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Original Article

Study of the Major Pathogens That Lead to Apple Fruit Decay During Storage

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Abstract

Different pathogenic fungi (e.g. *Penicillium* spp., *Monilinia fructigena*, *Venturia inaequalis*, *Glomerella cingulata*, *Diaporthe eres* etc.) can cause apple rot by producing pectic enzymes that break down apple pectin to expose the nutrients of the cells to the fungi. This study aimed to identify the pathogens that lead to the degradation of apples from five different varieties ('Granny Smith', 'Topaz', 'Imperial Gala', 'Jonagold' and 'Golden Reinders') and also the incidence of those pathogens under different treatment conditions. The results reveal different frequent attacks on distinct varieties ranging from 5 to over 50%. Of the pathogens that infect and occur in vegetation and deposit it can be seen that *Venturia inaequalis* has been identified in all varieties in most test variants. The highest frequency was recorded in the variant where during the vegetation period no treatments with fungicides against apple diseases were applied. Of the pathogens that infected and appeared during storage, isolated on the fruits, only *Fusarium* spp. and *Penicillium* spp. had a higher frequency. Applying treatments during the growing season reduced the rotting attack degree of apple fruits during storage. The best response to rot attack in the warehouse was 'Topaz' and 'Jonagold', the attack degree ranged between 0.3 and 10% on treated variants. By applying chemical treatments, the spectrum and the share of pathogens that lead to fruit degradation is different. This means that chemical treatments must be chosen depending on the nature of the pathogens and the apple variety.

Keywords: apple rot; decay; *Penicillium* spp.; plant disease; treatment; *Venturia inaequalis*

Introduction

The cultivation of the apple begins some millennia ago, as evidenced by the archaeological evidence found in the Östergötland area of Sweden, in the form of dried apples that were date with approx. 2500 years (Sandskär, 2003).

Many infectious diseases have been reported in apple orchards, most of which are produced by pathogenic fungi, but the apple is susceptible to the attack of viruses, phytoplasmas and bacteria (Ferree and Warrington, 2003; Bowen *et al*., 2010).

One of the limiting factors that influence the economic value of apples and reduces the period of consumption is the pathogen infection (González-Domínguez *et al*., 2017). These pathogens can infect apple before harvesting, under field conditions (Passey *et al*., 2017) or after harvest, during the period of storage or handling (Louw and Korsten, 2014). Symptoms of disease can occur in different phenological phases during vegetation, but many pathogens have the ability to remain latent until conditions become favorable for their manifestation (Ammar and El-Naggar, 2014). During the storage period, the most common cause of apple decay is fungal load (Sever *et al*., 2012).

Some pathogens are able to penetrate the unbroken skin of products and others require an injury in order to cause infection. (MacHardy, 1996). These damages are probably the major cause for the loss of fresh products (Köhl *et al*., 2015). During storage there are three types of pathogens that occur: pathogens that infect and occur in vegetation and deposits like *Venturia inaequalis* (Cooke) Winter (González-Domínguez *et al*., 2017; Passey *et al*., 2017) and *Glomerella cingulata* (Stoneman) Spaulding & von Schrenk (Wang *et al*., 2015; Zhang *et al*., 2016); pathogens that infect in vegetation and occur only in storage (*Neofabraea* spp. (anamorph *Gloeosporium* spp.) (Michalecka *et al*., 2016; Cameldi *et al*., 2017)) and pathogens that infect and occur in storage like *Penicillium* spp. (da Rocha Neto *et al*., 2016), *Fusarium* spp. (Kou *et al*., 2014; Wenneker *et al*., 2016) .

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In addition to the obvious symptoms of pathogens such as *Fusarium, Penicillium, Botrytis, Fusicladium, Alternaria* etc. (MacHardy *et al*., 2001; Xu and Berrie, 2005; Ikeura *et al*., 2011; Fernández *et al*., 2013; Louw and Korsten, 2014; Caiazzo *et al*., 2014; Fan *et al*., 2015) some fruits exhibited rotting symptoms without fructifications like *Phomopsis mali* (Schulzer & Saccardo) Roberge (Ko and. Sun, 2003), or *Trichothecium roseum* (Persoon) Gray (Hamid *et al*., 2014).

Some researchers have proposed biological approaches that means the usage of natural compounds (Doolotkeldieva and Bobusheva, 2017), so new disease control methods will be based on cultural practices and biotechnology (Odeh, 2006). To develop the most effective prophylactic or therapeutic methods it is necessary to know precisely the pathogens present on the fruit (Hinze and Kunz, 2010), this being the desideratum of this work.

Biological control products are much more specific, and this requires accurate knowledge about pathogens (Köhl *et al*., 2015). This study aimed to identify the spectrum of pathogens encountered on apple fruit during storage, so that can be a starting point in selecting and testing biological control products against those pathogens.

Materials and Methods

Biological material

In order to identify the pathogens, present on the stored fruits, apple material belonging to the five cultivars ('Granny Smith', 'Topaz', 'Imperial Gala', 'Jonagold' and 'Golden Reinders') was harvested. The fruits were subject to the three treatment variants tested: copper hydroxide and sulfur (T1), copper sulfate and sulfur (T2), methyl thiophanate (T3) and the control without treatment (M). After harvesting, the fruits were stored in the warehouse with natural storage conditions.

To achieve the proposed objectives, the fruits have been harvested from a super intensive high-density orchard (5000 trees / hectare). The experiment was conducted over three years.

Copper and sulfur based products have been chosen due to their affordability in organic farming and their massive use in traditional farming. Methyl thiophanate has been chosen because of its recognized efficacy and its use in both intensive and extensive farming systems. The use of chemical treatments has attempted to diversify the possible pathogens that can be identified on apple fruit.

The 12 treatments were performed with the same product each time. In the three experimental years no fertilizers were applied to prevent the fruit breakdown during storage.

Research method

The following steps have been taken to achieve the proposed objective:

- Determination of the rotting levels;
- Macroscopic identification of pathogens;
- Identification of pathogens after the fructifications formed in the wet chamber;
- Identification of pathogens after colonies formed on different culture media (Potato-dextrose-agar or Malt-agar).

Determination of rotting levels related to stored apples depending on the treatment applied to the vegetation, was done in all the studied varieties by 10 observations, each performed every 15 days, on a sample of 60 apples (3×20 apple). Identification of the pathogens on the stored fruits was made by observations throughout the storage period (September - February).

During the three experimental years, the harvested fruit was stored, while recording the frequency and severity of the rot attack and calculating the degree of attack or rotting. Frequency (F%) is the relative value of the number of attacked fruits relative to the total number of fruits analyzed. Severity (intensity) (I%) is the percentage of attack of the fruit. The attack degree (AD%) is calculated based on the frequency and severity of the attack.

$$AD\% = \frac{F \times I}{100}$$

The macroscopic identification of the pathogens was based on the fruit-based symptomatology. During the process if macro-symptoms showed fructifications (mycelium, spores), a sample was taken and examined under the microscope.

In addition to the obvious symptoms of pathogens such as *Fusarium*, *Penicillium*, *Botrytis*, *Fusicladium*, *Alternaria* etc. some fruits exhibited rotting symptoms without fructifications. In this situation, the fruits were sliced for identifying the symptoms of *Phomopsis mali* (Schulzer & Saccardo) Roberge, or *Trichothecium roseum* (Persoon). Samples were taken for microscopic analysis and, for better confirmation the samples were placed on a wet chamber (plastic sealed containers, moisture being provided by wetted paper with distilled water). It should be noted that for all the pathogens mentioned in this work only the ananorph forms were identified.

If an exact identification could not be made after the fungal formation even after they were kept in wet chamber, they were poured on malt-agar and potato-dextrose-agar sterile medium on Petri dishes and incubated for 7 days at 25 °C. Malt-Agar (MA) medium (content: malt extract - 30 g, mycological peptone - 5 g, Agar - 15g); Potato-Dextrose-Agar medium (PDA) (content: clean and wash potatoes - 200 g, dextrose (glucose) - 20 g/l, agar - 12 g/l). Streptomycin 0.05 g/l was added to both mediums to inhibit bacterial growth. Identification of the pathogens was done using the binocular magnifier and the microscope.

Statistical procedures

Data were analyzed using STATISTICA 10 program. Significant differences were confirmed by analysis of variance (comparing their significant levels at $p < 0.05$) and Duncan test, due to different treatments or varieties and their interaction.

Results

On the fruits from the trees which were treated with copper hydroxide and sulfur, ten pathogens were isolated (Table 1).

Venturia inaequalis (Cooke) Winter (anamorph *Fusicladium dendriticum* (Wallroth) Fuckel) was isolated on all five varieties, but for 'Granny Smith' the pathogen

rate was 28.3%, unlike other varieties where the pathogen frequency did not exceed 25% (Table 1).

Glomerella cingulata (Stoneman) Spaulding & von Schrenk (anamorph *Gloeosporium fructigenum* (Berkeley)) was isolated on four of the five cultivars (Table 1).

A dangerous pathogen responsible for producing mycotoxins, *Penicillium* spp. was isolated on the fruits of the 'Topaz', 'Imperial Gala' and 'Jonagold'. On 'Imperial Gala' the frequency of this pathogen reached 43.3% (Table 1).

On the 'Topaz' fruits, *Alternaria* spp. was isolated, with a frequency of up to 25%, and *Botryosphaeria obtusa* (Schweinitz) Shoemaker (anamorph *Sphaeropsis malorum* Peck) (Table 1) was isolated on the 'Jonagold' and 'Golden Reinders' varieties.

In the case of copper sulfate and sulfur treatment, 12 pathogens were isolated on the fruit of the tested varieties (Table 2). *Venturia inaequalis* (Cooke) Winter was isolated again, on the fruit of all studied varieties. *Glomerella cingulata* (Stoneman) Spaulding & von Schrenk was isolated on four of the five studied varieties, with the lowest frequency on the 'Granny Smith' variety (Table 2).

Another pathogen, present both during the growing season and on the stored fruit was *Monilinia fructigena* (Aderhold & Ruhland) Honey (anamorph *Monilia fructigena* (Persoon) Persoon). It was isolated not only on the fruits of 'Granny Smith' and 'Topaz', where it had the lowest frequency, but also on the 'Jonagold' variety. This pathogen was not identified in the variant where the trees were treated with copper hydroxide.

Neonectria ditissima (Tulasne & C. Tulasne) Samuels & Rossman (anamorph *Cylindrocarpon mali* (Allescher) Wollenweber) was isolated on the fruits of 'Granny Smith', 'Imperial Gala' and 'Jonagold' varieties and showed the same frequency (Table 2).

Diaporthe eres Nitschke was isolated, up to a frequency of 25%, on 'Granny Smith' and 'Golden Reinders'. A frequency up to 25% of *Fusarium spp.* was reported on 'Granny Smith', 'Topaz' and 'Imperial Gala'. Also, the pathogen *Botryotinia fuckeliana* (de Bary) Whetzel (anamorph *Botrytis cinerea* Persoon), was isolated from the fruits of the 'Granny Smith', 'Topaz' and 'Jonagold' varieties (Table 2).

Penicillium spp. was isolated on the fruits of all studied cultivars, with the highest frequency for the 'Jonagold' variety. On this variety there have also been isolated *Trichothecium roseum* (Persoon) Gray and *Phytophthora*

Table 1. Pathogens isolated from the stored apples corresponding to the treatment schedule copper hydroxide and sulfur

The anamorph state	The teleomorph state	Frequency/Variety				
		Granny Smith	Topaz	Imperial Gala	Jonagold	Golden Reinders
Fusicladium dendriticum	*Venturia inaequalis*	+++	++	++	++	++
Gloeosporium fructigenum	*Glomerella cingulata*	++	+		++	++
Sphaeropsis malorum	*Botryosphaeria obtusa*				+	++
Gloeosporium spp.	*Neofabraea spp.*	++			+	+
Cytospora spp	*Leucostoma spp.*					+
Phomopsis mali	*Diaporthe eres*	+				
Fusarium spp.	*Gibberella spp*		++	++		++
Botrytis cinerea	*Botryotinia fuckeliana*				++	
Penicillium spp.			++	+++	++	
Alternaria spp.			++			

Legend: + (F%=1-5%), + + (F%=5-25%), + + + (F%=25-50)

Table 2. Pathogens isolated from the stored apples corresponding to the treatment schedule copper sulfate and sulfur

The anamorph state	The teleomorph state	Frequency/Variety				
		Granny Smith	Topaz	Imperial Gala	Jonagold	Golden Reinders
Fusicladium dendriticum	*Venturia inaequalis*	+++	++	+++	++	++
Gloeosporium fructigenum	*Glomerella cingulata*	+	++		++	++
Monilia fructigena	*Monilinia fructigena*	++	+		++	
Sphaeropsis malorum	*Botryosphaeria obtusa*				++	
Cylindrocarpon mali	*Neonectria ditissima*	++		++	++	
Gloeosporium spp.	*Neofabraea* spp.	+				++
Phomopsis mali	*Diaporthe eres*	++				++
Fusarium spp.	*Gibberella* spp	++	++	++		
Botrytis cinerea	*Botryotinia fuckeliana*	+	++		+	
Penicillium spp.		++	++	+++	++	++
Trichothecium roseum					+	
	Phytophthora cactorum				+	

Legend: + (F%=1-5%), + + (F%=5-25%), + + + (F%=25-50%)

cactorum (Lebert & Cohn) J. Schröter with a frequency up to 5% (Table 2).

Both the number and frequency of isolated pathogens was lower, in variants where trees were treated during the growth stage with methyl thiophanate. On 'Granny Smith' variety, there were isolated *Venturia inaequalis* (Cooke) Winter having up to 50% frequency (Table 3).

In the 'Jonagold' variety, the frequency of all pathogens reported did not exceed 5% (Table 3).

On the fruit that came from the variant where treatments were not applied during the vegetation period, the frequency and number of reported pathogens were much higher (Table 4).

Venturia inaequalis (Cooke) Winter, *Glomerella cingulata* (Stoneman) Spaulding & von Schrenk, *Penicillium* spp. and *Monilinia fructigena* (Aderhold & Ruhland) Honey were isolated on the fruits of all studied varieties, with the highest frequency in the 'Granny Smith' variety (Table 4). *Neonectria ditissima* (Tulasne & C. Tulasne) Samuels & Rossman was isolated only on the fruits of the 'Jonagold' variety.

Neofabraea spp. (anamorph *Gloeosporium* spp.) was reported on four of the varieties studied with the highest frequency on 'Granny Smith' and 'Imperial Gala' (Table 4).

Alternaria spp. and *Aspergillus niger* van Tieghem were isolated on the fruits of the 'Topaz', 'Imperial Gala' and 'Jonagold' varieties with an attack frequency of up to 5% and *Phytophthora cactorum* (Lebert & Cohn) J.Schröter was isolated on the fruits of the 'Jonagold' and 'Golden Reinders' varieties (Table 4).

Fusarium spp. was isolated on the fruits of 'Topaz', 'Imperial Gala' and 'Golden Reinders'. *Penicillium* spp. was isolated on all varieties studied, with the highest frequency in the 'Imperial Gala' variety, more than 50% (Table 4).

Of the pathogens identified on total samples (Fig. 1), five had a frequency smaller than 5% (green bars). Most had

Table 3. Pathogens isolated from the stored apples corresponding to the treatment with methyl thiophanate

The anamorph state	The teleomorph state	Frequency/Variety				
		Granny Smith	Topaz	Imperial Gala	Jonagold	Golden Reinders
Fusicladium dendriticum	*Venturia inaequalis*	+++		++	+	++
Gloeosporium fructigenum	*Glomerella cingulata*		+	++		++
Monilia fructigena	*Monilinia fructigena*				+	
Sphaeropsis malorum	*Botryosphaeria obtusa*				+	
Cylindrocarpon mali	*Neonectria ditissima*			+	+	
Gloeosporium spp.	*Neofabraea spp.*				+	
Phomopsis mali	*Diaporthe eres*	+				
Fusarium spp.	*Gibberella spp*		++	++	+	
Penicillium spp.			++	+++	+	
Alternaria alternata						++

Legend: + (F%=1-5%), + + (F%=5-25%), + + + (F%=25-50%)

Table 4. Pathogens isolated from the stored apples corresponding to the untreated variant

The anamorph state	The teleomorph state	Frequency/Variety				
		Granny Smith	Topaz	Imperial Gala	Jonagold	Golden Reinders
Fusicladium dendriticum	*Venturia inaequalis*	++++	++	+++	++	+++
Gloeosporium fructigenum	*Glomerella cingulata*	+++	++	+	++	++
Monilia fructigena	*Monilinia fructigena*	++	+	+	++	++
Sphaeropsis malorum	*Botryosphaeria obtusa*				++	++
Cylindrocarpon mali	*Neonectria ditissima*				++	
Gloeosporium spp.	*Neofabraea spp.*	++	+	++		+
Phomopsis mali	*Diaporthe eres*		+	+		+
Rhizopus stolonifer				+		+
Fusarium spp.	*Gibberella spp*		++	+++		++
Botrytis cinerea	*Botryotinia fuckeliana*			+	++	
Penicillium spp.		++	++	++++	++	++
Alternaria spp.			+	+	+	
Aspergillus niger			+	+	+	
	Phytophthora cactorum				+	+

Legend: + (F%=1-5%), + + (F%=5-25%), + + + (F%=25-50%), ++++ (F%=50-100%),

a frequency of between 5 and 25%. Among the pathogens with a high frequency of over 25% (yellow bars, Fig.1) there were *Glomerella cingulate, Fusarium* spp. and *Penicillium* spp. The pathogen with the highest frequency of attack is *Venturia inaequalis*, with an attack frequency of over 50%.

Levels of attack are very different from one variety to another (Fig. 2). Thus, it was noticed that the lowest level of rotting degree was recorded in the 'Topaz' variety. The average attack rate recorded for this variety was 3.71%. The most susceptible variety in the storage period proved to be the 'Imperial Gala'. In this variety, the minimum level of attack was over 25%. Between this variety and the other four, the differences are significant. Significant differences in rotting rates also occur between the 'Golden Reinders' variety and the 'Topaz' and 'Jonagold' varieties. There are significant differences in degree of rotting between the treated and the untreated fruits (Fig. 3). There were no significant differences between the three treatment variants. The average attack rate ranged between 5 and 10% in the treated variants and exceeded 25% in the untreated control.

Three of the five tested cultivars recorded attack values below the average of the experience ('Topaz', 'Jonagold' and 'Granny Smith') (Fig. 4). The sensitivity of the 'Imperial Gala' variety is also confirmed by the analysis of this graph, where only untreated variants of the 'Jonagold' and 'Granny Smith' varieties are found to approach the minimum levels of attack of this variety. For 'Topaz', the maximum attack rates do not exceed the average of the whole experience.

Treatments applied during vegetation have substantially reduced the degree of attack on apple fruit. This is evidenced in the 'Jonagold', 'Granny Smith' and especially the 'Golden Reinders' cultivars. Probably due to the 'Topaz' resistance to attack, any differences between treated and untreated control variants are not spectacular (Fig. 4).

The 'Golden Reinders' cultivar best responded to applied chemical treatments. We recommend treatment during the growing season if the fruit of this cultivar is intended for storage. The 'Imperial Gala' variety is not recommended for long-term storage, losses during storage period ranging from 25 to 75% (Fig. 4).

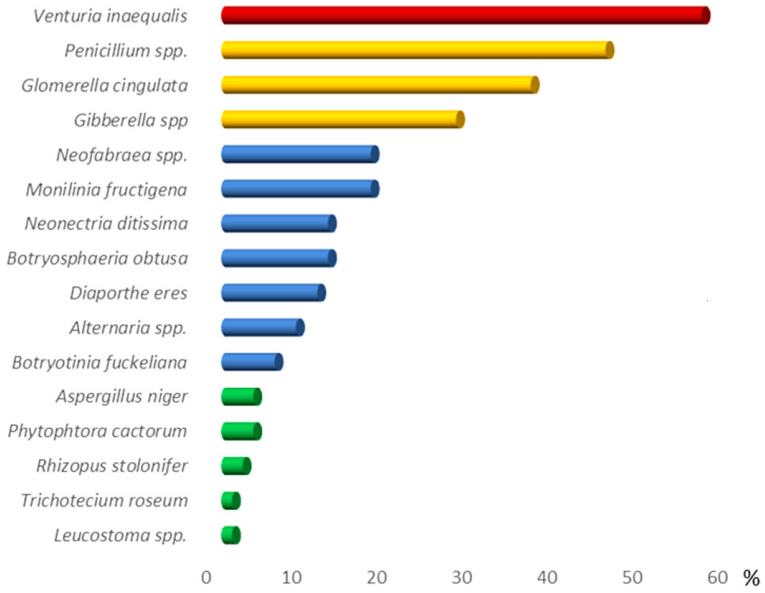

Fig. 1. The frequency of pathogens identified per total of samples analyzed

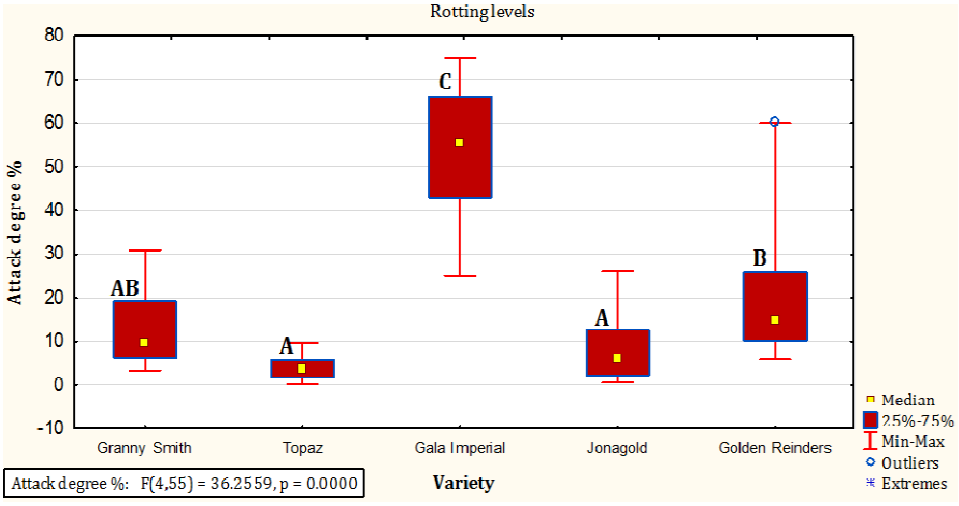

Fig. 2. Rotting attack degree of apple fruits during storage

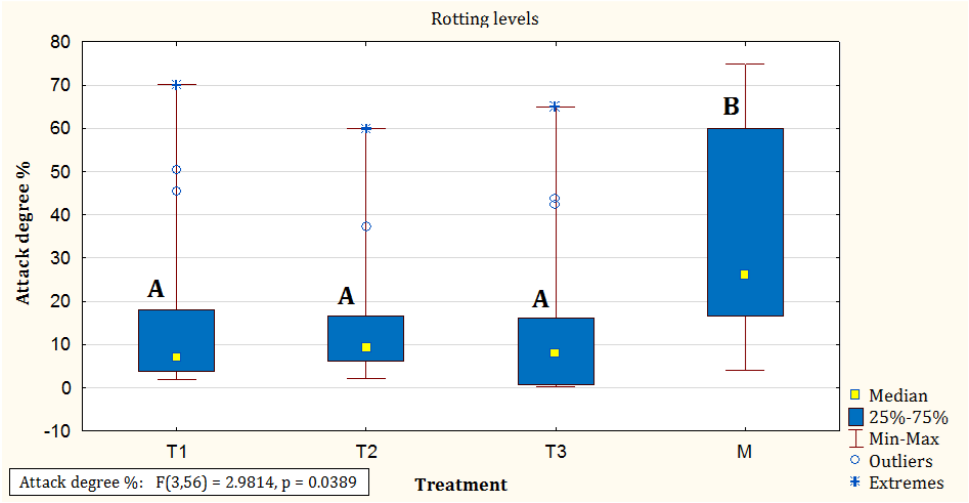

Fig. 3. Rotting attack degree of apple fruits, under different treatments applied during the growing season (T1- copper hydroxide and sulfur, T2- copper sulfate and sulfur, T3- methyl thiophanate, M-Control untreated)

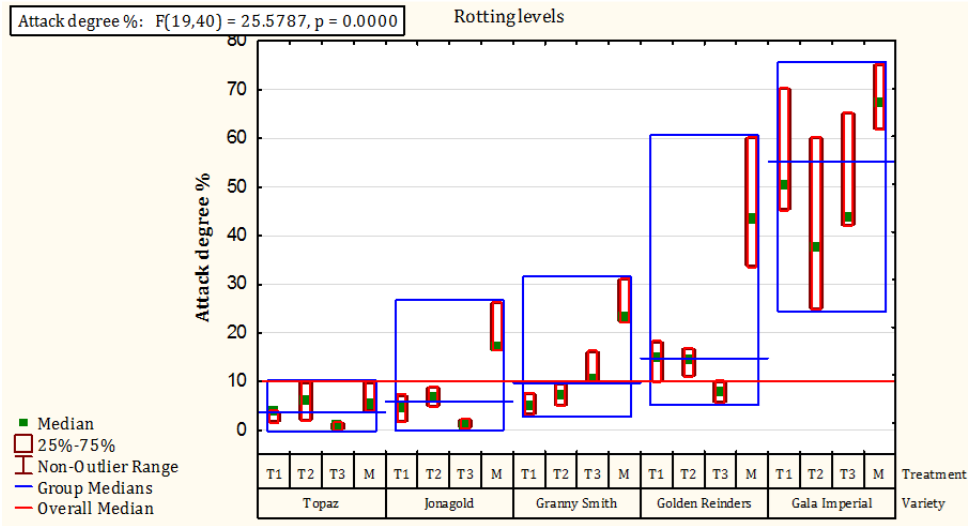

Fig. 4. The behavior of different apple varieties under different treatment conditions applied during the growing season (T1- copper hydroxide and sulfur, T2- copper sulfate and sulfur, T3- methyl thiophanate, M-Control untreated)

Discussion

Of the pathogens that infect and occur in vegetation and deposits *Venturia inaequalis* (Cooke) Winter has been identified in all varieties studied in most test variants. Previous studies confirmed as the dangerous pathogen of apples (González-Domínguez *et al*., 2017; Passey *et al*., 2017). The highest frequency is recorded in the variant where during the vegetation period no treatments were applied. Another pathogen in this category was *Glomerella cingulata* (Stoneman) Spaulding & von Schrenk, which manifested itself at quite high frequency. Wang *et al*. (2012) reported also a great attack of this pathogen. The lowest frequency of the attack was recorded in apple trees treated with methyl thiophanate during vegetation, data confirmed by Wang *et al*. (2017). In the case of the 'Jonagold' variety, in the treated variants, the rate of attack of this pathogen was higher than in the untreated control variant. In the case of pathogens infecting in vegetation that occurs during the storage, with the highest frequency was *Gloeosporium spp*. It was isolated from all the apples of the control and from four of the five cultivars under study of the treated variants. High susceptibility to this pathogen is the 'Jonagold' cultivar, the variety in which this pathogen is present in all the test variants.

Pathogens that infect and appear during storage, isolated on the fruits of the varieties studied, were *Fusarium* spp., *Penicillium* spp., *Alternaria* spp. and *Trichothecium roseum* (Persoon) Gray. Of the four pathogens, only *Fusarium* spp. and *Penicillium* spp. had a higher frequency. *Penicillium* spp. has manifested in all varieties in most variants studied. Sever *et al*. (2012), in a complex study succeeded to identify 32 species of *Fusarium* in four varieties studied. The 'Imperial Gala' variety, was the one where the frequency of *Penicillium* spp. attack was the highest. Louw and Korsten (2014) report high levels of attack *Penicillium expansum* Link and *Penicillium crustosum* Thom species, on the same variety. This pathogen was isolated on the 'Imperial Gala' fruit in all the experimental variants.

If we discuss the contribution of each pathogen to the decline of apple fruit, we can say that *Venturia inequalis* is the pathogen with the highest share of 17.7% to 25%. *Penicillium* spp. is one of the storage pathogens with the highest participation rate of 15.9 to 18.8%.

Phytosanitary treatments during vegetation influence the spectrum of pathogens responsible for the degradation of apple fruit. It was noted that in the no-treat variant 14 pathogens were identified while in variants treated with copper hydroxide or methyl thiophanate it did not exceed 10. It seems that copper sulfate fails to protect the fruit, the pathogens identified on the samples treated with it are virtually the same as those found on untreated apples. Knowing the spectrum of pathogens and the effect of fungicides is very important in choosing the right plant protection products and the number of treatments. Current trends in disease management aim to avoid this high number of treatments, which involve risks to human health and the environment (Rossi *et al*., 2012).

The degree of rot attack for the five monitored varieties recorded very different values. The rotting level of apple fruit may have been influenced by the storage conditions, but especially by the sensitivity of the cultivar.

Although nine different pathogens, on the 'Topaz' variety, were identified during the experiment, the intensity of their attack was very low, which also determined the low values of the degree of attack.

The results show that the highest values of the attack are recorded in the 'Imperial Gala' variety. This is certainly due to the large number of pathogens (11) identified on the fruits of this variety. Also, the high frequency of the attack, especially for *Penicillium* spp., *Fusarium* spp. and *Venturia inaequalis*, has led to a significant increase in the degree of decay of fruit of this variety.

Another cultivar with many identified pathogens (ten) was 'Golden Reinders', with average rates of attack frequency in three of the treatment variants. However, the average attack rate had lower values than the 'Imperial Gala' variety, due to the fact that only three pathogens were identified in the variant treated with methyl thiophanate. Good results in the control of apple pathogens using methyl thiophanate also obtained Wang *et al*. (2017).

Conclusions

This study succeeds in making an inventory of the pathogens that lead to the degradation of apple fruit during storage, as well as their involvement in this degradation process. Based on data on the frequency of pathogens, we can conclude that among the pathogens that produce infections during storage, *Penicillium* spp. has the largest share in the decline of apples. Of the pathogens that produce infections in the growing season and occur in the deposit, we notice *Venturia inequalis* with a high level of attack. This study is noted for the considerable number of pathogens found on apples and can be a starting point for future research, especially in the biological control of pathogens that lead to fruit decay.

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