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Fungi Causing Storage Rot of Apple Fruit in Integrated Pest Management System and their Sensitivity to Fungicides

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Abstract. Apple fruit rot can be caused by several fungi. In Northern Europe, the most common storage rot, Bull's eye rot, is caused by *Neofabraea* spp., bitter rot by *Colletotrichum* spp., brown rot by *Monilinia fructigena*, grey mould is caused by *Botrytis cinerea* and *Fusarium* rot by several *Fusarium* species. Blue mold decay caused by *Penicillium expansum* is an important disease in several European countries. Incidence of different causal agents may vary depending on cultivar, climate during growing season and agricultural practices. The main objective of the study was to obtain baseline information about apple rot-causing fungi, their incidence during fruit storage and to evaluate the fungicide sensitivity of most of isolated fungal species. The study was performed during the storage period of apples after the growth season of 2013. Rotten apples were sorted in the storage and part of them was brought to the laboratory in order to obtain fungal isolates. Fungi were identified according to the morphological characteristics and sequencing of the ITS1-5.8S-ITS2 region. During storage in February and March the total percentage of rotten apples in various cultivars varied from 3.6 to 58.9%. All post-harvest diseases described in Northern Europe were detected. In part of the storehouses apple rot caused by *Cadophora luteo-olivacea* was observed. *Alternaria* spp. and *Cladosporium* spp. were detected on few apples as secondary infection agents. Using the most often isolated fungal species, sensitivity tests were performed against five commonly used fungicides. In general, the sensitivity of tested fungi to the fungicides was high with exception of several *Neofabraea* and *Alternaria* isolates.
Key words: *Colletotrichum* spp., *Monilinia fructigena*, *Neofabraea alba*, *Neofabraea malicorticis, Penicillium* spp.

Introduction

Apple rot is an economically significant disease on apple (*Malus´ domestica* Borkh) and can be caused by several filamentous fungi with worldwide distribution like *Penicillium expansum* Link. and *Botrytis cinerea* Pers. that are common and causing significant economic losses in the USA and Europe (Sutton *et al*., 2014). It has been shown that in Northern Europe, particularly in Norway, the most important storage diseases in organically grown apples were caused by *Colletotrichum acutatum* J. H. Simmonds (bitter rot) and *Neofabraea* spp. (Bull's eye rot) up to 64 and 30% respectively from all rotten apples (Borve, Roen, & Stensvand, 2013). Grey mould caused by *Botrytis cinerea*, *Fusarium* rot caused by several *Fusarium* species, brown rot caused by *Monilinia fructigena* (Aderh & Ruhland) Honey and blue mold decay caused by *P. expansum* were found more rarely (Borve, Roen, & Stensvand, 2013). Apple rot studies in Denmark and Germany about organic orchards or orchards not treated with fungicides after petal fall have shown that *Neofabraea alba* (E.J. Guthrie) Verkley and *Neofabraea perennans* Kienholz were the most common storage-rot fungi (up to 62%). Other fungi, such as *Neonectria galligena* (Bres.) Rossman & Samuels, *M. fructigena*, *Cladosporium* spp., *P. expansum*, *Phacidiopycnis washingtonensis* Xiao & J.D.Rogers, *C. acutatum*, *Gibberella avenacea* R.J. Cook, *B. cinerea* were present up to 5% (Maxin *et al*., 2012a). The *Fusarium* rot has been detected on 9 to 30% of apples depending on cultivar stored in Ultra Low Oxygen (ULO) conditions in Croatia (Sever *et al*., 2012). Blue mold decay caused by *P. expansum* is damaging 30 to 60% of cold-stored apples in France, and it is important disease not only in other European countries but also in the USA (Morales *et al*., 2010). It is reported that *Alternaria alternata* causes core rot (Niem *et al.*, 2007) and rot around injuries or at calyx (Sutton *et al*., 2014). The rubbery rot caused by *P. washingtonensis* is considered as a new emerging disease in Northern Europe, and it is reported to cause rot in up to 10% of apples stored in ULO conditions (Weber, 2011).

Apple rot incidence may vary depending on cultivar (Sever *et al*., 2012); Weber, 2011) and harvest time (Borve, Roen, & Stensvand, 2013). Climate conditions during growing season can also have a significant impact, for example, on spore dispersal and following brown rot incidence as it is proved in the conidial dispersal rates in the case of *M. fructigena* in organic and integrated apple orchards in Hungary (Holb, 2008).

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Apples from integrated orchards have a lower disease incidence in comparison to organic orchards in the case of brown rot (Holb, 2008). Similarly, apples from conventional orchards have been reported to have lower levels of storage rots (blue mold, brown mold and Bull's eye rot) in comparison with organic orchards (DeEll & Prange, 1993).

Two mechanisms of infection can be determined, primary and secondary infection. The primary infection usually happens in orchards or during sorting before storage. The secondary infection happens when fungal mycelium and spores spread from infected apples to uninfected apples (Dutot, Nelson, & Tyson, 2013).

The sensitivity of above mentioned apple rot-causing fungi to the fungicides is various. It is reported that *N. alba* shows low sensitivity to fungicides. For instance, in the investigation in the USA it was shown that in *in vivo* conditions from three tested fungicides (copper sulfate, trifloxystrobin, ziram) only the copper sulfate reduced the conidial production by *N. alba* but none of the fungicides used reduced the germination of conidia (Henriquez, Sugar, & Spotts, 2006). Boscalid with pyraclostrobin had shown high efficacy in preharvest application against *P. expansum* and *B. cinerea* (Xiao & Boal, 2009). High resistance of several *P. expansum* isolates has been recorded against benomyl, thiabendazole and diphenylamine (Sholberg *et al.*, 2005). Azoxystrobin, difenoconazole, Polyoxin B and trifloxystrobin have been effective against *A. alternata* causing core rots (Reuveni & Sheglov, 2002).

In the present investigation apples from integrated orchards were examined at the end of the storage period in February and March. The aim of the study was to determine the main causal agents of apple rot during storage and to evaluate the sensitivity of most isolated fungal species to commonly used fungicides.

Materials and Methods

Inspected Apple Storehouses

Apples grown in seven orchards (Table 1) were stored in four storehouses: (1) a farm in Beverina region (northern part of the country) with average

Table 1

Applications of fungicides and other preparations in apple orchards involved in the investigation

Orchard and location	Treatments with fungicides and calcium preparations, dose (kg or L ha^{-1}) and application time in 2013	Total treatments with fungicides
I Beverina region	Funguran OH 1.5 L (06.-07.05.), Score 250 EC 0.2 L (22.05.), Dithane NT 2 kg (28.05.), Chorus 50 WG 0.46 kg (08.-10.06.), Score 250 EC 0.185 L (20.-21.07.), Chorus 50 WG 0.45 kg (12.08.), five treatments with $CaCl_2$, in total 19 kg (18.07.-28.08.)	6
II Bauska region	Funguran OH 1.5 L (03.05.), Dithane NT 3 kg (08.05), Effector 0.3 kg / Chorus 50 WG 0.25 kg (20.05.), Effector 0.3 kg /Score 250 EC 0.25 kg (26.05.), Effector 0.3 kg /Score 250 EC 0.2 L (02.06.)	5
III Pure region A	Champion 4 kg (03.05.), Effector 0.25 kg / Score 250 EC 0.15 L (14.05.), Chorus 50 WG 0.4 kg (20.05.), Score 250 EC 0.15 L /Dithane NT 2 kg (29.05.), Chorus 50 WG 0.4 kg (06.06.), Dithane NT 2 kg (28.06.), Score 250 EC 0.15 L /Dithane NT 2 kg (17.07.)	7
IV Pure region B	Funguran OH 1.5 L (04.05.), Difo 250 EC 0.2 L /Dithane NT 2 kg (11.05.), Chorus 50 WG 0.4 kg (19.05.), Difo 0.2 L /Dithane 2 kg (27.05.), Dithane NT 2 kg (04.06.)	5
V Talsu region	Funguran OH 1.25 L (05.05.), Chorus 50 WG 0.2 kg (13.05.), Chorus 50 WG 0.2 kg/ Effector 0.25 kg h^{-1} (30.05.), Score 250 EC 0.15 L / Effector 0.25 kg (18.06.), Chorus 50 WG 0.2 kg/ Effector 0.25 kg (15.08.)	5
VI Vandzene region	Champion 4 kg (03.05.), Dithane NT 2 kg / Score 250 EC 0.2 L (20.05.), Dithane NT 2 kg / Score 250 EC 0.2 kg (28.05.), Difo 250 EC 0.2 L (14.06.) or Dithane NT 2 kg/ Score 0.2 L (14.06.)	4
VII Jelgava region	Champion 1% (01.05.), Effector 0.3 kg (11.05.), Chorus 50 WG 0.33 kg (30.05.), Dithane NT 2 kg (15.06.), Chorus 50 WG 0.33 kg (27.06.), Effector 0.3 kg/ $CaCl_2$ 1 % (07.07.), Chorus 50 WG 0.33 kg/ $CaCl_2$ 1 % (28.07.)	7

air temperature in the storage 1–3 °C and average relative air humidity 80–90% (orchard I), (2) a farm in Bauska region in the central part of Latvia (2–3 °C, 90%; orchard II); (3) a joint storage for several farmers in Pure region in Western part of Latvia (4–4.5 °C, 80–90%); (4) a farm in Vandzene region in Western part of Latvia (4 °C, 85–90%; orchard VI). In the joint storage in Pure region, the inspected apple cultivars had been grown in various orchards: 'Sinap Orlovskij' in the orchard III, 'Belorusskoje Malinovoje' on the farm in Pure region (orchard IV) and 'Antej' on the farm in Talsi region (orchard V). From apples originating from the orchard VII few fungal isolates were obtained for comparison in the fungicide sensitivity tests, the storage site itself was not inspected.

The apple storehouses were inspected in February and March, 2014. In the storage, healthy and rotten apples in the containers were counted, one container for each cultivar containing from 600 to 27000 apples. Diseases were identified according to their symptoms on the location or later in the laboratory. The following apple cultivars were monitored: 'Ligol', 'Tellissaare', 'Antej', 'Sinap Orlovskij', 'Belorusskoje Malinovoje', 'Auksis', 'Lobo' and 'Forele'.

The applications of fungicides and calcium preparations in the mentioned apple orchards in 2013 are listed in Table 1. It has to be admitted that farmers in Latvia only occasionally use fungicides targeted specially against apple fruit rot. Only treatments against apple scab are usually carried out.

Isolation and Identification of Fungi

The fungal material (mycelium and spores) from rotten apples was directly subjected to light microscopy or after a few days long incubation of rotten apples in the incubator (20 ± 2 °C). From the rotten apples pure cultures of fungi were isolated on the potato dextrose agar (PDA) (Biolife, Italy). Fungal cultures were identified using morphological characteristics (Crous *et al*., 2019; Sutton *et al*., 2014); Watanabe, 2002) or with molecular biology methods sequencing part of the ITS1-5.8S-ITS2 region. Genomic DNA from approximately 0.25 g of mycelia was extracted using the E.Z.N.A. Fungal DNA Mini kit (Omega Bio-Tek, USA). The following primers were used: ITS4, universal for eukaryotes (White *et al*., 1990); ITS1F, specific for fungi (Gardes & Bruns, 1993). The PCR reactions in SensoQuest Labcycler 48 (SensoQuest, Germany) were carried out in 25 µl volume. The mixture contained 12.5 µl Hot Start Master Mix 2X, 0.375 µl Bovine Serum Albumin 20 mg ml^{-1} (all reagents from Thermo Scientific Fermentas Molecular Biology Solutions, Lithuania), 0.5 µl of each 10 µM primer (Integrated DNA Technologies, USA), 10.125 µl sterile distilled water and 1 µl of DNA template. The PCR conditions were as follows: the initial denaturation step of 4 min at 95 °C, 40 s of denaturation at 95 °C, 40 s of annealing at 52 °C, 1 min of primer extension at 72 °C (30 cycles) and final extension 10 min at 72 °C.

Amplified DNA fragments were treated with FastAP™ Thermosensitive Alkaline Phosphatase and Exonuclease I (Thermo Scientific Fermentas Molecular Biology Solutions, Lithuania) and sequenced by Macrogen Europe (Amsterdam, the Netherlands). Double stranded sequences of PCR amplicons were assembled using Staden Package 1.6.0. (Bonfield, Smith, & Staden, 1995). The resulting consensus sequences were used in BLASTN homology search against the National Centre for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov).

Identification of such fungi as *Cadophora luteo-olivacea* (J.F.H. Beyma) T.C. Harr. & McNew and *Alternaria alternata* (Fr.) Keissl. apple pathotype (*A. mali* Roberts) was further proved by specific PCR using the following primers: Clm-REV and Clu-FOR or Cma-FOR specific to ITS1-5.8S-ITS2 region of *C. luteo-olivacea* or *C. malorum*, respectively (Spadaro *et al*., 2011) and LinF1 and LinR specific to *AMT* gene of *A. alternata* apple pathotype (*A. mali*) (Johnson *et al.*, 2000).

Sequences of the ITS1-5.8S-ITS2 region of 59 fungal isolates were submitted to the GenBank (accession numbers KP903568-KP903626, KT923785-KT923789). All isolates were preserved in –86 ºC freezer in the collection of Latvian Plant Protection Centre.

Application of Koch's Postulates

With most often isolated fungal species from this and previous investigation of Latvian Plant Protection Centre (Volkova & Juhnevica-Radenkova, 2015) the Koch's postulates were applied using the basic principles of the methodology described in other investigations (Mari *et.al*., 2012; Xiao & Rogers, 2004). Inoculation was performed on apples sterilized for 2 min in 2% hypochlorite solution and rinsed with deionized water. In apples, the inoculation, a manual cut with a sterile scalpel up to 3 mm deep, with a small piece of fungal mycelia and/or spores from the cultures grown 14 days on PDA was done. After inoculation the apples were placed in sterile plastic boxes on the wet paper towel and incubated at 20 ± 2 °C up to 39 days. After incubation re-isolation of the pathogenic fungi was performed and these were identified according to the morphological characteristics.

Fungicide Sensitivity Tests

With most often isolated fungal species (*N. alba*, 11 isolates; *N. malicorticis*, five isolates; *C. luteo-olivacea*, three isolates; *A. alternata*, six isolates) representing various orchards fungicide sensitivity tests were carried out against five fungicides (Table 2)

Table 2

Fungicides and their concentrations used in the fungicide sensitivity tests

Fungicide, active substance, concentration	Application target	Recommended dose	Concentration used in the experiment (%)				
			1	2	3	4	5
Effector, dithianon, 700 g kg^{-1}	Apple fruit rot, apple scab	0.05-0.1%	0.025	0.05	0.075	0.1	0.2
Score 250 EC, difenoconazole 250 g l^{-1}	Apple scab, powdery mildew	0.01-0.05%	0.005	0.01	0.03	0.05	0.1
Chorus 50 WG, cyprodinil 500 g kg^{-1}	Apple scab, fruit rot of plums	0.3-0.45 kg ha^{-1}	0.03	0.06	0.075	0.09	0.18
Dithane NT, mancozeb 750 g kg^{-1}	Apple scab	2.0 kg ha^{-1}	0.1	0.2	0.3	0.4	0.6
Signum, boscalid 26.7 %, pyraclostrobin 6.7%	Fruit rot of plum and cherry	0.75-1.0 kg ha^{-1}	0.075	0.15	0.17	0.2	0.4

Table 3

List of isolates used in the fungicide sensitivity tests

Species	Orchard, cultivar	Accession number in Genbank	Species	Orchard, cultivar	Accession number in Genbank
N. alba	I, 'Beloruskoje Malinovoje'*	Not submitted	*N. malicorticis*	I, 'Beloruskoje Malinovoje'	KP903612
N. alba	I, 'Beloruskoje Malinovoje'	KT923785	*N. alba*	V, 'Antej'	KP903587
N. malicorticis	I, 'Sinap Orlovskij'	KP903615	*N. malicorticis*	VI, 'Forele'	KP903625
N. alba	I, 'Tellissaare'*	Not submitted	*C. luteo-olivacea*	III, 'Sinap Orlovskij'	KP903599
N. alba	II, 'Ligol'	KT923786	*C. luteo-olivacea*	IV, 'Beloruskoje Malinovoje'	KP903596
N. alba	II, 'Lobo'	KP903582	*C. luteo-olivacea*	VI, 'Forele'	KP903623
N. alba	II, 'Tellissaare'	KT923787	*A. alternata*	II, 'Auksis'	KP903568
N. alba	III, 'Sinap Orlovskij'	KT923788	*A. alternata*	II, 'Ligol'	KP903579
N. alba	IV, 'Beloruskoje Malinovoje'	KT923789	*A. alternata*	II, 'Tellissaare'	KP903586
N. alba	VII, 'Auksis'	KP903614	*A. alternata*	II, 'Auksis'	KP903571
N. alba	VII, 'Auksis'	Not submitted	*A. alternata*	VI, 'Beloruskoje Malinovoje'	KP903621
N. malicorticis	I, 'Sinap Orlovskij'	KP903618	*A. alternata*	III, 'Sinap Orlovskij '	KP903601
N. malicorticis	I, 'Sinap Orlovskij'	KP903591	-	-	-

*- isolate obtained from the previous investigation (Volkova & Juhnevica-Radenkova, 2015).

using *in vitro* agar plate test (Russell, 2002). Two additional *N. alba* isolates originated from the orchard VII (Table 1). A complete list of isolates is given in Table 3. Each fungal isolate was grown in two replicates on PDA supplemented with particular fungicide in each concentration after autoclaving just before the test. Concentrations 1 and 5 were twice lower or twice higher than minimal and maximal doses recommended by a producer. Other concentrations were within the recommended range. The recommended dose for Dithane NT corresponded to the concentration 4. The theoretical spraying volume used for calculations was 500 l ha^{-1}. Fungi were incubated at 25 °C for three weeks; after that the diameter of fungal colony was measured.

Statistical Analysis

The statistical analysis was performed with data from the fungicide sensitivity tests comparing the average colony diameters of isolates at presence of various fungicides and in control plates, and within isolates from the same species and among various species. The F-test, t-test ($\alpha = 0.05$) and correlation analysis were done with *Excel* (Microsoft, USA). Both Pearson correlation coefficients (*r*) and determination coefficients (R^2) were calculated to determine the correlation strength among fungicide doses and average growth results of every species.

Results

In February and March, the total percentage of rotten apples of various cultivars and in different storage sites ranged from 3.55 to 58.88% (Table 4). Both types of infections - primary and secondary, were observed,. From infected apples *Neofabraea* spp. ranged from 0.33 to 50.88% depending on the cultivar. The most severely affected cultivar was 'Forele'. *Fusarium* spp. caused rot ranged from 0 to 2.22%, *Penicillium* spp. from 0.04 to 5.34%, *Colletotrichum* spp. from 0.12 to 3.13%, *Botrytis* spp. from 0 to 3.37%, *Monilinia* spp. from 0 to 21.08% (the most affected cultivar was 'Lobo'). In

Table 4

Total percentage of rotten apples of various cultivars and in different storage sites and percentage of particular rot type

Cultivar	*Neofabraea spp.*[1]	*Fusarium spp.*[2]	*Penicillium spp.*[3]	*Colletotrichum spp.*[4]	*Botrytis cinerea*	*Monilinia fructigena*	*Alternaria alternata*	*Cladosporium* spp.	*C. luteo-olivacea*	Other[5]	Total number of apples	Rotted apples (%)
Storage in Bauska region												
'Tellissaare'	0.33	0.15	0.37	0.33	3.37	0.18	0.04	0.18	0.00	0.00	2728	4.95
'Auksis'	3.29	2.22	5.34	1.51	0.27	0.00	0.31	0.00	0.00	0.00	2249	12.89
'Lobo'	0.90	0.30	0.60	0.15	0.00	21.08	0.00	0.30	0.00	0.00	664	23.34
'Ligol'	0.95	0.24	2.25	0.12	0.06	0.12	0.06	0.00	0.00	0.00	1690	3.55
Joint storage for several farmers in Pure region												
'Antej'	3.80	0.00	0.29	1.58	0.06	0.29	0.06	0.00	0.06	0.00	1709	6.14
'Beloruskoje Malinovoje'	6.38	0.00	0.51	2.42	0.05	0.10	0.00	0.00	0.00	0.41	1945	9.87
'Sinap Orlovskij'	4.24	0.19	0.64	2.44	0.51	0.51	0.06	0.26	0.06	0.32	1557	9.31
Storage in Vandzene region												
'Beloruskoje Malinovoje'	9.26	0.04	0.53	1.24	0.89	0.09	0.04	0.00	0.00	0.75	2258	12.84
'Forele'	50.88	0.25	1.00	3.13	2.88	0.50	0.00	0.13	0.13	0.00	800	58.88
Storage in Beverina region												
'Sinap Orlovskij'	1.59	0.04	0.04	0.09	0.04	0.13	0.00	0.00	0.00	2.04	1562	5.76
'Beloruskoje Malinovoje'	8.00	0.13	0.13	0.00	1.00	1.13	0.38	0.00	0.00	4.38	1215	9.96

1 – *N. alba*, *N. malicorticis*; 2 – *F. avenaceum* (*G. avenacea*), *Fusarium* spp.; 3 – *P. brevicompactum* Dierckx, *P. echinulatum* Raper & Thom ex Fassat., *P. expansum, P. solitum* Westling; 4 – *C. acutatum*, *C. gloeosporioides* (Penz.) Penz. & Sacc.; 5 - *Aureobasidium pullulans* (de Bary) G. Arnaud, *Diaporthe eres Nitschke* (*Phomopsis velata* (Nitzschke ex Sacc.) Traverso), *N. galligena*.

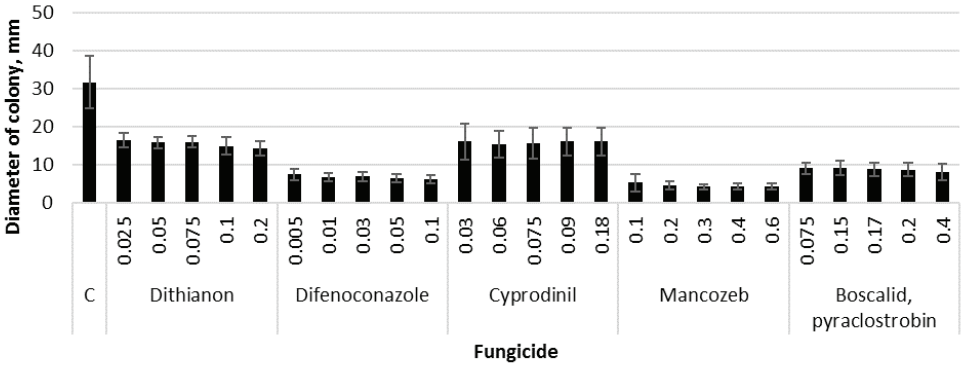

Figure 1. Sensitivity of *N. alba* isolates to five fungicides (C – control without fungicides). Error bars indicate standard deviations (±SD), $n = 11$.

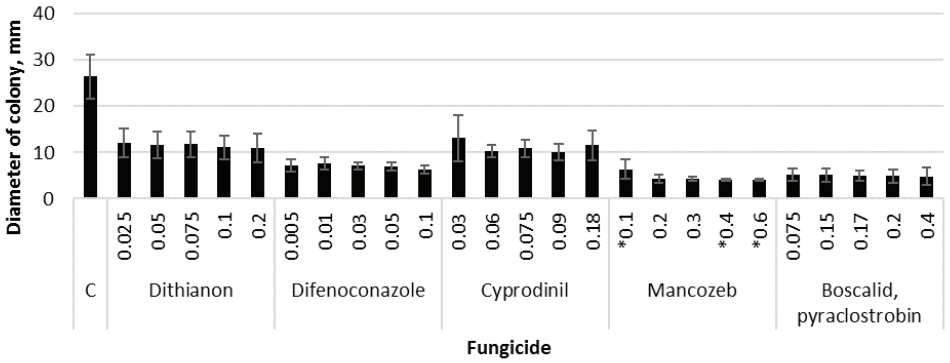

Figure 2. Sensitivity of *N. malicorticis* isolates to five fungicides (C – control without fungicides). Statistically significant differences in dose response are indicated with an asterisk. Error bars indicate standard deviations (±SD), $n = 5$.

two storehouses apple rot caused by *C. luteo-olivacea* was detected, and its identity was successfully proved by PCR with primers specific to this species (data not shown). *A. alternata* and *Cladosporium* spp. were detected on a few apples as secondary infection agents. None of the *A. alternata* isolates gave positive amplification products with primers specific to *AMT* gene.

Application of Koch's postulates was successfully carried out with *C. gloeosporioides*, *N. alba*, *F. avenaceum* and *Penicillium expansum* on six apple varrieties: 'Auksis', 'Belorusskoje Malinovoje', 'Iedzēnu', 'Sinap Orlovskij', 'Tellissaare' and 'Saltanat', and with *C. luteo-olivacea* on 'Auksis' and 'Zarja Alatau'.

Results of fungicide sensitivity tests are shown in Figure 1 to 4. Isolates of *N. alba* and *N. malicorticis* were sensitive to difenoconazole, mancozeb and boscalid with pyraclostrobin which reduced the growth of these fungi by 70–80%. Dithianon and cyprodinil reduced the fungal growth only by 50% (Figures 1 and 2). In general, isolates of *N. malicorticis* were significantly slowly growing ($p < 0.001$), but the average response to fungicides was not significantly different.

Three isolates of *N. alba* from the orchards IV, V and VII were significantly less sensitive to the tested fungicides in comparison to the rest of isolates ($p < 0.05$), especially to the dithianon and cyprodinil. The growth of these isolates in the presence of the dithianon was reduced only by 41, 45 and 43%, respectively, at the highest fungicide concentration. In the presence of the cyprodinil the growth was reduced only by 41, 36 and 35%, respectively, at the presence of the highest fungicide concentration.

From five isolates of *N. malicorticis* one isolate originating from the orchard I (KP903612) showed less sensitivity to dithianon in comparison to the rest of isolates. The growth of this isolate was reduced only by 51 *vs*. 64% in the presence of the highest fungicide concentration.

Differences of dose response of *N. alba* isolates to tested fungicides were not statistically significant. In the case of *N. malicorticis* statistically significant

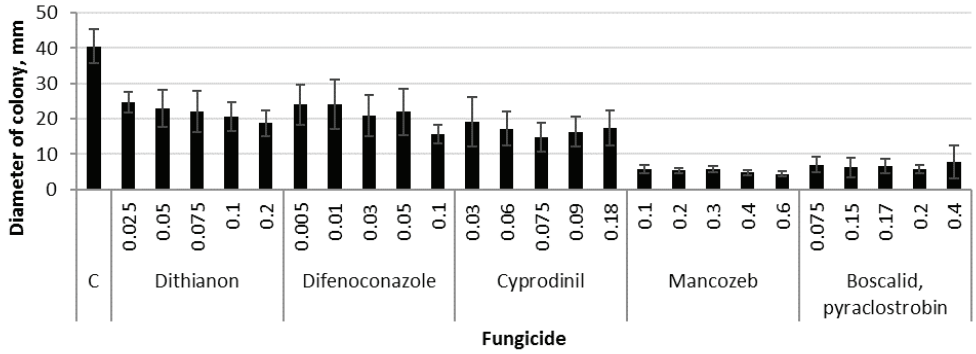

Figure 3. Sensitivity of C. *luteo-olivacea* isolates to five fungicides (C – control without fungicides). Error bars indicate standard deviations (±SD), $n = 4$.

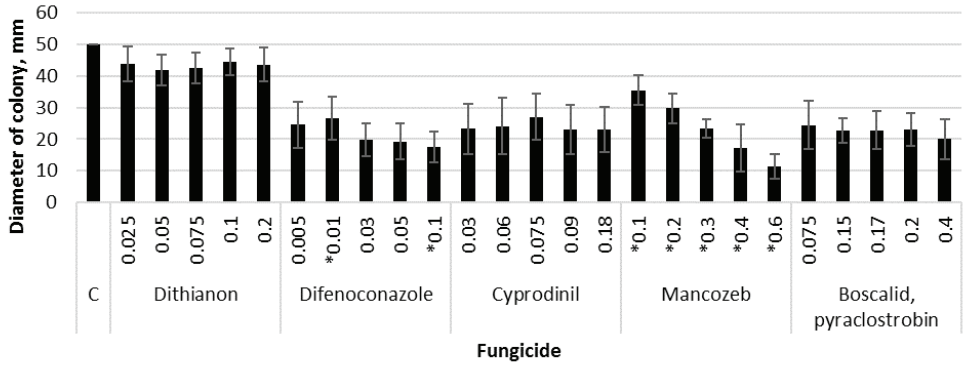

Figure 4. Sensitivity of *A. alternata* isolates to five fungicides (C – control without fungicides). Statistically significant differences in dose response are indicated with an asterisk. Error bars indicate standard deviations (±SD), $n = 7$.

differences ($p < 0.05$) in the dose response were detected only in the case of mancozeb ($r = -0.70$, $R^2 = 0.49$).

Isolates of C. *luteo-olivacea* were sensitive only to mancozeb and boscalid with pyraclostrobin, which reduced the growth of these fungi on average by 85%. Other fungicides were less effective, reducing the growth of these fungi only by 50–60% (Figure 3). Differences of dose response of C. *luteo-olivacea* isolates against tested fungicides were not statistically significant although the correlation between dithianon and difenoconazole doses and average colony diameters was strong ($r = 0.95$, $R^2 = 0.92$).

Isolates of *A. alternata* were medium sensitive to difenoconazole, cyprodinil, mancozeb and boscalid and pyraclostrobin, on average reducing the fungal growth by 50%, the dithianon was the least effective reducing the growth only by 13% (Figure 4). One isolate from the orchard II was 100% resistant to all concentrations of the dithianon. On average, *A. alternata* isolates showed statistically significant differences in dose response against mancozeb ($r = -0.98$, $R^2 = 0.97$) and particularly against difenoconazole ($r = -0.85$, $R^2 = 0.72$).

Discussion

The species spectrum of apple rot-associated fungi identified in the present study was mainly identical to the causing agent spectrum described in the literature. The exception was the rubbery rot caused by *P. washingtonensis* that is considered as a new emerging disease in Northern Europe, and it is reported to cause rot in up to 10% of apples stored in ULO conditions (Weber, 2011), but it was not detected in the present investigation. The second exception was side rot caused by *C. luteo-olivacea* that is not so common in other studies, but it was detected in two storage sites in Latvia. This pathogen is reported as important kiwi pathogen especially during long storage periods, but it has also been found on apples (Spadaro *et al*., 2011).

From the apples in three storage sites several isolates of *Phomopsis velata* (imperfect stage of *Diaporthe eres*) were isolated. This fungus has been previously described as common pathogen of peach

(*Prunus persica* (L.) Stokes) causing shoot blight and canker disease of peach in Greece (Thomidis & Michailides, 2009) and minor disease of apple and pear in North America, Europe and Japan causing cankers and fruit decay (Sutton *et al*., 2014).

Several *Penicillium* species (*P. brevicompactum*, *P. echinulatum*, *P. expansum, P. solitum*. *P. expansum* and *P. solitum*) were isolated, which are common apple rot-causing pathogens (Sholberg *et al.*, 2005). Isolates of *P. brevicompactum* have been isolated from rotten apples in Canada (Sholberg & Haag, 1996). *P. echinulatum* have been found on lipid-rich substrates and wood residues (Frisvad & Samson, 2004).

Aureobasidium pullulans was isolated from the cultivar 'Beloruskoje Malinovoje' (orchard I) only once and has been characterized as causing agent of miscellaneous postharvest diseases of apples (Sutton *et al*., 2014).

N. galligena was isolated from the cultivar 'Lobo' (orchard II) only once and is reported as rot -causing agent of apples (Maxin *et al*., 2012a, 2012b).

The proportion of rotten apples varied from 3.55 to 58.88% and was more cultivar-specific, not related to the number of fungicide treatments or storage conditions. For example, in the storage of orchard II four cultivars were examined having the same fungicide treatments and storage conditions, but the respective proportion of rotten apples varied from 3.55 ('Ligol') to 23.34% ('Lobo'). Differences of cultivars in infection level have been described in other investigations, for instance, in the case of infections with *Fusarium* spp. (Sever *et al*., 2012).

It is reported that *N. alba* shows low sensitivity to fungicides. For instance, in the study in Southern Oregon it was detected that in *in vivo* conditions only copper sulfate reduced the conidial production by *N. alba* but none of the fungicides used (copper sulfate, trifloxystrobin, ziram) reduced the germination of conidia (Henriquez, Sugar, & Spotts, 2006). In the present investigation isolates of *N. alba* as well *N. malicorticis* showed high sensitivity to the tested fungicides: difenoconazole, mancozeb and boscalid with pyraclostrobin reduced the growth of these fungi by 70–80%, dithianon and cyprodinil reduced the fungal growth by 50%.

Three isolates of *N. alba* from the orchards IV, V and VII were less sensitive to tested fungicides, especially to dithianon and cyprodinil. The orchard VII had one of the highest numbers of fungicide treatments in 2013 (seven), but the orchards IV and V had an average number of treatments (Table 1). On the orchard VII the dithianon-containing fungicides were used twice and cyprodinil-containing fungicides were used three times in the growing season of 2013.

From five *N. malicorticis* isolates one isolate originating from the orchard I showed less sensitivity to dithianon and cyprodinil in comparison with the rest of the isolates. Orchard I had one of the highest numbers of fungicide treatments in 2013 (six). Orchard VI from which one isolate of *N. malicorticis* originated had smaller numbers of treatments – four. In the orchard I the dithianon was not used but cyprodinil-containing fungicides were used twice. In orchard VI fungicides containing dithianon or cyprodinil were not used in 2013.

Sensitivity of *A. alternata* isolates to tested fungicides was medium high by comparison to other fungi and also to other investigations. For example, in the study in Lithuania, it was detected that the growth of *A. alternata* isolated from lovage and cabbage was reduced by 80% in the case of boscalid with pyraclostrobin (Signum) in comparison with 55% in the present study (Surviliene & Dambrauskiene, 2006). One isolate from the orchard II was 100% resistant to all concentrations of the dithianon. The dithianon in this orchard was applied three times during 2013 and up to five times in the previous two years.

In fungicide efficacy trials in Czech Republic, it was stated that strobilurines were the most effective fungicides against the storage rot of cultivar 'Golden Delicious' caused by *N. alba*, *N. malicorticis* and *Penicillium* spp. (Minář, 2006). Slightly lower efficacy was shown by tolylfluanid, dodine and captan. Dithianon-containing fungicide was the least effective as it was observed in the present investigation. Since 2015 dodine is registered in Latvia against apple scab. Captan is registered against apple scab and fruit rot, but the strobilurin-containing fungicide Signum which is registered against fruit rot of plum and cherry can be used with special individual permissions on apple.

In the present investigation, the cyprodinil-containing fungicide showed the lowest inhibition effect on tested fungal species. However, it has shown efficacy against postharvest apple rot caused by *B. cinerea* in Canada (Sholberg, Bedford, & Stokes, 2003).

A few isolates with reduced sensitivity to tested fungicides found in the present study illustrate the tendency that larger numbers of fungicide applications are causing lesser sensitivity of pathogenic fungi against fungicides. Development of fungicide resistance of pathogens is one of the problems demanding for new control strategies (De Capdeville *et al*., 2002).

Conclusions

1. The most common apple fruit rot causing agents were *Neofabraea alba*, *Neofabraea malicorticis*,

Fusarium spp., *Penicillium* spp., *Colletotrichum* spp., *Botrytis cinerea*, *Monilinia fructigena*. The following species *Cadophora luteo-olivacea*, *Phomopsis velata* and *Alternaria alternata* were considered of minor importance.

2. The most severely affected cultivar by *Neofabraea* spp. was 'Forele' but the most affected cultivar by *Monilinia* spp. was 'Lobo'. The least infected cultivars in general were 'Tellissaare' and 'Ligol'.
3. In general, the sensitivity of tested fungi to the fungicides was high with exception of particular *Neofabraea* and *Alternaria* isolates. The most effective fungicides were difenoconazole, mancozeb and pyraclostrobin-containing fungicides. Recent changes in the list of fungicides that are registered in Latvia on apples can help growers to diversify the fungicide spectrum including in the treatments dodine- and captan-containing fungicides which have shown good efficacy against storage diseases of apples in other countries.
4. The investigation should be continued increasing the number of orchards, storehouses and cultivars.

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