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Physiological disorders affect apple susceptibility to *Penicillium expansum* infection and increase probability for mycotoxin patulin occurrence in apple juice

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Abstract. *Penicillium expansum* infection of apples and mycotoxin patulin (PAT) production has previously been associated with many pre- and postharvest factors other than physiological disorders. In the current study, 'Antei' and 'Krameri tuviõun' apples with and without bitter pit (BP) symptoms and 'Talvenauding' apples with and without superficial scald (SS) symptoms were used in order to determine if the named physiological disorders may influence susceptibility to *P. expansum* infection and PAT production. Apples were inoculated with 10 µL *P. expansum* spore suspension with the concentration of 1×105 conidia mL^{-1} and stored at 24 °C with relative humidity (RH) 80%. After 7 and 11 days, lesion diameters were measured, and apples were pressed into juice. PAT content was determined in pasteurized juice. Two cultivars out of three showed that in fruit with physiological disorders, *Penicilllium* infection and PAT production proceeded significantly faster compared to apples, which did not have physiological disorders. SS increased the risk for PAT occurrence in juice more than BP: while the juice pressed from BP–affected apples with no visual signs of fungal diseases did not contain PAT, juice pressed from apples with SS contained PAT three times above legislative limits defined by the World Health Organization (50 µg L^{-1}).

Key words: bitter pit, blue mould, lesion diameter, superficial scald.

INTRODUCTION

Physiological disorders like bitter pit (BP) and superficial scald (SS) have commonly been described affecting visual quality of apple fruit and therefore decreasing their market value. Less attention has been paid to study whether physiological disorders can be possible infection points for mycotoxin-producing fungi, especially *Penicillium expansum*, the major producer of patulin (PAT) (Morales et al., 2007). SS is caused by the accumulation of oxidative breakdown products of α-farnesene, which results in the death of the hypodermal and epidermal cells (McGlasson, 1996). The disorder is characterized by an uneven browning or bronzing of the skin along with the development of skin wrinkling and pitting with increasing severity (Rupasinghe et al., 2000; Savran & Koyuncu, 2016). The cause of BP has been associated with calcium (Ca) deficiency:

Ca sprays and Ca applications to soil during growing season or Ca solution dips at postharvest are widespread practices to supply Ca and decrease BP in apples (Ferguson & Watkins, 1989; Fallahi et al., 1997; Blanco et al., 2010; Torres et al., 2017). BP is a physiological disorder that is defined as brown, corky and roundish lesions, which can develop in apples before and after harvest (Jarolmasjed et al., 2016). In Estonia, apples 'Krameri tuviõun' and 'Antei' are susceptible to BP among commercially grown cultivars. In other countries, several apple cultivars are susceptible to BP like 'Sinap Orlovskij', 'Ligol', 'Free Redstar', 'Rajka', 'Topaz', 'Sampion', 'Honeycrisp', 'Golden Delicious' and 'Granny Smith' (Lanauskas & Kviklienė, 2006; Bryk & Broniarek-Niemiec, 2008; Valiuškaitė et al., 2009; Lanauskas et al., 2012; Zúñiga et al., 2017).

Earlier experiments in Estonia have shown that BP in 'Krameri Tuviõun' was not reduced by Ca treatment; instead it correlated negatively with Mg and P content and Mg:Ca ratio in apples (Moor et al., 2006). It appears that BP is a complex disorder, which is not easy to eliminate. Saure (2002) stated that BP is essentially the result of a gibberellin-induced increased susceptibility of the cell membranes to stress, and Ca only reduces the effect of gibberellins.

Besides reducing the market value of apples, BP can also be responsible for apple susceptibility to different storage diseases. For instance, an earlier study by Holb et al. (2012) showed that pre-harvest Ca sprays resulted in significant brown rot reduction on apple fruit over a 6-month storage period. So far the named disorders have not been associated with susceptibility to patulin-producing fungi and therefore fruits with BP or SS are considered to be safe for juice processing. However, in our previous research (Heinmaa et al., 2019) we found PAT in juice pressed from apples affected with BP, but not showing any visual signs of fungal infection.

The aforementioned finding led us to the hypotheses of the current study: 1) apples with physiological disorders are more susceptible to *P. expansum* infection; 2) apple juice pressed from fruits with physiological disorders has higher PAT content.

MATERIALS AND METHODS

Apple cultivars and inoculation experiments

At first, the pilot experiment was carried out in order to determine the most virulent *Penicillium* isolate out of three and to find out if there is a tendency that apples with BP symptoms are more susceptible to *Penicillium* than healthy apples. For the pilot experiment, *Penicillium spp* spores were collected from three different apple cultivars that had visible blue mould rot symptoms. Isolates were separately cultured in PDA plate for 2 weeks at 25 °C in the dark. Further on, the procedure described by Chen et al. (2017) was followed for inoculum preparation. Conidia were harvested with 0.05% Tween and counted with a hemocytometer using an optical microscope and diluted to a concentration of 1×105 conidia mL^{-1}. In the pilot experiment, 36 'Antei' apples were used: six apples with and six without BP symptoms were inoculated with three different *P. expansum* isolates. Apples were surface disinfected for 2 min with 1% sodium hypochlorite solution, rinsed three times with deionized water, and air dried on a clean bench. Each apple was inoculated in two places with 10 µL of the spore suspension (the suspension was mixed using vortexer before inoculation) using a pipette. Sterile distilled water with 0.05% Tween 20 was used as the control. Apples were stored in plastic baskets at 24 °C with RH 80%.

Lesion diameters were measured 7 and 11 days after inoculation (DAI). Two diameter values of each lesion in two mutually perpendicular directions were recorded. The average of the two values was defined as the diameter of the lesion. No apple juice was pressed in the pilot experiment. The most virulent *Penicillium* isolate out of three was used in further inoculation experiments following the same inoculation and lesion diameter measuring procedure as described above. The isolates identity as *P. expansum* was confirmed through molecular analysis as described by Adamson et al. (2015).

For further experiments, conventional 'Antei' and 'Krameri tuviõun' apples and organic 'Talvenauding' apples were harvested from South Estonian apple orchards in autumn 2017. 'Talvenauding' apples had no signs of SS and 'Antei' and 'Krameri tuviõun' had slight BP symptoms when harvested. BP symptoms develop during first two months of storage. SS symptoms usually develop during shelf life, when apples are removed from cool storage and transferred to room temperature. 100 kg of apples were harvested per cultivar and stored at 3 ± 2 °C and relative humidity 95%. The storage duration for 'Krameri tuviõun' was 12 weeks, for 'Antei' 11 weeks and for 'Talvenauding'14 weeks. Since development of BP continued during storage, 'Antei' and 'Krameri tuviõun' fruits were inspected after two months and divided into two categories: sound apples and apples with BP. Since SS develops slowly at low temperatures and rapidly at room temperature, half of the 'Talvenauding' apples were placed at 20 ± 2 °C for one week before the inoculation experiment. Flesh firmness was measured from 10 apples per category by using TMS-Pro Texture Analyser (Food Technology Corporation, USA) before inoculation. Since 'Krameri tuviõun' has conic shape and Talvenauding' is ribbed, fruit were cut in half for achieving steadiness during measurement. A small skin area was removed from two opposite sides of each fruit around the equator corresponding to the blushed and shaded sides as previously described by Saei et al. (2011). The penetration force was measured by pressing a 10 mm diameter plunger 5 mm deep into peeled fruit at a speed of 1.7 mm s^{-1}. The two readings taken on opposing sides of each fruit were averaged to obtain a mean flesh firmness value for each fruit. The readings were given in Newton (N).

In the second experiment 30 'Antei' and 30 'Krameri tuviõun' apples with and without visible BP symptoms (altogether 120 apples) were inoculated with the most virulent *P. expansum* isolate at the beginning of December 2017. Apples were stored and lesion diameters were measured as described in the pilot experiment. Half of the inoculated apples from each treatment were pressed into juice 7 DAI and the other half after 11 DAI in order to determine if and how much PAT had been produced at each time point. By 11 DAI 'Krameri tuviõun' apples were too rotten to press juice and PAT was not determined.

The third inoculation experiment was carried out at the end of January 2018 when 30 'Talvenauding' apples with and without SS symptoms were inoculated. The lesion diameters were measured 7 DAI and apples were pressed into juice on the same day. Since the lesions were already large 7 DAI, it was decided that 11 DAI the deterioration of apples was too large to press juice.

Juice pressing and patulin analyses

Non-inoculated apples with and without physiological disorders were separately pressed into juice 10 weeks after harvest. All apples with visual symptoms of fungal infection were rejected before juice pressing. 20 kg of apples in each treatment were washed and disintegrated with Voran centrifugal mill RM2.2 (Voran Maschinen GmbH, Pichl bei Wels, Austria) and pressed by water-press (WP) Lancman VSPX 120 (Gomark d.o.o., Vransko, Slovenia). Juices were pasteurized at 85 °C for 1 min by a tubular system and packed immediately into airtight 1.4-litre aluminium foil bags with Bag-in-box filler BBF6 (Gebhardt Anlagentechnik GmbH & Co. KG, Germany). Approximately 10 kg of apples for each cultivar were cut into halves in order to inspect apple cores for possible visual symptoms of fungal infection. Inoculated apples (approximately 1.5–2 kg in each category) were pressed into juice with a laboratory press Sencor Juice Extractor SJE 1005. Juices were pasteurized at 85 °C for 1 min and packed immediately into airtight 1.4-litre aluminium foil bags. PAT analyses were carried out in the Estonian Health Board laboratory, which is accredited by the Estonian Accreditation Centre. Juices were pre-treated with pectinase enzyme. The sample preparation was based on Solid Phase Extraction. PAT was eluated from the cartridge with ethyl acetate. After evaporation of the solvent, the residue was dissolved in mobile phase, and PAT was quantitatively determined by HPLC with UV detection. The level of quantification was 4 μg L^{-1}.

Statistical analysis

Data obtained in this study were subjected to statistical analysis using Dell Statistica version 13 (Dell Inc., USA) and were expressed as the means ± standard errors. The effect of *P. expansum* isolates and BP on lesion diameter was compared by analysis of variance (two-way ANOVA) (Fig. 1). Apple flesh firmness and juice PAT content were compared by one-way ANOVA (Tables 1 and 2) and significant differences among the variables for 7 and 11 DAI separately were determined according to Duncan's multiple range test ($p \leq 0.05$). Mann-Whitney U tests were performed to compare lesion diameters between categorical variables (Tables 1 and 2).

RESULTS AND DISCUSSION

In the pilot experiment, isolate 3 was the most virulent one as the average of (Fig. 1). The average effect of BP was also significant: the lesion diameters of apples with BP symptoms were significantly larger compared to apples with no disorders, which indicates that *Penicillium* develops more rapidly in apple tissues with BP symptoms.

Apple flesh firmness was not influenced by the presence of BP nor SS symptoms in any of the three cultivars studied (Tables 1 and 2). Juices pressed from non-inoculated 'Antei' apples did not contain PAT, irrespective of whether they had BP or not (Table 1). Seven DAI lesion diameters of 'Antei' apples with BP symptoms were significantly larger than the lesion diameters of sound

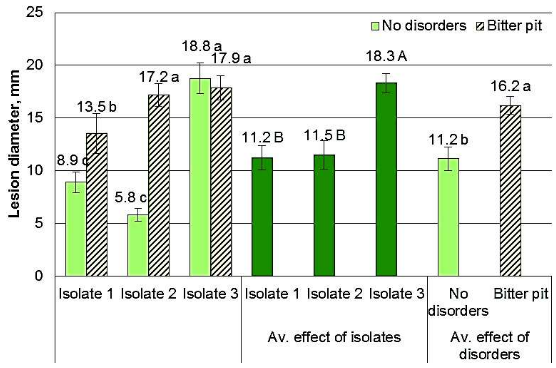

Figure 1. Mean lesion diameters of 'Antei' apples with BP symptoms and no physiological disorders inoculated with three different *Penicillium* isolates. Values with different letters for the same parameter are significantly different (two-way ANOVA).

apples. Guerrero-Prieto et al. (2017) reported that the greater the fruit Ca content, the lower the severity of *P. expansum* infection. At 11 DAI the lesion sizes were not significantly different. Among the inoculated apples, juice pressed from fruits with BP symptoms had significantly higher PAT content than juice pressed from sound apples at both 7 and 11 DAI. Also, the PAT content in the juice pressed from inoculated 'Antei' apples, which had no physiological disorders, contained PAT below the legal limit of 50 μg L^{-1}. PAT content in juice pressed from apples with BP symptoms was more than three times above the legal limit. Although no significant differences in flesh firmness were detected, the results indicated that *P. expansum* develops more rapidly and starts to produce PAT significantly sooner in 'Antei' apples with BP symptoms compared to sound apples. Since it is widely known that fungal conidia of *P. expansum* invade through wounds and bruises of the fruit (Spotts et al., 1998) it can be assumed that even though BP affected areas are below the skin, somehow the skin above disordered tissues is also weaker and makes it possible for *P. expansum* to invade the fruit.

Table 1. 'Antei' and 'Krameri tuviõun' apples' flesh firmness (N) before inoculation, mean lesion diameters of inoculated apples (mm) and juice PAT content (μg L^{-1}) ± SE; DAI = days after inoculation; BP = Bitter pit; NI = not inoculated

		'Antei'			'Krameri tuviõun'		
Dis-order	DAI	Flesh firmness, N	Lesion diameter, mm	PAT, μg L^{-1}	Flesh firmness, N	Lesion diameter, mm	PAT, μg L^{-1}
none	7	43.8 ± 1.3a	8.8 ± 0.8b	22 ± 5b	31.7 ± 0.9a	13.3 ± 1.2a	609 ± 28a
BP	7	41.3 ± 1.1a	11.1 ± 1.1a	182 ± 16a	31.2 ± 1.3a	11.0 ± 0.9a	478 ± 34b
none	11	43.8 ± 1.3A	16.1 ± 2.9A	373 ± 19B	31.7 ± 0.9A	35.0 ± 4.6A	-
BP	11	41.3 ± 1.1A	17.3 ± 2.4A	769 ± 32A	31.2 ± 1.3A	24.6 ± 3.1A	-
none	NI	43.8 ± 1.3*a*	-	< 4	31.7 ± 0.9*a*	-	< 4
BP	NI	41.3 ± 1.1*a*	-	< 4	31.2 ± 1.3*a*	-	< 4

Values with different letters for the same DAI and parameter for each cultivar are significantly different. Apple flesh firmness and juice PAT content were compared by one-way ANOVA and lesion diameters by Mann-Whitney U tests.

Table 2. 'Talvenauding' apples' fruit flesh firmness (N) before inoculation, mean lesion diameters of inoculated apples (mm) and juice PAT content (μg L^{-1}) ± SE; DAI = days after inoculation; SS = Superficial skald; NI = not inoculated

Disorder	DAI	Flesh firmness, N	Lesion diameter, mm	PAT, μg L^{-1}
none	7	39.4 ± 1.0a	23.5 ± 0.3a	4,203 ± 103b
SS	7	39.8 ± 0.6a	20.4 ± 0.6b	4,922 ± 128a
none	NI	39.4 ± 1.0A	-	< 4
SS	NI	39.8 ± 0.6A	-	162.5 ± 33A

Values with different letters for the same parameter are significantly different. Apple flesh firmness and juice PAT content were compared by one-way ANOVA and lesion diameters by Mann-Whitney U tests.

Juice pressed from non-inoculated 'Krameri tuviõun' apples with or without BP symptoms did not contain PAT. The lesion diameters of 'Krameri tuviõun' apples with and without BP symptoms were not significantly different at 7 or 11 DAI (Table 1). At 7 DAI, the content of PAT was higher in the juice pressed from initially sound inoculated apples compared to juice pressed from inoculated apples with BP symptoms. The results from ‘Krameri tuviõun’ apples were not in accordance with the findings of the other two cultivars: the lesion diameters of the apples with no physiological disorders were significantly larger and juice PAT content was higher compared to the apples with BP symptoms. The reason for that might be that ‘Krameri tuviõun’ apples were much softer than the other two cultivars studied (Tables 1 and 2) and therefore more susceptible to different competing fungi like *Botrytis* and *Gloeosporium*, which were developing alongside with *P. expansum* and suppressing its development. Earlier studies by Morales et al. (2013) have proven that interactions between *Botrytis cinerea* and *P. expansum* in grape juice medium enhanced *B. cinerea* growth and prevented PAT accumulation, which indicates that *P. expansum* is a weak competitor compared other common postharvest pathogens in apples.

‘Talvenauding’ flesh firmness was not affected by SS (Table 2). Juice pressed from apples without SS did not contain PAT over detection limit (< 4 μg L^{-1}), whereas juice pressed from non-inoculated ’Talvenauding’ apples with SS symptoms had PAT contamination of 162.5 μg L^{-1}, which is above the legislative limit of 50 μg L^{-1} (World Health Organization, 1995). At 7 DAI, apples with SS had significantly smaller lesion diameters than initially sound inoculated apples. However, PAT content was higher in the juice pressed from apples with SS symptoms than sound apples (4,922 and 4,203 μg L^{-1}, respectively). This may indicate that the lesion diameter on the fruit surface does not definitively describe the development phase of *P. expansum* and production of mycotoxin PAT. The finding is also in accordance with the results of Drusch & Ragab (2003), who reported that no correlation between the size of the lesion and the PAT concentration was found. Although ‘Talvenauding’ apples had similar flesh firmness compared to ‘Antei’ apples, the lesion diameters were approximately twice as large compared to other two cultivars and PAT content was extremely high.

Harris (2007) reported that ’Jonagold’ and ’Red Delicious’ apple juice extracted from rotten tissue of fruits inoculated with *P. expansum* in the laboratory typically contained > 1,000 μg of PAT L^{-1} by 6 days after inoculation. In our study ’Antei’ and ’Krameri tuviõun’ apple juice pressed after 7 days of inoculation contained less than 1,000 μg L^{-1} of PAT, but ’Talvenauding’ apple juice contained more than 1,000 μg L^{-1} of PAT.

Based on current study it can be stated that the lesion diameters of ‘Talvenauding’ apples affected by SS were about twice as big and the patulin content in AJ was many times higher compared to AJ pressed from ‘Krameri tuviõun’ and ‘Antei’ apples affected by BP. This indicates, that *P. expansum* develops in the fruit and starts to produce PAT faster in apples with SS compared to apples with BP. The cause for this may be because BP formation starts internally in the flesh of apples and pit appears on the fruit surface with time (Jarolmasjed et al., 2016). SS formation starts on the apple surface due to the death of cells in the epidermal or hypodermal layers of the skin (Colgan et al., 1999), which at later stages can lead to the development of internal damage and pathological disorders (Paliyath et al., 1997). Since *P. expansum* is a wound pathogen and invades the fruit through the skin, it can be assumed that SS affected fruits are more susceptible to *P. expansum* development because the surface of the fruit is already damaged.

CONCLUSIONS

It can be concluded from the current study that among the physiological disorders, SS increases the risk for PAT occurrence in juice more than BP. While the juice pressed from BP-affected apples with no visual symptoms of fungal diseases did not contain PAT, juice pressed from apples with SS had PAT content three times above the legislative limit. We also conclude that apples with physiological disorders may be more susceptible to *P. expansum* infection, but cultivar differences also play a role. In the current study, two cultivars out of three showed that if *P. expansum* infection occurred in fruit with physiological disorders, PAT production occurred significantly sooner compared to initially sound apples even though lesion diameters were not always larger. Since apples with BP and SS are often used for juice pressing all over the world, further studies with apple cultivars susceptible to these disorders should be carried out in order to increase the knowledge about the risk factors causing PAT contamination in apple juice.

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