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New non-chemical postharvest technologies reducing berry contamination

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Abstract

One of the most important postharvest diseases of strawberry and raspberry is grey mould, caused by *Botrytis cinerea* Pers.: Fr. New non-chemical food safety technologies reducing berry rots and extending storage time were tested. Photosensitization is an innovative method for inhibition of pathogens in berry and is based on the interaction of light (λ = 400 nm, 20 mW cm^{-2}) and photoactive compound (chlorophyllin derivative). The aim of this study was to evaluate the effect of the chlorophyllin-based photosensitization on the reduction of grey mould (*B. cinerea*) in postharvest strawberry and raspberry fruits. The berries were soaked in a treatment solution for 1 hour and then exposed to prototype (light) for 30 minutes. The obtained data indicated that chlorophyllin derivative inhibited grey mould infection on fruits. The infection of *B. cinerea* in strawberries after a 6-day storage decreased by 8% and in raspberries after 1-day storage it decreased by 3%. The chlorophyllin had no impact on the visual quality and nutritional properties of strawberries and raspberries.

Key words: *Botrytis cinerea*, chlorophyllin derivative, photosensitization, raspberry, strawberry.

Introduction

Raspberries and strawberries, which are consumed fresh and processed, are the most popular soft fruits in the world and Lithuania (Uselis et al., 2008). Berries contain many vitamins, minerals, and a high content of phenolic compounds, other antioxidants and bioactive compounds (Viskelis et al., 2010; Bobinaitė et al., 2013). Phenolic compounds and anthocyanins are regarded as healthy, natural and functional components in human nutrition (Bobinaitė et al., 2012; Luksiene etal., 2013).

Fungal and bacterial infections are the main reason for 50% or greater yield losses of soft fruits during postharvest storage (*Botrytis*..., 2007). The initial strategy for reducing postharvest losses is appropriate agrotechnical handling and chemical pesticides (Droby, Lichter, 2007; Droby et al., 2009).

One of the most important postharvest diseases of soft fruits is grey mould caused by *Botrytis cinerea* Pers.: Fr., which results in a yield reduction ranging from 15% to 50% (Staats et al., 2005; Droby, Lichter, 2007). The *B. cinerea* infects more than 200 host plants worldwide (Staats et al., 2005). Symptoms of grey mould vary depending on the host, infection starts through flowers, various injuries, cracks and cuts. The pathogen has the capability to remain quiescent in unripe tissue and disease symptoms develop in ripe fruit. In berries, grey mould causes preharvest and postharvest fruit losses (Staats et al., 2005; *Botrytis*..., 2007; Droby et al., 2009).

Conventionally grey mould (*B. cinerea*) in strawberries is controlled by routine applications of fungicides once a week during flowering. Significant amounts of pesticides against diseases are used during the growing season of strawberries (Staats et al., 2005; *Botrytis*..., 2007). This type of application has become unacceptable because the inadequate usage of pesticides leads to the pathogen resistance (*Botrytis*..., 2007; Droby et al., 2009). Using forecasting models, applications of fungicides are made only when the model shows favourable conditions for *B. cinerea* development (Valiuškaitė et al., 2010; Rasiukevičiūtė et al., 2013). The cultivation of strawberries on plastic mulch had a positive effect on reducing fruit rot (Uselis et al., 2008). It is very expensive to use chemical fungicides and they have a negative impact on human health and environment (*Botrytis*..., 2007; Luksiene, Zukauskas, 2009).

Photosensitization is an innovative method for eliminating fruit pathogens based on simultaneous use of light and a photosensitizer. Chlorophyllin-photoactive compound is known as a photosensitizer, it is a water-soluble food additive (E140), and chlorophyllin derivative is safe as well (Luksiene, 2005; Luksiene, Zukauskas, 2009; Luksiene et al., 2010; Luksiene, Brovko, 2013; Luksiene, 2014). The data presented in Luksiene and Paskeviciute (2011) research clearly indicated that food pathogens and moulds in lab-scale experiments were effectively removed from the surface of strawberries. The aim of this study was to evaluate the effect of the chlorophyllin-based photosensitization on the reduction of grey mould (*B. cinerea*) in postharvest strawberry and raspberry fruits.

Materials and methods

Experiments. The experimental protocol for chlorophyllin-based photosensitization decontamination of berries was elaborated in Vilnius University, Institute of Applied Research. The effect of the photosensitization of chlorophyllin derivative (Chld) ("Alfa Aesar", Germany) on decontamination of berries was investigated at the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry in 2012 and 2013. In the experiments, we evaluated the effect of postharvest photosensitization on strawberry (*Fragaria* × *ananassa* Duch.) cv. 'Darselect' and raspberry (*Rubus idaeus* L.) cv. 'Polka'. The illumination experiments and assessment of diseased fruit were conducted in the Laboratory of Plant Protection. The quality tests were done in the Laboratory of Biochemistry and Technology. The raspberry (August 2012) and strawberry (June 2013) fruit samples were collected in the experimental field of Institute of Horticulture. The experimental design of treatments is provided in Table 1.

Table 1. The experimental design of postharvest treatment of strawberries and raspberries

No.	Treatment	Treatment abbreviation	Rate
1.	Untreated and not illuminated	control	–
2.	Treated with water solution of chlorophyllin derivative + illuminated by prototype	Chld	1.5×10^{-3} M
3.	Treated with sterile distilled water + illuminated by prototype	water	–

Illumination. The LED-based light source prototype for photosensitization was constructed in the Institute of Applied Research of Vilnius University (Fig. 1 B). The InGaN light emitting diode (LED) array LZ1-00UA00 (LED Engine Inc., USA) was used as a source of the light. The source of the light consisted of an illumination chamber and a supply unit (Luksiene, Zukauskas, 2009; Luksiene, 2014). The berries used in the experiments were of the same maturity, size and free of physical damage. Visually healthy fruits were soaked in chlorophyllin derivative (Chld) or water solution for 1 hour. Dehumidified fruits were placed on a sterile tray in a chamber of light source and were exposed for 30 minutes (15 min one side and 15 min – the other side). LED-based prototype emitted light in the λ = 400 nm with the intensity of 20 mW cm^{-2} on the surface of fruits. The InGaN light emitting diodes in the prototype ranged from the top of the illumination chamber. The control treatment was not soaked in Chld or water and not illuminated. After treatment, fruits were stored in a climate chamber KBF720 ("Binder", German) at a temperature of 5–7°C.

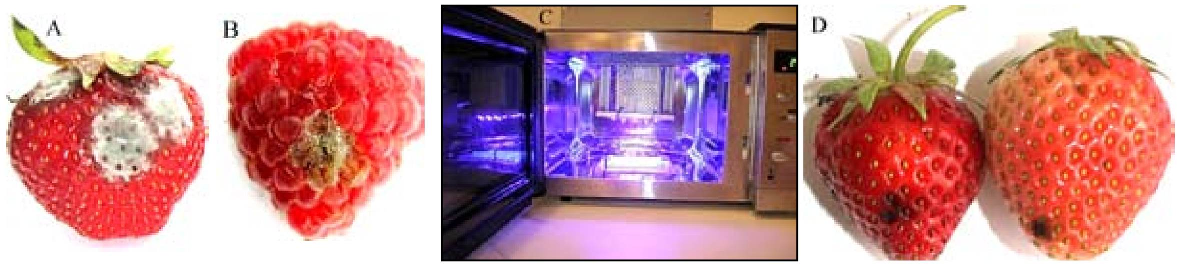

Figure 1. Strawberries and raspberries infected by *Botrytis cinerea* (control – untreated) (A, B), light source (illumination chamber) (C), strawberries after the application of chlorophyllin derivative and illumination (D)

The evaluation of B. cinerea decontamination efficiency. The effect of chlorophyllin derivative-based photosensitization on decontamination of *B. cinerea* was evaluated according to the EPPO standards PP 1/16(2) (EPPO, 1996). The *B. cinerea* frequency of occurrence (%) was estimated by the percent ratio of the number of samples where the genus was detected to the total number of tested fruits (Gonzalez et al., 1999). The incidence of *B. cinerea* was calculated based on berry surface browning and/or presence of mycelium. Visually detected *B. cinerea* was proved microscopically. Disease incidence (%) is the number of infected fruits per sample (Žemės ūkio augalų kenkėjai..., 2002). The assessments of strawberries were made until the 9th day of storage and of raspberries until the 4th day. One replication consisted of 50 fruits of strawberry and 90 fruits of raspberry with four replicates per treatment. For each replication, fruits were classified into healthy (visually without infection) and diseased (with disease symptoms).

Nutritional quality of strawberries. Berries were analysed immediately after treatment to find out if illumination and Chld had an influence on their appearance.

Firmness of strawberries was measured by a texture analyser TA.XTPlu ("Stable Micro Systems", England) and the data were processed by the program *Texture Exponent*. A flat head stainless cylindrical probe of 2 mm diameter was used for the penetration test. The speed of a probe was 1 mm s^{-1} the depth of the penetrated tissues was 10 mm.

The colour coordinates (L*, a*, b*) in the CIEL*a*b* (CIEL*a*b* color scale, 1996) scale of the strawberry surface was measured with a spectrophotometer MiniScan XE Plus (Hunter Associates Laboratory Inc., USA). The L* value indicates the ratio of white to black colour, a* value indicates the ratio of red to green colour, and b* value indicates the ratio of yellow to blue colour. The chroma (C* = (a*2 + b*2)½) and hue angle (h° = arctan (b*/a*)) were also calculated (McGuiere, 1992).

The content of soluble solids in berry was measured with a digital refractometer Atago PAL 3

(Atago Co. Ltd., Japan). Ascorbic acid (vitamin C) was determined by a titrimetric method using 2,6-dichlorphenolindophenol sodium salt solution (AOAC, 1990). The total quantity of phenolic compounds, expressed as a gallic acid equivalent, was determined spectrophotometrically (Slinkard, Singleton, 1997).

The antioxidant DPPH (2.2-diphenyl-1-picrylhydrazyl hydrate) activity was evaluated spectrophotometrically by a Brand-Williams method. DPPH radical methanol solution (2 mL 6×10^{-5} M) was mixed with 20 mL of strawberry extract. The results obtained at the absorption of 515 nm were recorded by a spectrophotometer Genesys-10 UV/Vis (Brand-Willians et al., 1995). The results were expressed as Trolox equivalents (μmol 1 g^{-1} berry).

The amount of anthocyanins in berries was determined by a pH differential method and expressed as cyanidin-3-glucoside equivalents (mg 100 g^{-1}) (Giusti, Wrolstad, 2001). Anthocyanin concentration was calculated by the formula:

$$\frac{(A_{510\,\mathrm{pH}1} - A_{700\,\mathrm{pH}1}) - (A_{510\,\mathrm{pH}4.5} - A_{700\,\mathrm{pH}4.5})}{\varepsilon \cdot L} \cdot MW \cdot k,$$

where ε – molar absorption (the coefficient of molar extinction), L – thickness of the test solution (cm), MW – molecular weight of cyanidin-3-glucoside, k – dilution factor, A – absorption. All measurements were carried out three times and the results were presented as mean.

Statistical analysis. The pathogens were identified according to the morphological traits, which were typical of the colonies (Vidhyasekaran, 2004; *Botrytis...*, 2007). The data were analysed with a module *ANOVA* of the software *STATISTICA 7.0*. The standard deviation was calculated and marked in a figure as bars.

Results and discussion

Postharvest diseases cause a great loss of fruits during the process of transportation and storage. Chemical fungicides are the main strategy for controlling postharvest losses (Sharma et al., 2009). Various studies of non-chemical treatments, including modified atmosphere with high CO_2, volatile compounds, edible coatings, UV-C and others, were conducted in order to prolong the postharvest period of soft fruits. Nigro et al. (2000) found that UV-C 254 nm reduces postharvest decay on strawberries. Neri et al. (2014) ran a test on thirty-five strawberry volatile organic compounds on *B. cinerea in vitro* and found that 14 compounds significantly influenced the conidial germination of *B. cinerea*. Tezotto-Uliana et al. (2014) ran a test on pre and post-harvest chitosan coating in order to prolong the shelf life of raspberries.

The data obtained in our previous study revealed that chlorophyllin-based treatment reduced food pathogen *Listeria monocytogenes* by 7-log *in vitro* (Luksiene et al., 2010). Luksiene and Brovko (2013) found that chlorophyllin reduced *Listeria* and *Bacillus* populations by 4.5-log and 5-log cycles, respectively. The data obtained in previous experiments clearly indicate that food pathogens can be easily destroyed by photosensitization on the surfaces of food products, also on strawberry fruits (Luksiene, 2005; Luksiene et al., 2010; Luksiene, Paskeviciute, 2011; Luksiene, Bovko, 2013). It is known that *B. cinerea* can rapidly adapt to the environment and develop resistance, but it is susceptible to photosensitization (Luksiene, 2005; Luksiene, Paskeviciute, 2011). Benzimidazoles fungicides were introduced for plant disease control in the 1960's, but within a few years resistance emerged (Kretschmer, Hahn, 2008; Debieu et al., 2013; Rodríguez et al., 2014). Our research was carried out to test the new non-chemical measures reducing *B. cinerea* on berry fruits. The photosensitization or volatile compound-based plant protection does not cause pathogen resistance (Neri et al., 2014; Rodríguez et al., 2014).

According to our analysis, treatment with photosensitization can reduce the contamination of berries with *B. cinerea* and prolong the disease-free period of fruits (Figs. 2–3). In our investigations, *Botrytis cinerea* was determined as only one causal agent of fruit rots. Experimental data revealed that 6 days after the Chld treatment, the number of *Botrytis* infected strawberries reduced by 8% and the number of disease-free strawberries increased by 6% compared with the control (Fig. 2). After 6 days of storage, in the Chld treatment there were 15% less *Botrytis*-infected strawberries compared with water treatment. After an 8-day storage, in the Chld treatment there were 20% and 5% more healthy fruits compared with the control and water treatment. In water treatment, the incidence of *B. cinerea* after a 6-day and 8-day storage was 17% and 16% higher compared to the Chld treatment (Fig. 2). The experimental results revealed that in Chld treatment there were less infected fruits (8% and 13%) compared to the control.

Experimental data showed that after a 6-day and 8-day storage in water treatment, the number of *Botrytis* infected strawberries increased by 9% and 3% in comparison with the control (Fig. 2). After a 6-day and 8-day storage, the *B. cinerea* frequency of occurrence in Chld treatment was slightly lower (2% and 3.25%) compared with the control. In Chld treatment, after a 6-day and 8-day storage, *B. cinerea* frequency of occurrence was 4.25% and 2.5% lower compared with the water treatment. According to our experiments, we can conclude that in Chld treatment, after 6 days of storage, *B. cinerea* was reduced by 8%.

Raspberries are highly perishable fruit. During storage, they lose firmness and change colour. The nutrition value of raspberries is very high, phenolic compounds are a major group of phytochemicals found in berries (Verde et al., 2013).

The experiments on raspberries revealed, that Chld treatment reduced *B. cinerea* by 3% (after 1-day storage) and by 4% (after 3-day storage), respectively, compared with the control (Fig. 3). There were 6% (after 1-day storage) and 54% (after 3-day storage) less infected fruits in Chld treatment, compared with water treatment. The comparison of Chld treatment with the control and water treatments revealed, that there were 3% and 5% more healthy fruits after 1-day of storage. Our experimental data indicated that after a 3-day of storage, in Chld treatment, there were 7% and 55% more healthy fruits, compared with the control and water treatment. After 1-day and 3-day storage *B. cinerea* frequency of occurrence in Chld treatment was lower (1% and 2%) compared with the control. After 1 and 3 days, in Chld treatment *B. cinerea* frequency of occurrence was 2% and 13% lower compared with water treatment (Fig. 3). The analysis of both experiments showed the same tendency for raspberries and strawberries, – the Chld treatment reduced *B. cinerea* and water treatment stimulated it.

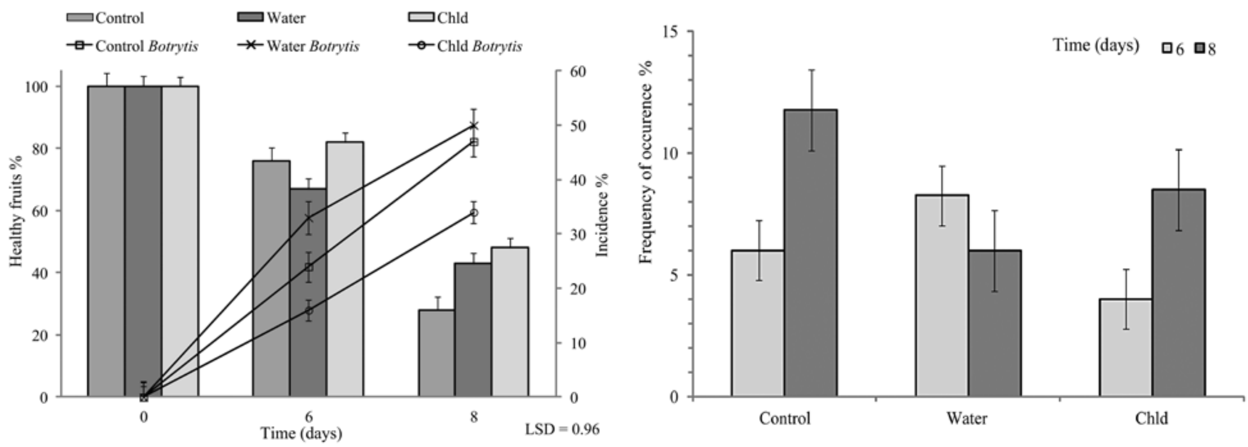

Figure 2. The inactivation of *Botrytis cinerea* by non-chemical chlorophyllin derivative (Chld) treatment in strawberries

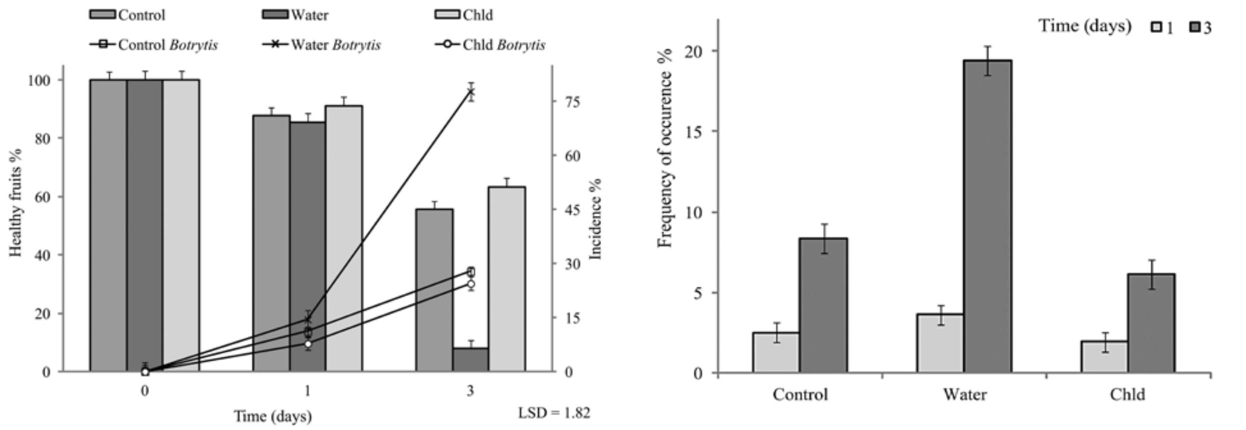

Figure 3. The inactivation of *Botrytis cinerea* by non-chemical chlorophyllin derivative (Chld) treatment in raspberries

The control of postharvest infections caused by *B. cinerea* relies on the preharvest applications. Nowadays chemical pesticides are major products used for applications against *B. cinerea* (Droby, Lichter, 2007; Droby et al., 2009). Due to the nontoxic properties of photosensitization, this treatment can be applied for microbial control as well (Luksiene et al., 2010). Recent studies of Tezotto-Uliana et al. (2014) showed that different chitosan concentrations inhibit the growth of pathogens by up to 88.9%.

The amount of soluble solids, vitamin C, total phenolic, anthocyanins content, antioxidant activity and firmness in strawberries were evaluated as well (Table 2). The results presented in Table 2 suggest that the chemical composition and firmness of strawberries among the treatments varied a little, but the treatment itself did not have a statistically significant effect. The total phenolic compounds in the control strawberries was 273.7 ± 8.8 mg 100 g^{-1}, whereas in the Chld-treated it was 270.0 ± 12.5 mg 100 g^{-1}. The level of anthocyanins in strawberries after the Chld treatment was 158.83 ± 10.48 mg 100 g^{-1} and similarly in the control 153.83 ± 8.28 mg 100 g^{-1}. The data presented in Table 2 indicated that the average firmness of Chld-treated strawberries was 16.02 ± 0.33 N cm^{-2}.

The firmness of the control strawberries was 16.66 ± 0.55 N cm^{-2}. The colour coordinates of strawberries, compared with the control treatment, showed a very

Table 2. The influence of non-chemical treatment on the colour and chemical composition of strawberries

Treatment	Fruit firmness N cm^{-2}	Ascorbic acid mg %	Anthocyanins mg 100 g	Soluble solids %	Total phenolic compounds mg 100 g^{-1}	DPPH µmol 1 g^{-1} berries
Control	16.66 ± 0.55	70.3 ± 1.2	153.83 ± 8.28	10.9 ± 0.2	273.7 ± 8.8	14.85 ± 0.14
Chlorophyllin derivative (Chld)	16.02 ± 0.33	70.7 ± 2.5	158.83 ± 10.48	10.7 ± 0.2	270.0 ± 12.5	14.74 ± 0.06
Colour parameters						
		L* (lightness)	C* (chroma)	h° (hue angle)	a* (+ redness)	b* (+ yellowness)
Control		36.27 ± 3.98	38.58 ± 3.00	33.76 ± 4.12	31.93 ± 1.86	21.50 ± 3.62
Chlorophyllin derivative (Chld)		32.47 ± 3.03	38.01 ± 4.22	30.91 ± 3.99	32.44 ± 2.84	19.65 ± 4.07

Note. The results are expressed as mean ± standard deviation.

slight, statistically insignificant difference as well, which led to the conclusion that the use of Chld did not have any influence on the colour of strawberries. Colour is the main indicator of fruit quality, which has an influence on the appearance and price (Luksiene et al., 2013).

In order to estimate specific changes of nutritional quality of raspberries, we evaluated the amount of ascorbic acid, total phenolic compounds, soluble solids, DPPH and flavonoids anthocyanins (Table 3). The raspberry fruit firmness depends on the variety, berry maturity, environmental conditions, processing, transportation and others factors (Verde et al., 2013). It was difficult to evaluate the influence of treatments on the disease incidence because raspberries are very fast perishable fruits (data are not shown).

Table 3. The influence of non-chemical treatment on the chemical composition of raspberries

Treatment	Ascorbic acid mg %	Anthocyanins mg 100 g	Soluble solids %	Total phenolic compounds mg 100 g^{-1}	DPPH µmol 1 g^{-1} berries
Control	27.5 ± 1.8	81.4 ± 5.8	9.8 ± 0.3	311.4 ± 9.8	18.8 ± 2.2
Chlorophyllin derivative (Chld)	27.7 ± 1.7	81.0 ± 4.7	9.6 ± 0.4	314.6 ± 11.5	18.1 ± 2.6

Note. The results are expressed as mean ± standard deviation.

Concerning ascorbic acid content, total anthocyanin and soluble solids, the photosensitization treatment had no impact on their amount in treated raspberries. The amount of total phenolic compounds in raspberries in the control treatment was 311.4 ± 9.8 mg 100 g^{-1}, similar results were obtained in the Chld-treated it was 314.6 ± 11.5 mg 100 g^{-1}. The soluble solids content in the Chld-treated was 9.8 ± 0.3%, which is similar to the control 9.6 ± 0.4%. According to our investigation, no significant colour changes were detected in the treated raspberries (data not shown) in comparison with the control. Based on our experiments, we can conclude that Chld did not affect the appearance and nutritional value of strawberries and raspberries.

Conclusions

1. The chlorophyllin derivative-based photosensitization is an innovative postharvest treatment for the inhibition of *Botrytis cinerea* on strawberries and raspberries without any negative influence on the colour, antioxidant activity and phenolic compounds.

2. The chlorophyllin derivative-based photosensitization reduced the contamination of strawberries with *B. cinerea* by 8% (after a 6-day storage) and that of raspberries by 3% (after 1-day storage).

3. Photosensitization has potential as a non-chemical plant protection tool, which could be used for the development of new plant protection technologies in agriculture.

Acknowledgments

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Naujos necheminės technologijos, mažinančios uogų užkrėstumą jų laikymo metu

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Santrauka

Viena pagrindinių braškių ir aviečių uogų nuostolius sukeliančių ligų yra kekerinis puvinys (*Botrytis cinerea* Pers.: Fr.). Tirtos naujos necheminės maisto saugos technologijos, mažinančios uogų puvinius ir prailginčios jų vartojimo trukmę. Fotosensibilizacija yra novatoriškas metodas, taikomas slopinti puvinių patogenams uogose, pagrįstas matomos šviesos (λ = 400 nm, 20 mW cm^{-2}) ir fotojautrios medžiagos (chlorofilino darinio) sąveika. Tyrimo tikslas – taikant fotosensibilizacijos metodą įvertinti chlorofilino darinių įtaką mažinant aviečių ir braškių uogų užkrėstumą kekeriniu puviniu (*B. cinerea*) jų laikymo metu. Tyrimo metu uogos 1 val. buvo apdorojamos tiriamaisiais tirpalais ir 30 min. veiktos šviesos šaltiniu. Nustatyta, kad chlorofilinas laikymo metu mažina uogų užkrėstumą kekeriniu puviniu. Braškėse po 6 parų laikymo užkrėstumas sumažėjo 8 %, o avietėse po 1 paros – 3 %. Chlorofilinas neturėjo įtakos braškių ir aviečių uogų išvaizdai bei maistinėms savybėms.

Reikšminiai žodžiai: avietės, *Botrytis cinerea*, braškės, chlorofilino dariniai, fotosensibilizacija.