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RESEARCH PAPERS

Diaporthe as the main cause of hazelnut defects in the Caucasus region

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Summary. Production of hazelnuts is affected by defects, the incidence of which is year-dependent but also economically damaging. The objectives of the present study were to quantify the incidence of different fungal genera in hazelnuts, and define the causal agent/agents of nut defects. A 4-year study (2013 to 2016) was carried out in the Caucasus region, in an orchard planted in 2008 with hazelnut cv. Anakliuri. Hazelnuts were sampled at early and full ripening stages, observed for defects, and then associated fungi were isolated. Nuts with necrotic spots and internal browning were commonly found, and some collected in 2016 were completely rotted, wet and almost black. *Alternaria, Aspergillus, Cladosporium, Colletotrichum, Diaporthe, Fusarium, Penicillium, Pestalotiopsis* and *Phoma* spp. were regularly isolated from diseased and symptomless nuts. *Diaporthe* spp. was the dominant genus, with increasing incidence from early to full ripening, and were more isolated from defected compared to healthy kernels and in the years with the greatest incidence of defects. Rainfall was associated with the incidence of nut defects. The role of *Diaporthe* as a key cause hazelnut defects was confirmed by pathogenicity tests. Three isolates from the *Diaporthe* population were identified as *D. eres* on the basis of EF, ACT, TUB and ITS loci. This supports the crucial role of climate during the crop-growing season for the development of defects in hazelnuts caused by *Diaporthe.*

Key words: Fungi, *Corylus avellana,* rot, meteorological data, rainfall.

Introduction

Hazelnuts (the fruits of *Corylus avellana*) are high value products that suffer defects that are year dependent, and are sometimes associated with off flavours (www.fao.org; Pscheidt and Ocamb, 2017; Teviotdale *et al*., 2002). These defects and quality problems cause yield losses due to non-compliance with the required market quality standards. Quality control, regularly applied to shelled nuts before processing (i.e. http://www.turkish-hazelnut.org/hazelnut/pdf/1917.pdf), aims to detect externally visible defects on kernels and those that are apparent after kernels are cut. The causal agents of defects have not been defined, although there is agreement that biotic, rather than abiotic factors, are the main causes (Pscheidt and Ocamb, 2017).

Extensive research has been undertaken in Chile, to isolate and identify fungi associated with hazelnut diseases, focusing on stems, twigs and roots of hazelnut plants (Guerrero *et al*., 2014a). In northern Italy (Piedmont), gleosporiosis, caused by *Gleosporium coryli* (Desm.), was reported on different plant parts, as well as on fruits (Tavella and Gianetti, 2006), while in central Italy (Viterbo Province) *Alternaria* spp., *Colletotrichum* spp., *Fusarium* spp. and *Phomopsis* spp. have also been isolated (Librandi *et al*., 2006). In Iran, *Rhizopus* sp., *Aspergillus flavus*, *Penicillium* sp., *Trichothecium* sp. and *Cladosporium cladosporioides* were isolated from stored fruits (Mogghadam and Taherzadeh, 2007). *Aspergillus flavus* and *A. parasiticus* were also reported by Ozay *et al*. (2007; 2008), as potential aflatoxin producers. *Botrytis cinerea* has been detected

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inside and outside fruits (Sezer and Dolar, 2012a), whereas on empty and abnormal shaped nuts *Trichothecium roseum* was found (Guerrero *et al*., 2014b). *Alternaria alternata*, *Fusarium* sp., *Aspergillus niger*, *Penicillium* spp. and *Rhizopus stolonifer* were detected in Chilean hazelnut products (Guerrero *et al*., 2014a). A broad list of fungi was reported in Turkey (Sezer and Dolar, 2016), including *Acremonium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Pestalotiopsis*, *Phomopsis*, *Rhizopus* and *Trichothecium*. The most recent report identified *Sphaceloma coryli* as a re-emerging pathogen causing heavy losses in hazelnuts in southern Italy (Minutolo *et al*., 2016).

Association between visible defects on hazelnuts and causal agents has rarely been described. *Alternaria* spp. and *Fusarium* spp. have been the most commonly isolated fungi from brown-greyish spots on the bases of nuts and necrotic patches on the bracts (Belisario and Santori, 2009). *Fusarium lateritium*, a worldwide plant pathogen, has been reported as the causal agent of nut grey necrosis (NGN) on hazelnuts (Santori *et al*., 2010; Vitale *et al*., 2011). In Turkey, *Colletotrichum acutatum* was isolated from hazelnuts showing necrotic, sunken lesions and rot (Sezer and Dolar, 2012b), but further studies did not allow association of commonly observed symptoms with causal agents (Sezer and Dolar, 2016). *Pestalotiopsis* sp. was identified as the causal agent of hazelnut blight and fruit necrosis (Sezer and Dolar, 2015).

Adequate description of different defects observed on nuts was outlined by Pscheidt and Ocamb (2017), and this includes defects with biotic and abiotic/unknown causes. Symptoms observed and described in hazelnuts can be summarized in three groups: 1) mould, 2) brown stain, and 3) miscellaneous defects.

Four main mould symptoms have been reported, including; i) necrosis of kernel tips, which usually extends a few mm into kernels, commonly observed between nut maturation and drying, and is associated to *Mycosphaerella punctiformis*; ii) internal discolouration of kernels, that sometimes becomes opaque white to translucent, buttery yellow, and is associated with bitter, rancid flavour and is commonly associated with *Phomopsis* sp. and *Septoria ostryae*; iii) kernel spots, which are dark, sunken lesions on kernel surfaces, of variable size and shape, and are commonly associated with the yeast *Nematospora coryli*; and iv) kernel shrivelling, associated with sporulation by several fungi, the pathogenicity of which have not been proven.

The causes of brown stains are unknown. These symptoms are characterized by brownish liquid that soaks the side and tip of each affected nut. Internal areas are affected later. The entire interior of the shell, including the kernel, may become a soft, brown, watery mass.

Miscellaneous defects are also due to unknown causes. These include black discolouration of the external kernel surfaces is reported as "discolouration", while kernels or portions of kernels with decomposed areas are defined as "decay".

A role of fungi was also supposed in development of off-flavours in hazelnuts, where a terpenoid compound, prenyl ethyl ether, was identified as a key contributor (Amrein *et al*., 2010). Further studies confirmed *Penicillium digitatum* as responsible for a "solvent like" off flavour (Amrein *et al*., 2014).

Information available in the literature on identification of fungi from hazelnut fruits or clusters causing undesired symptoms is fragmented (Librandi *et al*., 2006), with very few scientific papers focusing on the identification of causal agents of visible defects, suggesting that a complex of fungi is commonly associated with hazelnuts. A systematic study with organized sampling at different hazelnut crop growth stages and in different years has not been previously carried out. Therefore, a 4-year study was carried out in the Caucasus region, with repeated sampling in one hazelnut orchard. The objectives were to: i) quantify the incidence of different fungal genera in healthy and defected hazelnuts, ii) define the causal agent/agents of nut defects to provide the basis for rational preventive action aimed at reducing the incidence of hazelnut defects.

Materials and methods

Hazelnut orchard

A 10 ha hazelnut orchard in Chitatskari (Georgia), in the Caucasus region, planted in 2008 with cv. Anakliuri, was selected for this study. The orchard was managed according to common hazelnut agricultural practices, including chemical treatments for principal pests and copper spray applications to prevent bacterial and fungal diseases.

A wireless weather station (Vantage Pro2™ Plus®, Davis Instruments) was placed close to the orchard (approx. 500 m distance; GPS coordinates: N 42.44047 and E 41.85992), and hourly data of air temperature

(T, °C), air relative humidity (%RH) and rainfall (R, mm) were recorded, from 1 January to 30 August each year from 2013-2016. Hourly data of air temperature, air relative humidity and rainfall were also obtained from a weather station placed in Zugdidi, 8 km from the hazelnut orchard, for the 30 year period 1986 to 2016, as a reference historic dataset for the general geographic area.

Sampling

Hazelnuts were collected at the early ripening stage, approx. 45 d after setting, and also at full ripening (harvest time, approx. 30 d later; kernel humidity ≅ 12%). The orchard area was divided into four large plots, of 2,500 m^2 each, which were managed as four replicates. In each plot, 100 trees were chosen (excluding the first two external rows) and 25 hazelnuts per tree were collected from several fruit clusters in order to sample a total of 2,500 hazelnuts per sampling.

All the hazelnuts were shelled, assessed for visible defects, cut in half with a Magra hazelnut cutting unit (Teserba GmbH, Rüti, Switzerland) and observed again to detect any within nut symptoms (Figure 1). Based on these assessments, the nuts were separated into two groups, as either apparently healthy (no defects) or defected, as for commercial quality evaluation.

A sub-sample of 30 cut nuts from each group was collected from all the four replicates in the orchard (120 half-healthy and 120 defected nuts for each sampling time). Sometimes, the number of defected nuts was less than this and all available defected nuts were included. Each sample of 30 half nuts was stored under vacuum in a bag at 5°C, and delivered to the laboratory within 36 h.

Fungal isolation and identification

Nut samples collected at early and full ripening stages were managed following the same protocol. Each sample was washed with running tap water for 1 min, disinfected with 1% sodium hypochlorite solution for 1 min, rinsed three times in sterile distilled water and dried under a sterile hood on sterile paper. Seed halves of fruits were then plated in Petri dishes (9 cm diam.) containing water agar (WA; 15 g microbiological grade agar, 1L double distilled water) with 0.15 g L^{-1} streptomycin sulfate added during cooling, and incubated at 25°C, with natural photoperiod, for 21 d. The dishes were checked twice each week to monitor fungal growth. Each fungal colony developed on WA was repeatedly transferred to potato dextrose agar (PDA; 15 g agar, natural potato broth obtained from 200 g potato, 10 g dextrose, 1 L water) with 0.15 g L^{-1} streptomycin sulphate added during cooling, incubated at 25°C, with natural photoperiod, for a maximum of 30 d, depending on colony growth, to allow fungi to develop reproductive structures.

Figure 1. Shelled hazelnuts cut in half to observe internal defects. Based on these assessments, the nuts were categorised into two groups, as healthy (no defects) or defected.

Morphological characterization was then applied with the support of a stereo microscope (Motic) at ×40 magnification, and an optical microscope (Leitz labor lux D) at ×500 magnification, following taxonomic keys to identify the isolates at genus level (Ellis, 1971; Ellis, 1976; Gomes *et al*., 2013; Krol, 2005; Leslie and Summerel, 2006; Maharachchikumbura *et al*., 2014; Pitt, 1979; Raper and Fennell, 1965; Rotem, 1994; Samson *et al*., 2014; Sutton 1980; Udayanga *et al*., 2011; Visagie *et al*., 2013). The results of the fungal identifications were recorded as incidence (%) of hazelnuts infected by each genus.

Three isolates of *Diaporthe* spp. (PH01, PH02 and PH03) were selected for further study. They were managed to obtain monospore cultures: conidia were picked from each colony, suspended in 1 mL of physiological solution and serially diluted to 10^{-5}, 10^{-4} and 10^{-5} dilutions were plated in triplicate on WA and incubated at 25°C in the dark for 2–3 d. Two growing colonies were subsequently cut from each

plate, transferred onto PDA and incubated at 25°C in the dark. The serial dilutions and growth on PDA were repeated three times. One monospore colony was then chosen for each isolate and eight plugs of mycelium were removed, placed in vials containing double distilled sterile water and stored at 4°C before use.

Confirmation of *Diaporthe* spp. identification and its role as the cause of hazelnut defects

Molecular identification of the representative fungal isolates and pathogenicity tests were carried out to confirm the *Diaporthe* and its role as the cause of hazelnut defects.

Molecular analysis

The three monosporic strains were treated for DNA extraction and amplification according to the method of Jayaramaiah *et al*. (2013). The primer pairs Bt2a/Bt2b (Glass and Donaldson, 1995), ACT-512F/ACT783R and EF1-728F/ EF1-986R (Carbone and Kohn, 1999) previously used for *D. foeniculina* (Annesi *et al*., 2016), and ITS1/ITS4 (White *et al*., 1990) and Phom. I/Phom.2 (Zhang *et al*., 1997), previously used for *D. phaseolorum* and *Phomopsis longicolla*, were used, applying the PCR conditions reported by these authors (Table 1). The primers were obtained from Sigma-Aldrich, and the amplifications were performed using a 5 Prime Grad Thermal Cycler. Each PCR product mixture (1.5 μL) was separated on 1.5% agarose gel containing 4 μL of Midori Green Advanced (Nippon Genetics), and visualised and documented in a Azure C150 gel imaging workstation. The representative bands were excised from the agarose gel and DNA was extracted and purified using NucleoSpin Extract II (Machinery Nagel) according to the manufacturer's instructions. The three representative isolates were custom sequenced, (BRM Genomics). Sequences were identified by BLAST analyses (Altschul *et al*., 1997) against GenBank sequences, using the National Center for Biotechnology Information nucleotide BLAST tool (http://ncbi.nlm.nih.gov/blast/).

Pathogenicity trial

One of the three monosporic isolates of *Diaporthe* spp. (PH01) was selected for pathogenicity studies. The isolate grown on PDA plates with natural photoperiod until pycnidial conidiomata with visible sporulating conidial masses had developed. Ten conidiomata were then selected. A conidial suspension was obtained, diluted in 10 mL of distilled water with 10 μL of Tween® 20 added, and the suspension was adjusted to 10^6 conidia mL^{-1}.

Sixty ripe hazelnut kernels without defects were selected for pathogenicity tests. They were washed with running tap water for 1 min, disinfected with 1% sodium hypochlorite solution for 1 min, rinsed three times in sterile distilled water and dried in a sterile hood on sterile paper. They were then random-

Table 1. Primer sets and corresponding amplification targets used for identification of fungi.

Target gene	Primer name	Primer sequence	Size of the PCR amplicon (bp)	Reference
β-Tubulin	Bt2a	5′ GGTAACCAAATCGGTGCTGCTTTC 3′	300	Glass and Donaldson, 1995
	Bt2b	5′ ACCCTCAGTGTAGTGACCCTTGGC 3′		
ACT	ACT-512F	3′ ATGTGCAAGGCCGGTTTCGC 5′	300	Carbone and Kohn, 1999
	ACT-783R	3′ TACGAGTCCTTCTGGCCCAT 5′		
TEF-1α	EF1-728F	3′ CATCGAGAAGTTCGAGAAGG 5′	350	Carbone and Kohn, 1999
	EF1-986R	3′ TACTTGAAGGAACCCTTACC 5′		
ITS	ITS5	3′ GGAAGTAAAAGTCGTAACAAGG 5′	600	White *et al*., 1990
	ITS4	3′ TCCTCCGCTTATTGATATGC 5′		
ITS	Phom. I	5′ GAGCTCGCCACTAGATTTCAGGG 3′	337	Zhang *et al*. 1997
	Phom. II	5′ GGCGGCCAACCAAACTCTTGT 3′		

ly assigned into three groups of 20. Each group was then treated as one of the following: i) not inoculated (negative control), ii) undamaged inoculated kernels, or iii) kernels slightly damaged with a sterile scalpel before inoculation. Ten μL of the inoculum suspension were deposited on each inoculated kernel, and 10 μL of sterile double distilled water was added to each negative control kernel.

Three glass boxes were used as moist chambers, and each contained a sterile grid to support the inoculated kernels to avoid contact with water added to each box. The boxes were closed into plastic bags and incubated at 20°C for 21 d, with a 12 h light/12 h dark period each day. At the end of the incubation period, kernels were observed for external and internal symptoms. Fungal isolation was carried out from all symptomatic kernels following the protocol described above.

Data analyses

Sample size was estimated setting the percentage of defect at 1%, 95% confidence level and a Power at 80% (upper and lower limits resulted, respectively, as 1.95 and 0.35), using the R statistical software (https://www.R-project.org/). In four different time periods (P1 = 1 January – 30 April; P2 = 1 May – 30 June; P3 = 1 July – 31 July; P4 = 1 August – 30 August), in each of the 4 years, different meteorological parameters were computed from field-collected data. The collected parameters were: mean air temperature (°C), Degree Day (DD; °C), summation of DD (°C), total rainfall (R; mm), summation of R (mm), and mean air relative humidity (RH; %).

The software SPSS (IBM SPSS Statistics vers. 24) was used for data analyses. Analysis of variance was applied to arcsine transformed data on fungi incidence in hazelnut kernels. Tukey's test was used to indicate statistically significant differences between means. Pearson correlation analysis was run for data of defect incidence in hazelnuts and the incidence of fungi at harvest, and also between the incidence of defected nuts with the meteorological parameters computed for the four annual time periods, each year for the 4 years of the study.

Results

Sampling and meteorological data

The dates on which hazelnuts were sampled from orchards are reported in Table 2. Early ripening stage sampling was between 1 and 18 July, depending on the year, while the full ripening sampling was between 10 and 20 August.

Meteorological data are summarized in Table 3. Mean January to April temperatures were similar for the different years, with the lowest temperatures recorded for this period in 2015 and the highest temperatures in 2014, and 1,073 DD were recorded in that period in 2015 and 1,273 DD were recorded in 2014. The period May to June was very similar for T conditions in 2015 and 2016, cooler than 2013 and 2014. July was warmer in 2014 and 2016, compared to the other 2 years, while the coolest August was recorded in 2013. Based on the DD data for the full period considered each year (January to August), 2014 was the warmest of the 4 years.

Rainfall between January and April was abundant in 2013 and 2016, also in July and August of 2013, with a total amount of rainfall (1401 mm) in the full period considered (January to August) almost double that of 2014 (737 mm) or 2015 (722 mm), and 35% greater than in 2016 (904 mm). This is confirmed by the historical data series (30 year daily data) collected 8 km from Chitatskari, which confirms rainfall was abundant for the area in 2013 and 2016. Mean rainfall (30 years) was 512 mm for January to April, 56 mm for May to June, 45 mm for July and 34 for August, with a total rain fallen of 720 mm in the first eight months of the year, which was very similar to 2014 and 2015.

Table 2. Date of hazelnut sampling managed in Chitatskari, Caucasus region, during the 4 year study (2013 to 2016).

Hazelnut growth stage	2013	2014	2015	2016
Early ripening	18 July	1 July	8 July	15 July
Full ripening	20 August	10 August	18 August	11 August

Table 3. Summary of meteorological data collected during the 4 years study (2013 to 2016) at four time intervals (P1-P4) from 1 January to 30 August each year. Data are from a meteorological station situated approx. 500 m from the hazelnut orchard used for the present study.

Time intervals	Start date	End date	Mean air temperature (Tm, °C)	Degree Day (DD, °C)	Summation Degree Day (ΣDD, °C)	Total rainfall (R, mm)	Summation rainfall (ΣR, mm)	Mean air relative humidity (RH, %)
2013								
P1	01-Jan	30-Apr	9.2	1100	1100	630	630	79
P2	1-May	30-June	20.4	1245	2345	177	807	73
P3	1-July	31-July	22.7	705	3050	432	1239	85
P4	1-Aug	31-Aug	23.4	727	3776	162	1401	83
2014								
P1	01-Jan	30-Apr	10.3	1237	1237	346	346	70
P2	1-May	30-June	20.7	1265	2501	192	537	78
P3	1-July	31-July	24.3	754	3256	143	680	83
P4	1-Aug	31-Aug	25.8	799	4055	57	737	80
2015								
P1	01-Jan	30-Apr	8.9	1073	1073	365	365	79
P2	1-May	30-June	19.4	1180	2253	182	548	85
P3	1-July	31-July	22.7	705	2958	104	652	89
P4	1-Aug	31-Aug	25.2	782	3740	70	722	85
2016								
P1	01-Jan	30-Apr	9.6	1160	1160	568	568	79
P2	1-May	30-June	19.2	1171	2331	227	795	87
P3	1-July	31-July	23.3	745	3076	81	876	86
P4	1-Aug	31-Aug	25.4	788	3864	28	904	88

Defect observations, and fungal isolation and identification

Hazelnuts with defects were found in all the sampling years, but only sporadically in 2015. Therefore, in 2015, only healthy hazelnuts were processed. At harvest, the planned number of hazelnuts for defect assessments (30 for each replicate) were always collected, while at the early ripening stage the number collected was sometimes lower, but still representative (minimum of ten nuts).

The main symptoms observed on nuts were necrotic spots, sometimes only at the nut apex or distributed on the whole kernel. Occasionally the kernels were almost covered by white-grey mould. Further, dark brown spots were frequently visible only when the kernels were cut. In 2016, an additional symptom was observed; some kernels were completely rotten, wet and almost black (Figure 2).

Many different fungi were isolated by plating shelled hazelnuts, partly corresponding to those reported previously. Limiting the list to those regularly isolated, the fungal genera isolated were *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Diaporthe*, *Fusarium*, *Penicillium*, *Pestalotiopsis* and *Phoma*. These fungi were found both in healthy and defected hazelnuts. As with fungi reported elsewhere, *Botrytis*, *Rhiz-*

Figure 2. Symptoms observed in hazelnut kernels described as necrotic spots (A), mouldy (B), or black rotted (C), and visible after nuts were cut (D).

opus, *Septoria*, *Sphaceloma* and *Tricothecium* spp. were also isolated, but only occasionally.

The ANOVA was run for all defect incidence data collected at the two nut sampling times and for the four years, using symptoms (healthy / defected), years (2013 to 2016) and sampling dates (early and full ripening) as factors, for all the fungi mentioned (Table 4). Taking into account the whole dataset, fungal incidence was different ($P \geq 0.80$) between healthy and defected hazelnuts for *Cladosporium* ($P \geq 0.87$), *Colletotrichum* ($P \geq 0.87$), *Diaporthe* ($P \geq 0.83$), *Fusarium* ($P \geq 0.81$) and *Penicillium* ($P \geq 0.83$).

The greatest incidence of all fungi considered was observed in hazelnuts with defects, up to double the incidence in defected nuts compared to healthy ones, except for *Penicillium* spp.

Diaporthe was the most commonly isolated genus, with twice the incidence in defected compared to healthy nuts, followed by *Alternaria*, with less than half incidence, *Cladosporium* and *Fusarium*. *Colletotrichum* was rarely isolated, but five times greater incidence in rotten compared to healthy nuts.

Alternaria showed relevant incidence in 2013, the greatest incidence in the four years of the study. *Diaporthe* had greatest incidence in 2013 and 2016, significantly greater than in the other two years. The most relevant incidence of *Penicillium* and *Pestalotiopsis* was in 2016, but incidence of these two genera was less compared to *Alternaria*.

For the two sampling dates, the greatest incidence of all fungi occurred at full ripening, except for *Aspergillus* (no difference between the two sampling dates) and *Penicillium* (greatest incidence at early ripening).

The interaction between sampling date and years was significant for *Diaporthe* ($P = 0.001$); in 2015 no significant difference was found between early and

Table 4. Mean incidence of the nine most isolated fungal genera in healthy and defected hazelnuts, sampled at early and full ripening in the 4 year period of 2013 to 2016 in Caucasus region. Symptoms, year and nut maturity stage were considered as factors in ANOVA.

Anova factors	*Alternaria*	*Aspergillus*	*Cladosporium*	*Colletotrichum*	*Diaporthe*	*Fusarium*	*Penicillium*	*Pestalotiopsis*	*Phoma*
Symptom									
Healthy	14	10	9	1	28	11	14	15	3
Defected	26	15	18	5	59	18	7	20	3
Year									
2013	55 b	2 a	15	7 b	71 c	13	5 a	3 a	7 b
2014	18 a	21 c	13	3 b	19 ab	10	7 a	6 a	1 a
2015	8 a	2 ab	4	0 a	2 a	16	2 a	6 a	5 b
2016	3 a	18 bc	14	0 a	48 c	16	19 b	33 b	1 a
Growth stage									
Early ripening	17 a	13	5 a	0 a	16 a	11 a	18 b	16 a	2 a
Full ripening	20 b	11	21 b	5 b	67 b	17 b	4 a	18 b	4 b

Different letters indicated significant differences ($P \leq 0.05$), based on the Tukey's test.

late ripening, while sampling date was statistically significant for the other three years (Figure 3).

The ANOVA was run also including data only collected from defected nuts (Table 5). The greatest incidence of *Diaporthe* was confirmed, which was not significantly different between years, but significantly more frequently isolated at full ripening. The role of years was significant for *Alternaria*, *Aspergillus*, *Pestalotiopsis* and *Phoma*, with the greatest incidence in 2013 for *Alternaria* and *Phoma*, and in 2016 for *Pestalotiopsis*, even though this was not significantly different than 2014. Sampling date was also significant for *Colletotrichum,* not significant for *Cladosporium,* although three-fold greater at full ripening compared to early ripening.

Because of the greater variability in symptoms observed in 2016 compared to the previous years, data analyses were also run accounting for the different symptoms observed, namely: i) kernel with necrotic spots; ii) kernel with necrotic spots and visible mould; or iii) rotten black kernels (Figure 2). *Cladosporium* spp. and *Diaporthe* spp. showed significantly different incidence based on symptoms observed: *Cladosporium* was isolated at significantly greater incidence (36%) from rotten black kernels than *Diaporthe* (3%), but *Diaporthe* was isolated from kernels with necrotic spots, irrespective of visible/non-visible mould occurring (*Diaporthe* incidence 49% *versus* 13% *Cladosporium*).

Incidence of defected kernels

Based on observation for 2,500 hazelnuts at full ripening, both for whole and cut nuts, defects occurred in all the 4 years of the study. Differences between years were observed for incidence of defects, with 7.5% of nuts affected in 2013, 1.1% in 2014, 3.3% in 2015 and 14.3% in 2016. A single mean value was available, so no statistical analysis of these data were carried out. The incidence of hidden defects, observed only after nuts were cut, was very variable, but accounted for 50% to 60% of defected hazelnuts (mean of all samples analysed).

Correlation analyses

Pearson correlation analysis, run between the incidence of hazelnuts with defects and the incidence of fungi at harvest, confirmed a significant correlation with *Diaporthe* ($\varrho = 0.844$; $P = 0.00$) and with *Colletotrichum* spp. ($\varrho = 0.529$; $P = 0.04$), but also with *Pestalotiopsis* ($\varrho = 0.631$; $P = 0.01$). When Pearson correlations were determined between the meteorological param-

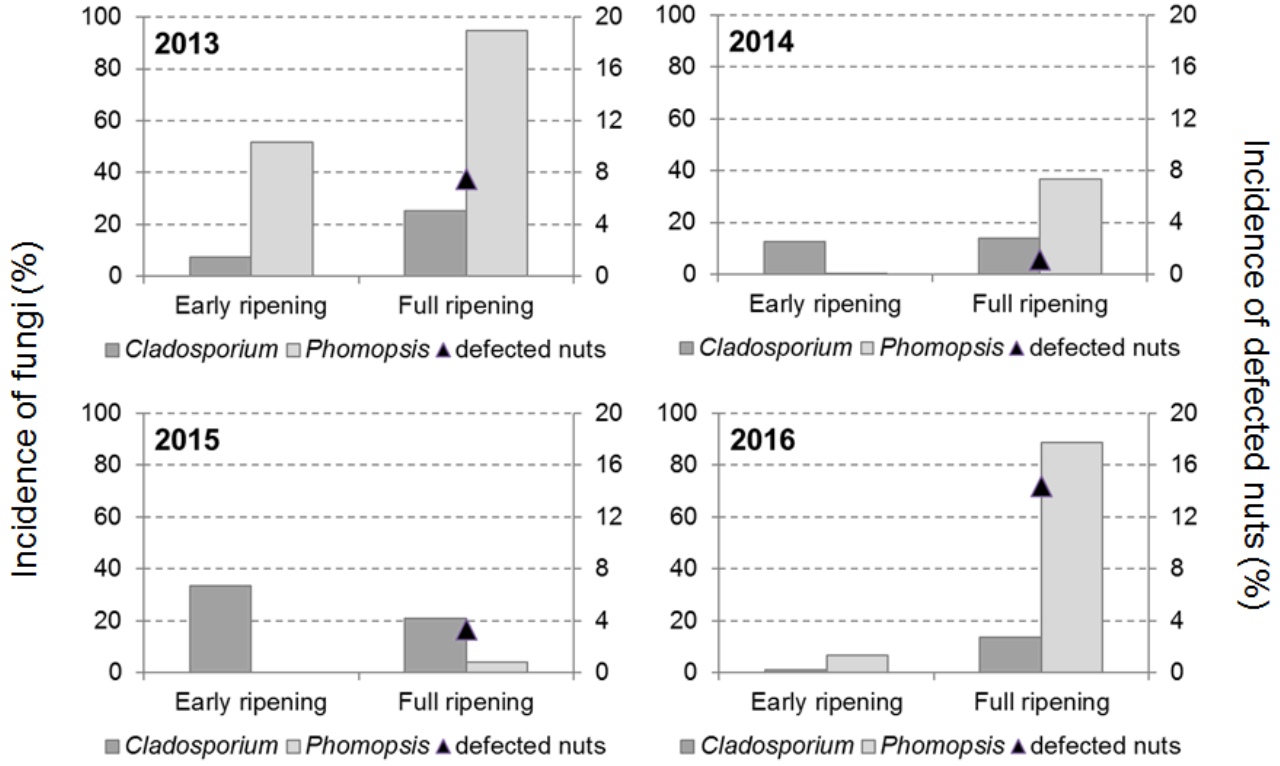

Figure 3. Incidence of *Cladosporium* spp. and *Diaporthe* spp. from hazelnuts at early or full ripening stages, and incidence of defects in nuts collected from 10 ha orchard.

eters and incidence of fungi at harvest, only summation of rainfall during the crop growing season gave a significant relationships only for *Diaporthe* ($\varrho = 0.545$; $P = 0.03$) and *Colletotrichum* ($\varrho = 0.596$; $P = 0.02$).

Confirmation of *Diaporthe* identification

The three monosporic *Diaporthe* strains showed very similar colony morphologies. Colonies of all these strains grown on PDA covered the Petri dishes after 1 week at 25°C. The colonies were "ropey" with abundant white-grey mycelium, were slightly raised, bluff and developed forming prominent growth rings, numerous black pycnidial conidiomata (each 0.5–2 mm diam.), from which mucilaginous light cream cirrhi containing alpha and beta conidia, were visible. The colony reverses were zonate with irregular lines.

Conidia were collected from pycnidia, and these were of typical *Diaporthe* form (Gomes *et al*. 2013). The alpha conidia were unicellular, non-septate, hyaline, biguttulate, and elongated, and beta conidia were non-septate, filiform, and hyaline (Figure 4). Alpha conidia were 8–12 × 2–3 µm, and beta conidia were 18–30 × 1.0–1.5 µm.

The conventional PCR amplification from the three representative isolates (PH01, PH02 and PH03) each gave amplicons of expected bp sizes. BLAST results indicated that the three isolates had 99 to 100% nucleotide sequence identity with *D. eres* for all the four loci considered. In particular, β tubulin (GenBank accession number KJ420781.1) showed 100% identity on 294 over 300 nucleotides, ACT (JN230372.1) 99% (coverage 100%), EF (JN192168.1) 99% (coverage 100%) and ITS (KY569368.1 and MH121513.1) 100% and 99% respectively for the primers pairs ITS1/ITS4 (coverage 100%) and PhomI/PhomII (294 over 300 nucleotides).

Pathogenicity test

No external or internal symptoms were observed in nil-inoculated (distilled water) hazelnut kernels.

Table 5. Mean incidence of the nine most isolated fungal genera in defected hazelnuts, sampled at early and full ripening stages in the 4 year period 2013 to 2016 in the Caucasus region. Year and sampling nut maturity stage were considered as factors in ANOVA.

Anova factors	*Alternaria*	*Aspergillus*	*Cladosporium*	*Colletotrichum*	*Diaporthe*	*Fusarium*	*Penicillium*	*Pestalotiopsis*	*Phoma*
Year									
2013	73 b	3 a	14	13	96	15	1	3 a	6 b
2014	17 a	23 b	17	5	28	11	11	10 ab	1 a
2016	3 a	19 b	21	0	50	22	8	33 b	2 a
Growth stage									
Early ripening	32	12	8	0 a	35 a	32	11	24	2
Full ripening	19	18	29	10 b	85 b	3	2	15	4

Different letters indicated significant differences ($P \leq 0.05$), based on the Tukey's test.

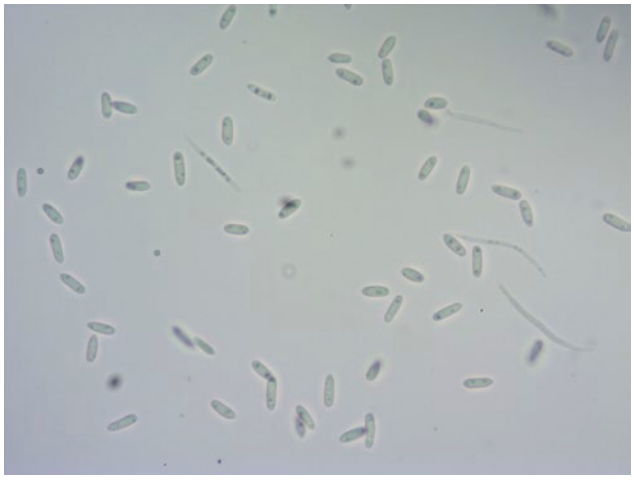

Figure 4. Alpha (α; 8 - 12 μm × 2-3 μm) and beta (β; 18-30 μm × 1.0-1.5 μm) conidia of *Diaporthe* spp., produced in PDA cultures.

All of the inoculated kernels showed necrotic spots on their surfaces, which were more evident in kernels slightly damaged before inoculation. Undamaged kernels frequently developed grey mould on their surfaces. After kernels were cut, internal browning was only observed in kernels slightly damaged before inoculation (Figure 5). *Diaporthe* sp. was re-isolated from all artificially inoculated kernels, and occasionally also in the nil-inoculated controls. Identification of the re-isolated *Diaporthe* was based on morphological characters.

Discussion

Hazelnut production and the occurrence of kernel defects show fluctuations depending on the climatic conditions from year to year (www.fao.org). Due to the global importance of the crop, hazelnut defects have key economic impacts. Understanding of the causes of these defects is essential in order to prevent their occurrence within the hazelnut value chain (Garrone and Vacchetti, 1994). In the hazelnut market, the commonly called "rotten hazelnut" is one of the major defects that affect commercial quality, yield losses and market values in the hazelnut industry. For commercial evaluation, "rotten hazelnuts" includes all kinds of defects, including brown spotted or mouldy nut kernels. These symptoms are frequently associated with negative sensory attributes, such as mould, old, bitter and earthy tastes that make the hazelnuts unsuitable for trade. These defects cause considerable problems to the value chain, because a major part of the hazelnut production goes to processing industries.

Sorting machines are currently used in industrial plants to exclude foreign objects and defected nuts from the processing chain, and efforts are made to improve sorting performance (Moscetti *et al*., 2015). Nevertheless, sorting operates mostly with visible defects that are, at least, based on the symptoms observed in the present study, and represent less than 50% of the uncompliant product; the complementary percentage consists of defects visible after the nuts are cut in half. Therefore, reducing occurrence of defects,

Figure 5. A, Necrotic spots on hazelnut kernel surfaces developed after artificial inoculation with *Diaporthe* spp. and incubation at 20°C for 21 d. B. Internal browning observed in cut kernels that were slightly damaged before artificial inoculation.

based on the identification of causal agents and on their control, is mandatory to improve the quality of raw nut products.

It is a commonly held view among stakeholders involved in the hazelnut value chain that inadequate post-harvest management strongly contributes to the formation of, or at least increases in, these defects. However, the data collected in the present study showed an important incidence of defects at harvest, at least in 2013 and 2016.

Kernel spots, with dark, sunken lesions on kernel surfaces, varied in size and shape, attributed to a yeast, are probably the description found in literature (Pscheidt and Ocamb, 2017) that best fits with the predominant defect observed in the present study. Nevertheless, the symptoms do not fit completely; in many cases, mould was visible on the brown areas of affected nuts, suggesting filamentous fungi as causal agents. In addition, a further severe symptom was noticed in 2016, of rotten black completely decayed nuts, possibly similar to the defect attributed to *C. acutatum* (Sezer and Dolar, 2012b).

Several fungi were isolated during this 4 year study, and at least nine different genera were regularly detected. Among them, *Diaporthe* spp. played a key role, and this was confirmed by the significant difference recorded in the incidence of this fungus between years. Supporting this was the high incidence of the fungus in 2013 (71%) and 2016 (48%), the years with the greatest incidence of defected nuts, but there was only 2% incidence in 2015, the year with almost no defects. Furthermore, *Diaporthe* incidence increased from early to full ripening, the growth stage where symptoms on nuts were the most severe. *Diaporthe* was closely associated with defected nuts, but not in all the cases analysed. This could be attributed to competition amongst ubiquitous fungi on artificial media during isolation, but the co-occurrence of other fungi could also have played a role as causes of visible symptoms. The co-occurrence of *Diaporthe* with several other fungi has been reported in other pathosystems, such as grapevines in South Africa (Mostert *et al*., 2001) or more recently in branch cankers and stem-end rot of avocado in Italy (Guarnaccia *et al*., 2016).

Diaporthe has been reported to be associated with internal discolouration of hazelnut kernels that sometimes become opaque, white to translucent, and buttery yellow, and that have bitter, rancid flavour (Teviotdale *et al*., 2002; Pscheidt and Ocamb, 2017). This description is comparable to defects we observed only after the kernels are cut in half, indicating that cutting is important to reveal this undesirable defect. The pathogenicity test conducted in this study confirmed *Diaporthe eres* can cause the observed symptoms, including internal kernel browning.

Phomopsis spp., now recommended to be named as *Diaporthe* (Rossman *et al*., 2015), is a complex paraphyletic genus (Gao *et al*., 2017), and is a genus involved in several important diseases of annual and perennial hosts, including grapevine, juniper, apple, peach, blueberry, strawberry, cantaloupe and eggplant (Agrios, 2005; Karaoglanidis and Bardas, 2006). Important recently studied diseases caused by *Diaporthe* are stem canker of soybean (Santos *et al*., 2011), melanose of citrus fruit (Guarnaccia and Crous, 2017), and wood disease of deciduous fruit trees (Sessa *et al*., 2017), and several others could be mentioned. The symptoms attributed to *Phomopsis*/*Diaporthe* are quite different, depending on the host plant or crop, but at least dark patches on citrus fruits or rot of eggplant fruits are not very different from those observed in hazelnuts, while taking account of the differences between the products.

Diaporthe asexual stages produce conidia in globose pycnidia, which represent important overwintering structures for the pathogen in all the diseases described. Conidia, spread by rain splashes, cause primary and secondary infections (Agrios, 2005). This conforms with our findings that rainfall is a crucial parameter for fungal dissemination and infection. 2013 and 2016 were the years with the greatest incidence of defected hazelnuts, with the greatest rain falling during the crop growing season, significantly and positively correlated with greatest incidence of *Diaporthe*.

A clear role was also attributed to *Cladosporium*, strictly associated with rotted black kernels regularly found only in 2016. This fungus, in particular *Cl. cladosporioides*, has only been reported in stored hazelnuts in Iran (Mogghadam and Taherzadeh, 2007). Nevertheless, this could be due to the focus of other researchers on defected, rather than completely rotten kernels.

In conclusion, the present study confirms that *Diaporthe* is a key candidate fungus as the cause of the most important hazelnut defects in the Caucasus region. Identification at species level, and confirmation of pathogenicity, indicate *D. eres* as the main species involved. However, as the pathogenicity test of the present study focused only on one representative isolate, this conclusion should be confirmed.

These result also emphasise the crucial role of the growing season for hazelnut defect development, *Diaporthe* being typical "field" fungi that are not reported as "postharvest" fungi. Further studies, including support from molecular tools, are necessary to improve the knowledge acquired, and description of the principal causal agent(s) of hazelnut defects. This will better address future efforts to understand *Diaporthe* infection cycles on hazelnuts, interactions with other co-occurring fungi, and to develop rational preventive actions to mitigate the impacts of these diseases. Predictive approaches for *Diaporthe* related disease occurrence, focused on the crop growing period, have been developed for soybean, based on crop growth stage at fungal infection (McGee, 1986), and for grapevine, based on weather conditions (Erincik and Madden, 2003; Nita *et al*. 2006; Anco *et al*., 2013). A similar approach should also be considered for hazelnut pathogens and diseases.

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