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Role of the Fungal Flora on Kernel Rot of Chestnuts

Kestane Meyve Çürüklüğünde Fungal Floranın Rolü

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Abstract: In order to determine the fungi causing kernel rot on chestnut, 150 chestnut kernels were collected from chestnut forests of Düzce province. Ninety-eight of the kernels showed necrosis at various sizes, while the rest of 52 were healthy in appearance. Twelve fungi were recovered from the necrotic kernels, while four from the healthy kernels. The most frequently isolated fungus from the necrotic kernels was *Gnomoniopsis smithogilvyi*, obtained from 24 of the kernels. It was also isolated from 5 healthy kernels and produced necrosis when inoculated on the intact kernels. Besides *G. smithogilvyi;* 11 fungi; *Diplodina castanea* (1), *Botrytis cinerea* (6), *Aureobasidium* sp. (4), *Alternaria alternata* (2), *Alternaria tenuissima* (1), *Penicillium* spp. (19), *Trichoderma* sp. (12), *Cladosporium* sp. (2), *Cylindrocarpon* sp. (2), *Mucor* sp. (10), *Rhizopus stolonifer* (5) and bacterial growth were also recovered from necrotic kernels. On the other hand, *G. smithogilvyi* (5), *Penicillium* sp. (9), *Trichoderma* sp. (6), *Mucor* sp. (3) and bacterial growth were also found out from the symptomless kernels. G. *smithogilvyi, D. castanea, B. cinerea, Cladosporium* sp. and *Penicillium* sp. produced necrosis at varying rates when inoculated on to intact kernels. Compared to other species, *Penicillium* sp. showed the lowest rate of pathogenicity, which was the dominant fungus on the healthy fruits and recovered from 9 of them.

Keywords: *Castanea sativa*, chestnut rot, fungal flora

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Öz: Meyve çürüklüğüne neden olan fungusları belirlemek için, Düzce ilinde bulunan kestane ormalarından 150 adet kestane meyvesi toplanmıştır. Toplanan meyvelerin 98 tanesinde farklı ölçülerde nekrozlar görülmüş olup, 52 adedinin ise semptomsuz olduğu gözlenmiştir. Nekrozlu meyveden 12 farklı fungus tespit edilirken, sağlıklı meyvelerden 4 farklı fungus tespit edilmiştir. Nekrozlu meyvede en sık rastlanan fungus *Gnomoniopsis smithogilvyi* (24) olmuştur. Ayrıca semptom göstermeyen 5 meyveden de bu fungus izole edilmiştir. *Gnomoniopsis smithogilvyi'nin* yanısıra, nekrozlu meyvelerden *Diplodina castanea* (1), *Botrytis cinerea* (6), *Aureobasidium* sp. (4), *Alternaria alternata* (2), *Alternaria tenuissima* (1), *Penicillium* spp. (19), *Trichoderma* sp. (12), *Cladosporium* sp. (2), *Cylindrocarpon* sp. (2), *Mucor* sp. (10), *Rhizopus stolonifer* (5) olmak üzere 11 fungus izole edilmiş ve bakteriyel gelişmeler tespit edilmiştir. Semptomsuz meyvelerde ise *G. smithogilvyi* (5), *Penicillium* sp. (9), *Trichoderma* sp. (6), *Mucor* sp. (3) ve bakteriyel gelişmeler tespit edilmiştir. Meyvelerden izole edilen G. *smithogilvyi, D. castanea, B. cinerea, Cladosporium* sp. ve *Penicillim* sp. olmak üzere 5 türün patojenitesi yapılmış olup, meyvede patojen olduğu saptanmıştır. *Penicillium* sp. diğer türlere göre az da olsa patojenite göstermiştir. Semptomsuz meyveden yapılan fungus izolasyonunda ise en sık tespit edilen *Penicillium* sp. (9) olmuştur.

Anahtar Kelimeler: *Castanea sativa*, meyve çürüklüğü, fungal flora

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INTRODUCTION

Sweet chestnut or European chestnut (*Castanea sativa* Mill.) not only grows in about 81.232 ha as a forest tree but is also grown as a fruit tree in Turkey (OGM, 2020). According to the FAO statistics, Turkey takes the fourth place on chestnut fruit production in the world (FAOSTAT D, 2023). Chestnut is a tree with multipurpose use and along with its edible fruits it has a wide range of beneficial uses such as timber, coppice, wood, honey production, etc. (Conedera et al., 2016).

Along with serious pests and diseases occurring on chestnut trees, fruit rots have also attracted attention of the researchers recently (Sieber et al., 2007) and *Ciboria batschiana* (Zopf) N.F was reported as the main causal agent of black fruit rot up to 2000s (Vettraino et al., 2005; Blaiotta et al., 2014).

After 2000s, an increase on the chestnut fruit rot in Europe and Australasia was observed by the growers and *Gnomoniopsis smithogilvyi* Tamietti (Synonym: *Gnomoniopsis castaneae*) (*Gnomoniaceae*, *Diaporthales*) was reported as the main cause of fruit rot (Smith and Agri, 2008; Gentile et al., 2009; Visentin et al., 2012; Tamietti, 2016). *Gnomoniopsis smithogilvyi* was found to cause necrosis on the endosperm and embryos of the fruits and described as the causal agent of brown rot on chestnut fruit (Shuttleworth et al., 2012a, 2015). In addition to *G. smithogilvyi*, other fungi such as *Phomopsis viterbensis*, *P. castanea*, *P. endogena*, *Phoma endogena*, *Gnomonia pascoe*, *Dendrostoma castaneum* were also recovered from chestnut fruits having brown rots (Maresi et al., 2013; Jaklitsch and Voglmayr, 2019).

Many other fungi were also mentioned to cause chestnut fruit rot. Using 350 fruits, Overy et al. (2003), isolated three mycotoxin-producing *Penicillium* spp., *Penicillium glabrum*, *P. crustosum*, and *P. discolour* as the dominant fungi in Canada while *Ciboria bastiana, Penicillium* sp., *Mucor hiemalis* were the prevailing species in Switzerland (Jermini et al., 2006). In China, Xiao-qing et al. (2009) determined 13 fungi; *Alternaria* sp., *Aureobasidium* sp., *Botrytis* sp., *Colletotrichum* sp., *Penicillium* sp., *Phoma* sp., *Phomopsis* sp., *Trichothecium* sp., *Fusarium* sp., *Fusicoccum* sp., *Rhizoctonia* sp., *Mucor* sp., and *Rhizopus* sp. on the fruits with and without decay symptoms and four of them; *Alternaria* sp., *Fusarium* sp., *Fusicoccum* sp. and *Trichothecium* sp. produced decay when inoculated on the intact fruits. Similar fungi; *Acrospeira mirabilis, Botryotinia fuckeliana, Botryosphaeria ribis, Coniophora puteana, Gibberella* sp., *Penicillium chrysogenum*, *P. griseofulvum, P. expansum*, and *Sclerotinia sclerotiorum* were also determined on chestnut fruits in Michigan (Donis-González et al., 2016). Apart from the above-mentioned fungi, *Colletotrichum acutatum* was detected from the chestnut fruits having pink discolorations (Gaffuri et al., 2017).

Gnomoniopsis smithogilvyi was admitted as the main agent of brown rots of chestnut fruits in Europe, Australia, and New Zealand by many authors (Shuttleworth et al., 2012a, 2012b; Visentin et al., 2012; Dennert et al., 2015; Lione et al., 2015; Tziros, 2018; Aguín-Casal et al., 2022) and it was also isolated from various trees belonging *Betulaceae*, *Fagaceae*, *Oleaceae* and *Pinaceae* including *Corylus avellana* L. (Linaldeddu et al. 2016), *Fraxinus ornus* L., *Quercus cerris* L., *Pinus pinaster* Aiton (Fernandez et al. 2017; Lione et al. 2019), and *Quercus ilex* L. (Shuttleworth et al., 2012b). Besides fruit rot, it was reported to cause cankers on chestnut and hazelnut, galls on chestnut, and necrosis on the leaves of chestnut and oak (Magro et al., 2010; Linaldeddu et al., 2016; Pasche et al., 2016; Jiang et al., 2021). The pathogen was also determined on chestnut fruit and boxwood trees in Turkey (Akıllı Şimşek et al., 2019; Çakar and Şimşek, 2022).

The aim of this work was; (1) to determine fungal flora on chestnut fruits collected from Düzce province, (2) to find out their roles on fruit rot.

MATERIAL AND METHOD

Sampling

Total of 150 chestnut fruits were collected from the ground under the 15 selected chestnut trees, 1-2 km apart, being 10 fruit from each, situated at forests administered by Düzce Forest Management Directorate of Bolu Regional Forestry Directorate situated at 40°47'47.92" North, 31°20'22.34" East coordinates.

Method

Isolation of Fungi from Nuts

The chestnut fruits were first examined under a stereomicroscope, and 98 fruits having necrosis and 52 asymptomatic ones were disinfected in 0.5% sodium hypochlorite (NaOCl) for 4 min. Fruit tissues from each of the necrotic fruits about 0.5 × 0.5 cm containing the necrotic and intact areas were dissected, plated on potato dextrose agar (PDA; Difco, Sparks, MD, USA, adjusted to pH 4.5 by 125µL L^{-1} lactic acid) and incubated at 25 °C for 4 days. Samples at the same size were taken from the centres of asymptomatic fruits and treated as mentioned above. Incubated plates were examined at 2-8 days intervals and mycelial tips from the growing fungi were removed and plated on PDA. The obtained fungal cultures were identified based on their morphological aspects described by the references (Barnet and Hunter, 1972; Sutton, 1980) and stored in the laboratory at the Biology Department, Science Faculty of Çankırı Karatekin University.

DNA Isolation

DNA isolations were performed by using ten days old fungal cultures grown on PDA and employing DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer directions. The obtained DNA was dissolved in ultra-pure water and stored 20 °C.

PCR Studies

For molecular identification of *G. smithogilvyi, Diplodina castanea*, *Penicillium* sp., *B. cinerea*, and *Cladosporium* sp. primer sets of ITS1/ITS4 (White et al., 1990) for Internal Transcribed Space region, Bt2a/Bt2b (Glass and Donaldson, 1995) for β-tubulin (*tub2*), EF-728F/1199R (Walker et al., 2010) for the translation elongation factor 1 α (*TEF*)were used for DNA amplification of the relevant gene regions. PCR was carried out in 50 µL reaction mix of 40 ng template DNA, 1× FirePol PCR Buffer BD (0.8 M Tris-HCl, 0.2 M (NH_4), 1 µL (10 µM) for each primer, 200 µM dNTPs, 1 unit of FIREPol *Taq* DNA polymerase (Solis Biodyne, Tartu, Estonia) by using T100 thermal cycler (Bio-Rad, Hercules, CA, USA). The reaction was started by denaturation at 94 °C for 5 min, run 45 cycle as denaturation at 94 °C for 45 sec, annealing at varying temperatures (52, 53, 55 °C for ITS,TUB, TEF, respectively) depending on the primer for 45 sec, extension at 72 °C for 90 s, and final extension at 72 °C for 6 min.

PCR products were run in 1.4% agarose gel in 10 µL 1× TAE buffer, stained with ethidium bromide, and visualized under UV by using trans illuminator (Vilber, Deutchland, Eberharedzell, Germany). Row DNA sequence data was processed by using MEGA X software (Kumar et al., 2018) and the obtained sequences were blasted into GeneBank for comparisons.

Pathogenicity of Fungi on Nuts

In order to determine the role of five fungi on fruit decay, one isolate from each of them was used as; *G. smithogilvyi* Gc_01 accessed to GenBank as ON326601 (ITS), ON337137 (TUB), ON337136 (TEF); *D. castanea* Dc_85 accessed to GenBank as OP837526 (ITS); *Penicillium* sp. Pc_70 accessed to GenBank as OQ354382 (ITS); *B. cinerea* Bc_31 accessed to GenBank as OQ354380 (ITS).; *Cladosporium* sp. C_53 accessed to GenBank as OQ354381 (ITS).

A sufficient number of asymptomatic chestnut fruits for the inoculation of 5 fungi were selected and first soaked in 75% alcohol for 1 min, then 1.25% sodium hypochlorite for 3 min and later on rinsed with sterile water for 2 min to disinfect them. Wells, seven mm in diameter were drilled on the disinfected fruits and the outer and inner skins were removed to see the interior fruit tissue to check their health status. Ten asymptomatic fruits were inoculated by each of the five fungi by placing culture disks taken from the peripheries of young cultures on to the wells and covering the inoculation point with a damp cotton wool, then sealing the inoculation points with Parafilm (Jiang and Tian, 2019) (Figure 1 a, b, c, d). The inoculated fruits were kept at 25 °C in dark and the decay was evaluated for 15 days by measuring the necrotic areas

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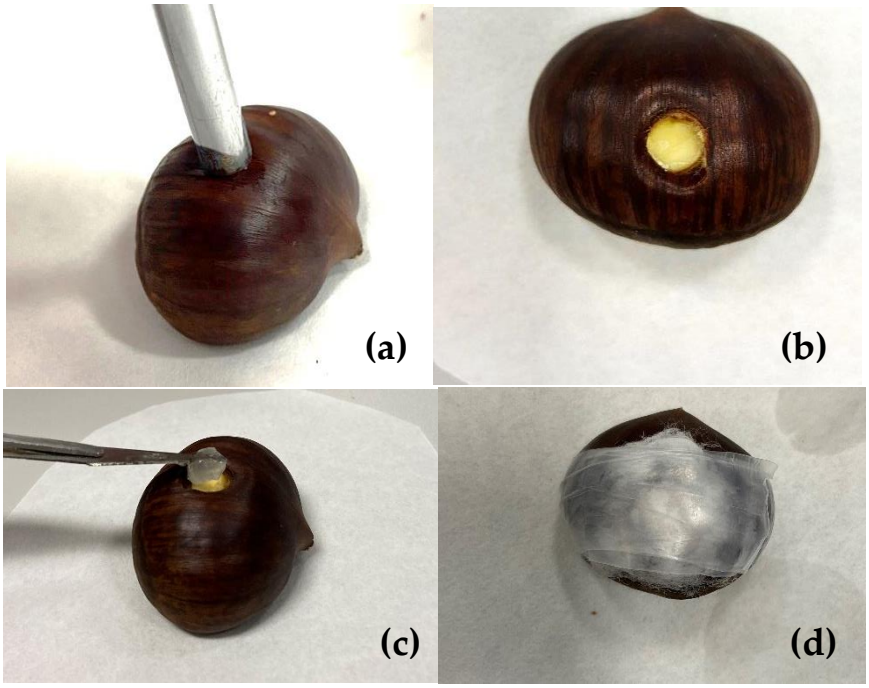

Figure 1. The pathogenicity test on nuts, a) drilling a well on the skin, b) healthy looking inner tissue of a nut c) placing the fungal culture on the wells d) wrapping the wounded nut with moist cotton and Para film.
Şekil 1. Meyvelerde patojenite testi, a) kabukta delik açılması b) bir meyvenin sağlıklı görünen iç dokusu c) deliklere fungal kültürün yerleştirilmesi d) yaralanmış meyvenin, nemli pamuk ve parafilm ile sarılması.

RESULTS AND DISCUSSION

Disease Symptoms

Necrosis in various colours; brown, white, green, cream and black, and sizes were seen on 98 of the 150 nuts (Table 1, Figure 2), and no symptoms were observed on the remaining 52 nuts. Almost two-third of the chestnut nuts collected from forests of Düzce province was found to have various types of necrosis, and twelve fungi were isolated from these fruits. Some of the fungi, such as *Alternaria alternata*, *Botrytis cinerea, Cladosporium* sp., *Trichoderma* sp., *Penicillium* spp., and *Rhizopus* sp. were also found by the other researchers previously (Akıllı et al., 2011; Dennert et al., 2015; Shuttleworth et al., 2015). The sizes and colours of chestnut fruit decay showed wide variations and mainly observed as brown, brown-black, green, cream and white discolorations. *Gnomoniopsis smithogilvyi* was the dominant fungus on fruit decay and it was generally isolated from brown and white rots. *Penicillium* sp. were the second most frequently recovered fungus and occurred on all types of decays.

Table 1. Symptoms observed on rotten fruits and the fungi obtained from them.
Çizelge 1. Meyvelerde görülen çürüme belirtileri ve bunlardan elde edilen etmenler.

Necrosis symptoms	The fungi recovered
Brown rot	*Gnomoniopsis smithogilvyi*, bacteria, *Penicillium* spp., *Rhizopus stolonifer*
Green rot	*Trichoderma* sp., *Alternaria alternata, Alternaria tenuissima, Aureobasidium* sp., *Penicillium* sp., *Trichoderma* sp.
Brown to black rot	*Gnomoniopsis smithogilvyi, Diplodina castanea, Penicillium* sp., *Botrytis cinerea, Trichoderma* sp., *Cladosporium* sp.
White rot	*Gnomoniopsis smithogilvyi, Penicillium* sp., *Cylindrocarpon* sp.
Cream colour decay	Bacteria, *Gnomoniopsis smithogilvyi, Aureobasidium* sp, *Penicillium* sp., *Trichoderma* sp.

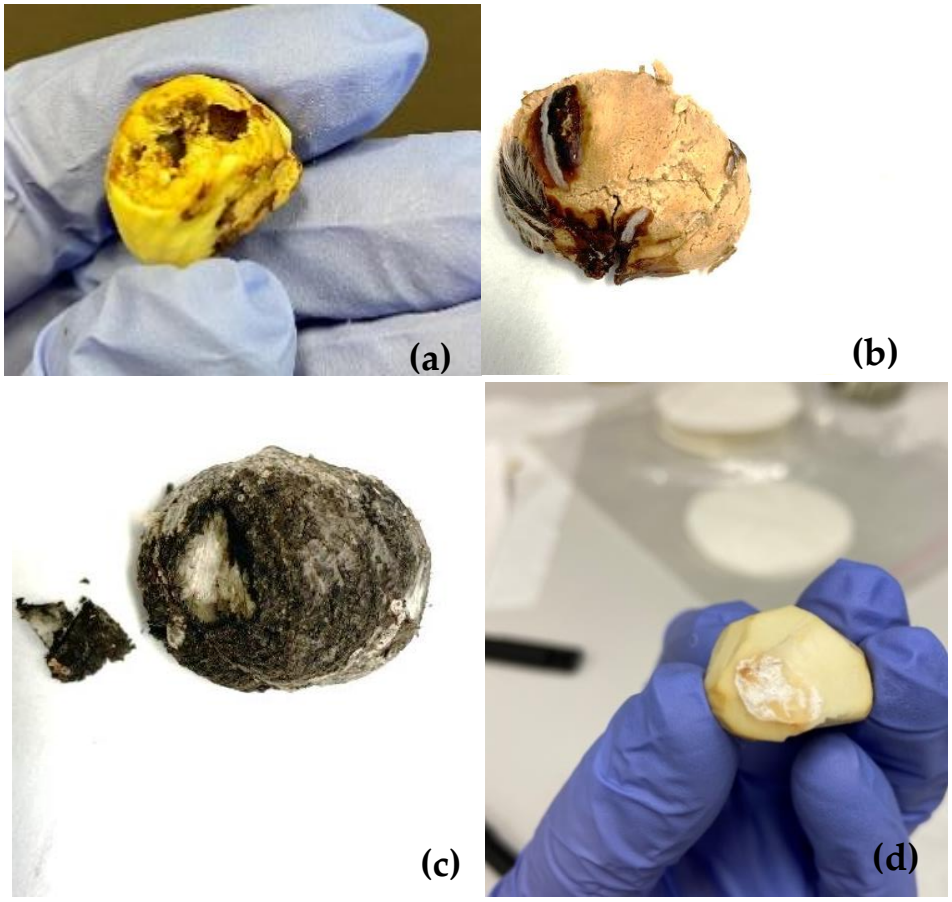

Figure 2. Symptoms caused by *Gnomoniopsis smithogilvyi* on the fruits; brown rot and cream colour softening (a, b) green decay and white rot caused by *Penicillium* sp. (c, d).
Şekil 2. Gnomoniopsis smithogilvyi'nin meyve üzerinde oluşturduğu kahverengi çürüklük ve bej renkli yumuşama (a, b), Penicillium sp.*'nin neden olduğu yeşil çürüme ve beyaz çürüme*(c, d).

Fungi Obtained from Necrotic Kernels

The twelve fungi obtained from 98 necrotic fruits are as following with their percentages; *G. smithogilvyi* (24), *D. castanea* (1), *B. cinerea* (6), *Aureobasidium* sp. (4), *A. alternata* (2), *A. tenuissima* (1), *Penicillium* spp. (19), *Trichoderma* sp. (12), *Cladosporium* sp. (2), *Cylindrocarpon* sp. (2), *Mucor* sp. (10), *R. stolonifer* (5), and bacterial growth (5). *Gnomoniopsis smithogilvyi* was generally isolated individually but sometimes it occurred simultaneously with *Penicillium* sp., *Trichoderma* sp. as mentioned by the other authors, the most widely encountered fruit rot agent was *G. smithogilvyi* in this study (Dennert et al., 2015; Visentin et al., 2012). *Diplodina castanea* has not been reported from chestnut fruits so far but from other tissues (Adamčíková et al., 2013). Occurrence on fruit rots is the first report for Turkey.

Identification of some of the well-known fungi were done by their morphological characteristics described in the related sources and internet. Identification of *G. smithogilvyi*, *D. castanea*, *Penicillium* sp., *B. cinerea*, and *Cladosporium* sp. was also confirmed by molecular tools. A BLASTn search of the sequences *G. smithogilvyi* [GenBank accession nos. ON326601 (ITS), ON337137 *(tub-2),* ON337136 (*TEF*)] showed 99.82, 100.00, and 100.00% nucleotide identity with the ITS (Accession no. NR_166040), *tub-2* (Accession no. KX929733), and *TEF* (Accession no. LN999975); *D. castanea* [OP837526 (ITS)] displayed 99.79% nucleotide homology with the ITS (KX929760). *B. cinerea* [OQ354380 (ITS)] displayed 99.80% nucleotide homology with the ITS (MK748141). *Cladosporium* sp. [OQ354381 (ITS)] displayed 100% nucleotide homology with the ITS (HG008746). *Penicillium* sp. [OQ354382 (ITS)] showed 100% nucleotide homology with the ITS (MK775828).

Gnomoniopsis smithogilvyi first grew pale brown on the culture plates, after, on the 10. days the colour became darker brown with the formation of intensive acervuli (Figure 3a). The conidia were hyaline, fusiform, one-celled and 5.0–7.5 × 1.7–3.2 µm (average 5.8 × 2.3 µm) (n=30) (Figure 3b). Colonies of *Diplodina castanea* were dark brown on the 10. day with undulate margins (Figure 3c), having guttulate, ellipsoid, hyaline, fusiform, dull brownish-grey conidia of 6.0–12.2 x 2.1–3.7 µm (average 9.2 × 2.7 µm) (n=30) dimensions (Figure 3d).

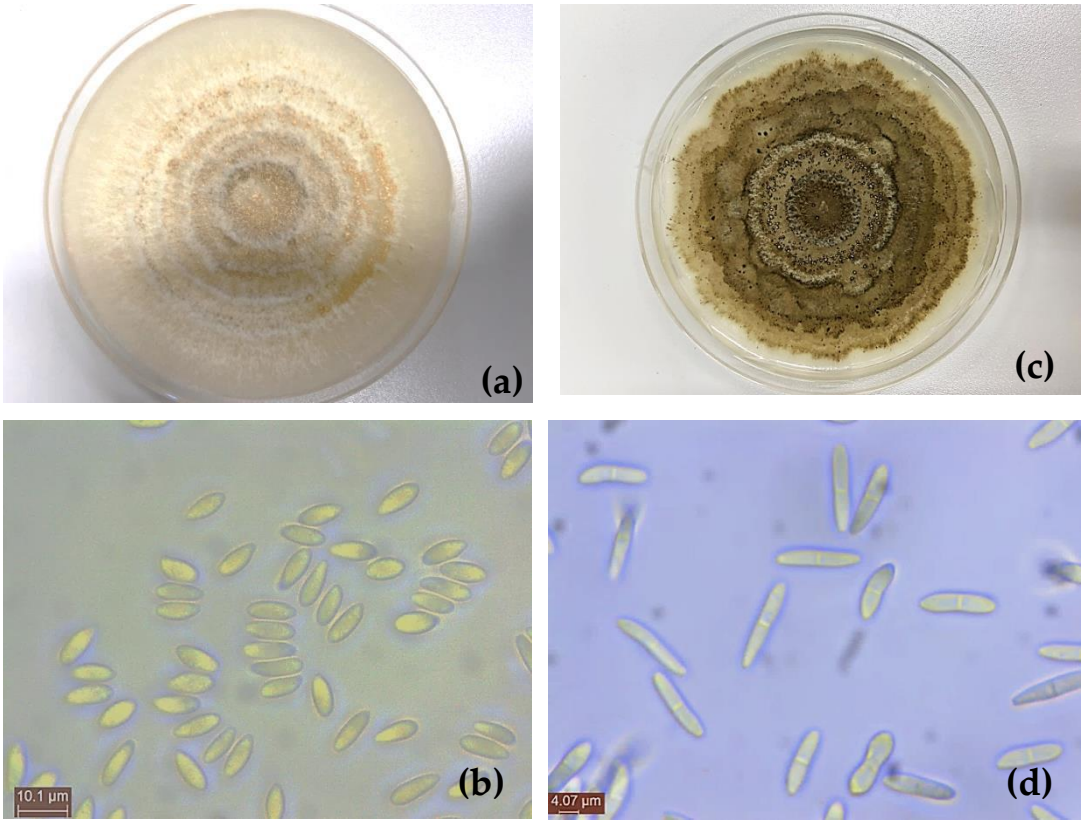

Figure 3. Growth of of *Gnomoniopsis smithogilvyi* on PDA medium (a), Its conidia (b), Growth of *Diplodina castanea* on PDA (c), İts conidia (d).

Şekil 3. Potato dekstrose agar ortamında gelişen (a) Gnomoniopsis smithogilvyi'nin (c) Diplodina castanea morfolojik görüntüsü, b) G. smithogilvyi, (d) D. castanea'nın konidiumları.

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Fungi Obtained from Symptomless Nuts

From the samples taken from the interior parts of the asymptomatic nuts, four fungi; *G. smithogilvyi, Penicillium* sp., *Trichoderma* sp., *Mucor* sp. and bacterial growth were isolated at 5, 9,6, 3, and 4 of the samples respectively. *Gnomoniopsis smithogilvyi* which is admitted to be endophytic on chestnuts (Dennert et al., 2015) was also recovered from asymptomatic fruits along with *Penicillium* sp., *Trichoderma* sp., and *Mucor* sp. The presence of *G. smithogilvyi* in asymptomatic fruits implies that symptomless fruit could develop fruit rot during storage.

Pathogenicity of The Fungi

The isolates of *G. smithogilvyi* Gc_01, *D. castanea* Dc_85, *B. cinerea* Bc_31, and *Cladosporium* sp. C_53 recovered from the necrotic fruits produced an average of 1.7, 1.5, 2, and 2 cm necrosis on the fifteenth days of the inoculations (Figure 4a, b, c, d). *Penicillium* sp. Pc_70 isolate produced smaller decays, an average of 0.7 cm. on the 15. days then the other isolates tested. No rot was observed on the control fruits. When inoculated on to the intact chestnut fruits, *G. smithogilvyi* and *D. castanea* produced rapid decay. The other fungi also produced necrosis on the intact fruits, *Penicillium* sp. being the weakest, as mentioned by Liang and Wang, 2003.

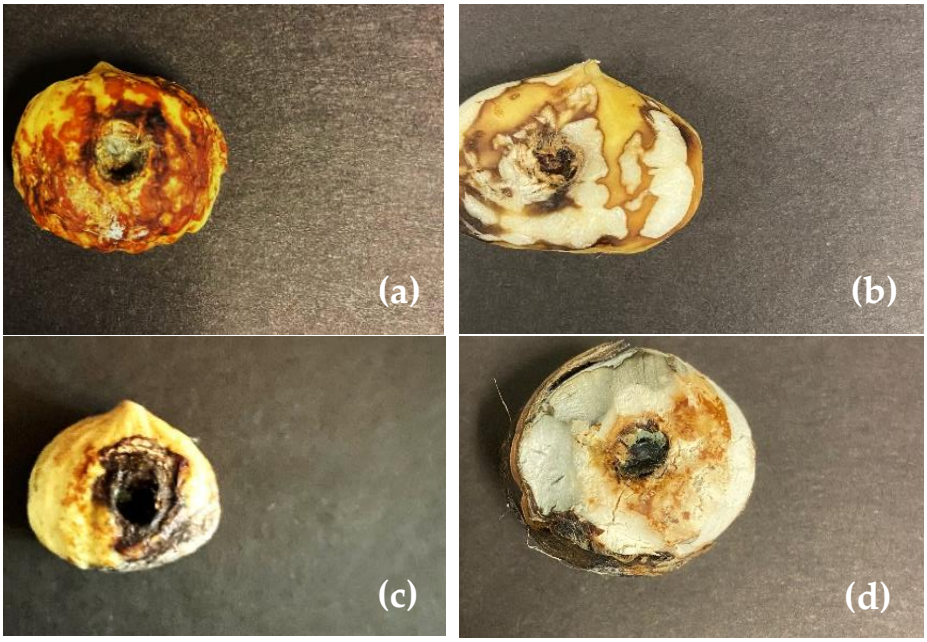

Figure 4. Decay formed by (a) *Gnomoniopsis smithogilvyi*, (b) *Botrytis cinerea* (b), *Diplodina castanea* (c) *Cladosporium* sp. (d) isolates of Gc_01, Dc_85, Bc_31, and C_53 respectively on the 15. days on the intact chestnut fruits.
Şekil 4. Patojenite testlerinde Gnomoniopsis smithogilvyi Gc_01 (a,) Botrytis cinerea Bc_31 (b), Diplodina castanea Dc_85, (c) ve Cladosporium sp. C_53 (d) izolatlarının sağlam kestane meyvelerinde oluşturduğu çürümeler.

CONCLUSION

Chestnut is an economically important tree due to its multiple benefits for edible fruits, nectar source for honey production and durable wood. Like the other vegetative parts, chestnut fruits also encounter various damage from of diseases and pests. Together with the fruit pests, significant fruit damage is also caused by fungi (Sieber et al., 2007).

As mentioned by the other authors; the most widely encountered fruit rot agent was *G. smithogilvyi* in this study, but other pathogens such as, *Phomopsis viterbensis*, *P. castanea, P. endogena*, *Phoma endogena*, *Dendrostoma castaneum*, *Gnomonia pascoe* have also been recorded from decaying chestnut fruits by them (Dennert et al., 2015; Lione et al., 2015; Visentin et al., 2012). In this study, the other species such as *Botrytis cinerea*, *Cladosporium* sp., *D. castanea,* and *Penicillium* sp. were also found as pathogens of chestnut nut.

Gnomoniopsis smithogilvyi was also reported from asymptomatic leaves, branches, symptomless fruits and dead plant materials (Crous et al., 2012; Visentin et al., 2012). Its endophytic nature was also found out by our work. Recently, this pathogen has also been found to be a pathogen of chestnut wood (Dar and Rai, 2013; Pasche et al., 2016; Lewis et al., 2017; Trapiello et al., 2018; Lione et al., 2019). Besides fruit rot, it was reported to cause cankers on chestnut and hazelnut, galls on chestnut, and necrosis on the leaves of chestnut and oak (Magro et al., 2010; Pasche et al., 2016; Linaldeddu et al., 2016; Jiang et al., 2021). The pathogen was also determined on chestnut fruit and boxwood trees previously in Turkey (Akıllı Şimşek et al., 2019; Çakar and Şimşek, 2022). Its role on other forest trees should be taken into consideration for the next studies.

Authors claimed that rate of the chestnut fruit rots would increase during the storage period (Maresi et al., 2013; Dennert et al., 2015). Ruocco et al. (2016) tested a new strategy to control fruit rot of chestnuts. They applied the enzyme of *T. harzianum* Rifai strain T22, disrupting the cell wall, to the heated water where the fruits were kept 45-50°C in hot water and then 15-18°C in cold water for 50 min and a found significant reduction on the rate of fruit rot. Although some treatments, such as fungicide applications and organic fertilizers, are proposed to control fruit rot (Lione et al., 2019), some biocontrol methods deserve to be tested since Pasche et al. (2016) obtained hopeful results by the treatment of *T. atroviride* and *Bacillus*

amyloliquefaciens. This method was carried out as preventive biocontrol experiments by using chestnut scion. The endophytic and opportunistic pathogenic fungus *G. smithogilvyi* was not found on chestnut scions treated with *B. amyloliquefaciens*. With a similar experiment conducted by *T. atroviride* the result was similar. The entophytic behaviour of these biocontrol agents inhibited the growth of *G. Smithogilvyi.*

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DECLARATION OF AUTHOR CONTRIBUTION

Deniz Çakar: Conceptualization, Methodology, Investigation, Molecular analysis, **Seçil Akıllı Şimşek:** Conceptualization, Methodology, Investigation.

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