacks may have favored brown rot spread in central and northern Italy. Afflic-
ted and suffering chestnut trees are likely to be highly susceptible to infection by endo-
phytes or latent pathogens such as Gnomoni-
opsis sp. They have been to chestnut blight (Turchetti et al. 2010). In a context of
climate change, new interesting perspectives arise for the concept of parasitism: asympto-
matic fungi may become virulent and pro-
duce disease symptoms under certain envi-
rnmental conditions (Brown et al. 1998,
Slippers & Wingfield 2007, Rodriguez et al.
2009, Krabel et al. 2013). It is worth noting
that temperatures around 27 °C are consid-
ered optimal for the growth of Phoma endo-
gen, the causal agent of brown rot, ac-
ding to Ridé & Gudin (1960).
Gnomoniopsis sp. was isolated from initial
brown decaying tissue, white chalky tissue
and blackish fruiting bodies on decayed en-
dosperm. There is a close correspondence
between these symptoms and those of Pho-
ma endogena, first described by Spezazzini
(1879) on nuts in the Veneto region (nor-
thern Italy). Voglino & Bongini (1917) gave
an accurate account of the degradation of in-
fected nuts collected in Piedmont, and the
symptoms and pycnidia described, as well as
the photographs, closely match those of
Gnomoniopsis sp. Servazzi (1941) used the
term “mummification” for the same pheno-
menon observed on nuts from the same area
(Piedmont), while Ciferri (1951) reported that
Servazzi’s description corresponded with the
symptoms he observed on nuts col-
lected near Cuneo (Piedmont). He proposed
the new name Phomopsis endogena based
on the presence of a very few Phomopsis
type β conidia and suggested that P. endo-
gen may be identical with Phomopsis viter-
bensis from the Viterbo area described by
Camici (1948). Moreover, working with
Voglino & Bongini’s (1917) original strains,
he confirmed homology in morphology
between Phoma and Phomopsis endogena.
Ciferri also suggested that this fungus was
already present in the nut during fructifica-
tion and therefore in burrs. Remarkably,
all these reports refer to the areas where the
main attacks of Gnomoniopsis sp. are cur-
cently being observed. Ridé & Gudin (1960)
introduced the term “brown rot” (pourtrui
brune) and revived the name Phoma endo-
gen for the absence of type β conidia: their
descriptions of the mycelium and symptoms
closely match those reported by the afore-
mentioned authors and those described in
this paper. They also hypothesized that in-
fec tion occurred at early fructification stages,
that P. endogena was possibly endophytic,
and that there was a relationship between the
action of the fungus and warm temperature
dry conditions. Breisch (1995) and Pra-
tella (1994) showed clear pictures of P. endo-
gen attacks in their papers, which per-
fectly match the symptoms observed in the
present study. The same rot disease has also
been ascribed to Gnomonia pascoe in New
Zealand (Smith & Agri 2008). Ho (pers.
comm.) recently pointed out the genetic cor-
respondence between P. castanea, P. endo-
gen and Gnomoniopsis sp. in New Zealand,
while in Australia Shuttleworth et al. (2012,
2013) put forward the name Gnomoniopsis
smithogilvyi instead of G. pascoe for the
agent of brown rot damage. Eventually, the
same fungus was described in Piedmont as
Gnomoniopsis castanea (Visentin et al. 2012).
Unfortunately, no Phoma or Pho-
mopsis endogena cultures or DNA sequen-
ces are recovered for comparison with Gno-
moniopsis sp.
Montealegre & Gonzalez (1986) in Chile and
Washington et al. (1997, 1999) in Aus-
tralia both indicated Phomopsis castanea as
agents of brown rot in Castanea sativa or-
chards, though they did not describe or re-
cord type β conidia. Phomopsis castanea
was instead recovered from cankers on small
branches and shoots of European chestnut
but not on nuts (Saccardo 1879, 1884, 1931,
Petrak 1921, Moriondo 1963, Belisari &
Scortichini 1993). Recently, a Phomopsis
castanea mollissima was described on leaves
but not on fruits of Castanea mollissima in
China (Shu-Xia & Hong-Bing 2010). On
this basis, Smith & Agri (2008) clearly dis-
tinguished between Phomopsis or Diaporthe
castanea found on chestnut older branches
and Gnomonia pascoae agent of brown rot,
then described as Gnomoniopsis by Shuttle-
worth et al. (2012).
In conclusion, Phoma endogena, Phomop-
sis endogena, Gnomonia pascoe, Gnome-
niopsis castanea, Gnomoniopsis smitho-
gilvyi and Gnomoniopsis sp. have very si-
milar morphological characteristics and pa-
thogenic behavior, in that they have simi-
lar-sized pycnidia and conidia and are able
to produce the same array of symptoms in
chestnut nuts, being also all endophytic in
bark tissues. Therefore, the hypothesis that
they could be the same fungus is very plau-
sible. Anyway, further studies on the taxono-
my and genetics of these fungi are required,
given that DNA analyses clearly separate the
Phoma, Phomopsis and Gnomoniopsis
groups. Nonetheless, Gnomoniopsis sp. is
the agent involved in the current epidemic of
brown rot, and recovered on D. kuriphilus
galls in Italy, like the brown rot pathogen re-
corded in Australia. In the light of these find-
ings, further researches are desirable to find
new control strategies aimed to save produc-
tion in the most affected areas. The need is
urgent as there is a risk that a combination of
various factors, such as the resurgence of
blight due to climate stress, the expansion of
the Asian chestnut gall wasp and crop de-
struction by fungi or insects, could set off a
general decline in chestnut trees and or-
chards or, even worse, in the entire chestnut
production.

Acknowledgements
We would like to thank Giovanni Falchero,
Paolo Morreti, Luciano Massarotto and
Francesca Ugolini for technical assistance in
the field and data processing.

References
Belisario A, Scorticchini M (1993). Phomopsis castanea and Pseudonomonas songae pv. syr-
ingae associated with Castanea sativa seedling and stem cankers. In: “Proceedings of interna-
tional congress on chestnut”. Spoleto (Italy) 20-
23 Oct 1993. E. Antognozzi ed., Spoleto, Italy,
pp. 541-544.
Bernardze MM, De la Montana MJ, Garcia QJ
(2004). HPLC determination of sugars in varie-
ties of chestnut fruits from Galicia (Spain). Jour-
nal of Food Composition and Analysis 17 (1):
63-67. - doi: 10.1016/S0889-1575(03)00093-0
Bissegge M, Sieber TN (1994). Assemblages of
endothytic fungi in coppice shoots of Castanea
3760535
Bosio G, Gerbaudo C, Piazza E (2010). Dryocos-
mus kuriphilus Yasumatsu: an outline seven years
after the first report in Piedmont (Italy).
Breisch H (1995). Châteaignes et marrons. Cftf,
Paris, France, pp. 239. [in French]
Brown KB, Hyde KD, Guest DI (1998). Prelimi-
nary studies on endophytic fungal communities of
Musa acuminate species complex in Hong
Camici L (1948). “La mummificazione delle
castagne” da Phomopsis viterbensis sp. Annali
della Sperimentazione Agraria 2: 557-566.
[in Italian]
Ciferri R (1951). “La mummificazione” delle
castagne da Phomopsis endogena (Spec.) nobis,
n. comb. Notiziario delle Malattie delle piante 8:
36-37. [in Italian]
Conedera M, Jermini M, Sassella T, Sieber TN
(2005). Raccolta, trattamento e conservazione
delle castagne. Caratteristiche del frutto e prin-
cipali agenti infestanti. Prima parte. Sherwood
11(107): 5-12. [in Italian]
Deloutre C, Morelet M (1979). La pourriture noire
des glands. Revue Foretiere Française 3 (2):
plants. An identification handbook. The Rich-
Ferreira-Cardoso J, De Vasconcelos Mc (2009).
Caracteristicas comerciais e composição química
básica das castanhas provenientes de clones
híbridos da seleção do CENASEF. In: “Casta-
heiroes híbridos - Estudos de resistência à doen-
cia da tinta” (Gomes-Laranjo J, Peixoto F, Fer-
02, Programa INTERREG III A Cooperação
Transfronteiriça UTAD, Vila Real, Galicia-
Norte, Portugal, pp. 8-17. [in Portuguese]
Gentile S, Valentino D, Visentin I, Tarnetti G


