Effect of ozone treatment on strawberry pests

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EFFECT OF OZONE TREATMENT ON STRAWBERRY PESTS

MASTER THESIS

Lucija Purić

Zagreb, September 2022

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MASTER THESIS

Lucija Purić

Supervisor:
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STUDENT'S STATEMENT

ON ACADEMIC RECTITUDE

I, **Lucija Purić**, JMBAG 0178090057, born on 29^{th} of November 1992 in Rijeka, declare that I have independently written the thesis under the title:

EFFECT OF OZONE TREATMENT ON STRAWBERRY PESTS

With my signature, I guarantee:

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REPORT

ON EVALUATION AND MASTER'S THESIS DEFENSE

Master's thesis written by Lucija Purić, JMBAG 0178090057, under the title

EFFECT OF OZONE TRETMENT ON STRAWBERRY PESTS

Is defended and evaluated with the grade____________________ ,on__________________.

Committee for thesis evaluation and defense: Signatures:

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Summary

Of the master’s thesis – student **Lucija Purić,** entitled

EFFECT OF OZONE TREATMENT ON STRAWBERRY PESTS

Diseases and pests are a great concern for the human food industry. The appearance of diseases and pests results in large economic losses of manufacturers. When it comes to storing agricultural products, currently diseases and pests are treated by cleaning or treating with fungicides and insecticides. In order to avoid the use of pesticides, in society is currently necessary to find some new eco-friendly methods. One of the potential methods is the use of ozone, which has been studied in this work on strawberries. The strawberries have a very thin skin, and it is very problematic to store them because of their easy perishability. Main goal of this study was to determine the effectiveness of ozone in combating diseases and pests on strawberries. In the experiments carried out, different durations of ozone exposure and different number of days of treatments were examined. The results did not show the adverse effects of ozone on *Botrytis* assuming that this was due to adverse conditions during the conduct of the experiment. It is necessary for new research to be carried out with different concentrations of ozone and in controlled storage conditions.

Keywords: diseases, pests, strawberries, ozone, *Botrytis*

1. Introduction

Strawberries are perennial shrubby plants belonging to the genus *Fragaria* and are classified in the family Rosaceae. The most popular cultivated strawberry is the desert strawberry, *Fragaria* × *ananassa* Duchesne (Hancock et al., 2008). The first record of strawberries was fruits of the species *Fragaria vesca* L. Species *Fragaria vesca* started being cultivated in the 1300s in Europe, specifically in France, where it was mostly cultivated for the flower, it gave rather than the actual fruit because of its beauty (Darrow, 1966). Strawberry is considered as the most economically important type of berries in Republic of Croatia and in the world. It is grown because of the fruit, which is of exceptional quality, rich in sugars, minerals, and vitamin C. They can be consumed fresh or minimally processed, and they can also be frozen.

Annual world production of this fruit has steadily grown through time. In 2020, strawberries were grown on 384,668 ha with a yield of 8,861,381 tonnes (FAOSTAT, 2022). Strawberries, mainly for the fresh market, are grown commercially in Croatia on about 367 ha with a total production of 3,383 tonnes. Most strawberries in the area are grown under integrated pest management.

Strawberries are attacked by a variety of pests, including insects, mites, and pathogens which can cause economically significant damage. With the increasing prevalence of insects, diseases, and plant products, as well as the pressure of climate change, the number of harmful organisms causing problems in strawberry production is steadily increasing. The appearance of diseases and pests depends on the area of cultivation, the method of growing strawberries and the sensitivity of the variety itself (Zovko, 2021). The main factor, which limits the production of strawberry in many countries, is the loss of yield or the decay of entire plantations caused by fungal diseases (Kapytowski and Bojarska, 2005). Strawberries are particularly susceptible to the rapid development of postharvest rots caused by infestations of various pests. Because berries are pulpy fruits with soft skin and high moisture and sugar content, they are vulnerable to physical damage that accelerates their deterioration increasing water loss, which may increase contamination by microbial organisms (Previdi et al., 2016). In inadequate conditions of transportation, sale and especially storage, the risk of fruit decay is higher, therefore it is not surprising that the biggest problem with strawberries is their storage.

Strawberry is a very perishable fruit; therefore, it is necessary to find procedures that can prolong the shelf life of the strawberry and preserve its original properties during storage. Storage may affect the chemical composition and properties of strawberries (Mishra and Kar, 2014). When storing strawberries, although the fruits during harvesting were without visible symptoms of the disease, the biggest problem is the appearance of gray mold, which results in rapid rotting of the fruit.

The primary goal of producers is to extend the shelf life of strawberries from harvest to sale. Pesticide treatments are not allowed at this stage, so alternative methods must be explored, one of which is the use of ozone. Ozone effectively suppresses bacteria, viruses, fungi and numerous insects. Ozone is used in the processing of fruits and vegetables to inactivate pathogenic microorganisms that cause food spoilage, most often these are molds (*Botrytis cinerea*) and yeasts (Barth et al., 1995). It is not toxic to the environment, decays to oxygen in only 30 minutes under normal conditions, it leaves no residue, and no adverse health effects have been reported. There are no data on the effectiveness of ozone on strawberry pests but judging by the fact that it is used to combat pests and diseases on other berries it could also be effective on strawberries.

1.1. The aim of the work

The aim is to determine the efficacy of ozone in form of biofumigant as an alternative technology for strawberry pest control.

2. Literature review

2.1. Strawberry lat. *Fragaria* × *ananassa* Duchesne

Strawberry (Figure 2.1.) is a perennial herbaceous plant of the rose family (Rosaceae).

Figure 2.1. Strawberries (Photo: Darija Lemić)

Family Rosaceae is divided into four subfamilies regarding the morphology of the flowers, the location of the fruiting house and the type of fruit. Those are Spiroideae, Rosoideae, Maloideae and Prunoideae. Genus *Fragaria* (strawberry) belongs to the subfamily Rosoidae (Table 2.1.).

Table 2.1. Scientific classification of strawberry

Kingdom	Plantae
Order	Rosales
Family	Rosaceae
Sub-family	Rosoidae
Genus	*Fragaria*
Species	*ananasa*
Botanical name	*Fragaria ananasa*
Fragaria ananasa	*Fragaria chilonensis* x *Fragaria virginiana*
Origin	France

Source: Nikolić and Milivojević, 2015

The most important strawberry cultivar, *Fragaria x ananasa* Duch., is the octoloid with 56 chromosomes. It was formed in 18^{th} century as a hybrid of two types of strawberries, *Fragaria virginiana* and *Fragaria chiloensis* (Hancock, 1999). According to Nikolić and Milojević (2015), 47 varieties of strawberries are known, of which 12 are significant and they are divided based on their fertility.

Strawberry is considered the most economically important type of berries in Croatia and in the world. Its fruit is of exceptional quality, rich in sugars, minerals and vitamin C (nestovise.org). The fruit of strawberry is of significant nutritional and health value, and it has a positive effect on human health. The largest share of strawberry fruit is water, over 90%. It contains a high proportion of vitamin C, about 60 mg in 100 g of fruit, vitamins E, A and B are also represented. Of the minerals, potassium content is significant, an average of 153 mg per 100 g of fruit. Phosphorus, calcium, magnesium, sodium, and iron are also significantly represented (Maretić and Duralija, 2014).

2.1.1. Growing strawberries in Croatia and world

Strawberry cultivation (*Fragaria x ananassa*) in the world is increasing due to the prolongation of the consumption season (Figure 2.2.). For successful production, it is important to monitor improvements in breeding technology but also the choice of varieties depending on the purpose or area of production (Maretić and Duralija, 2014). We distinguish very early varieties, early varieties, medium-early varieties, and medium-late varieties. Some of the strawberry varieties grown in Croatia are *Alba, Albion, Antea, Arosa, Asia, Clery, Elsanta, Joly* etc. mainly in the period from July 10 to July 20 (www.fragaria.hr).

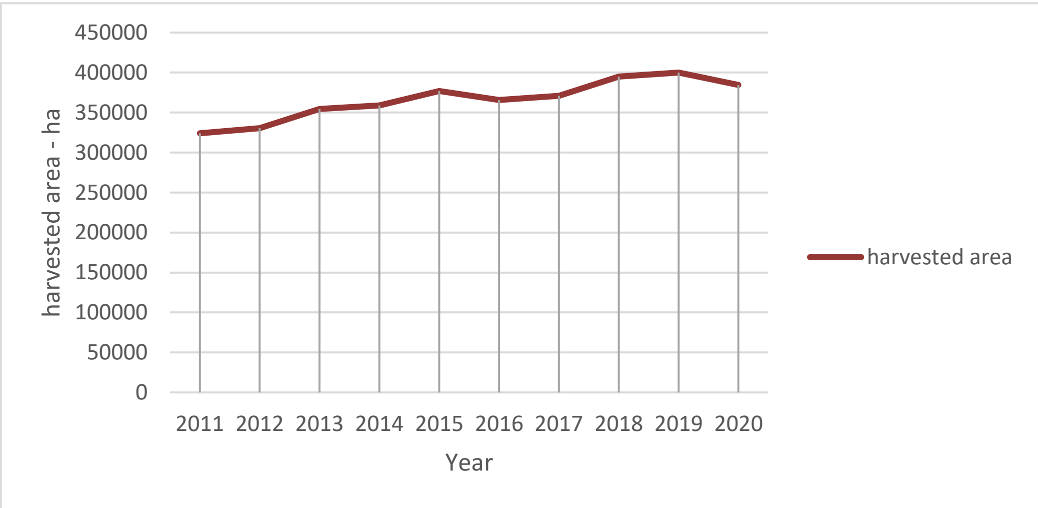

Figure 2.2. Harvested area of strawberries in world
Source: FAOSTAT, 2022

In accordance with the increase or decrease in strawberry growing areas, the amount of yield increases or decreases (Figure 2.3.).

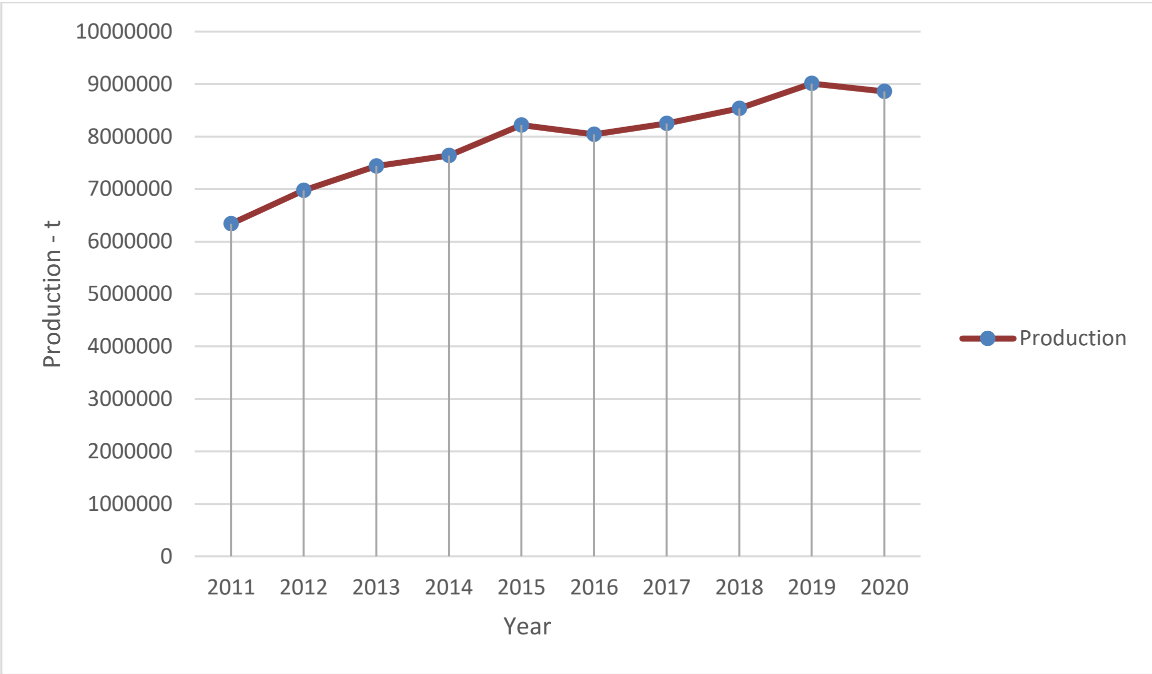

Figure 2.3. Production of strawberries in world

Source: FAOSTAT, 2022

Data for Croatia indicate that strawberries are produced on an area of about 300 ha (Figure 2.4.). This production is insufficient for domestic needs, especially considering good climatic and edaphic conditions, the availability and cleanliness of water and the proximity of the market.

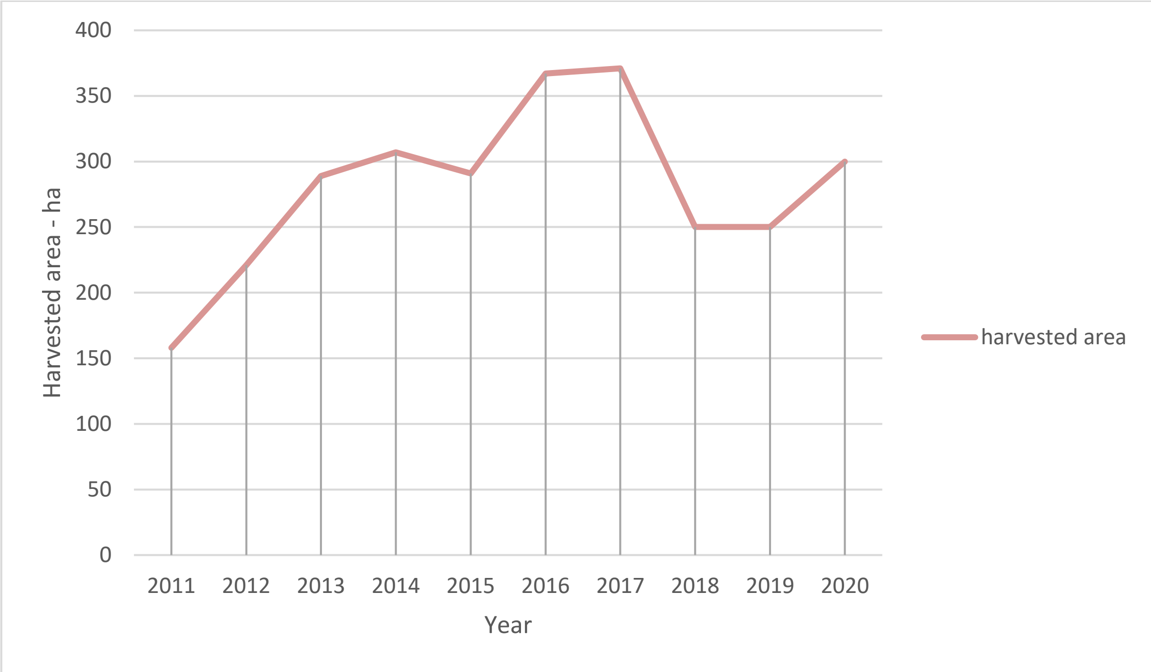

Figure 2.4. Harvested area of strawberries in Croatia
Source: FAOSTAT, 2022

According to FAOSTAT (2022), 2,630 tons of strawberries were produced in Croatia in 2022 (Figure 2.5). In last ten years the biggest production was in 2013.

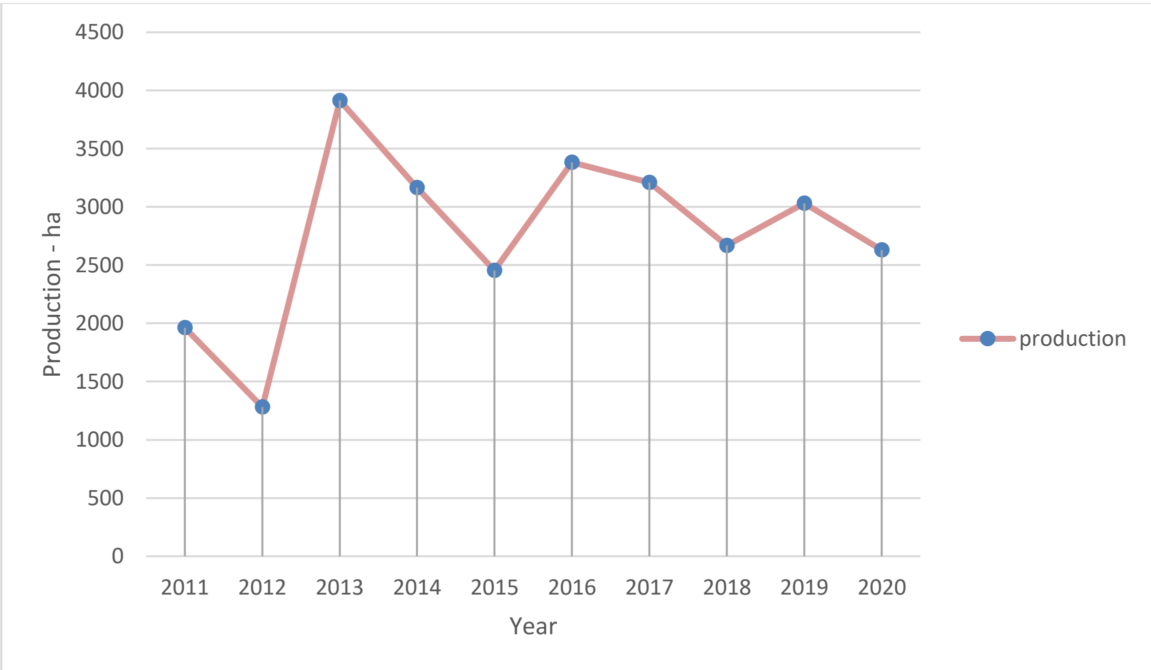

Figure 2.5. Production of strawberries in Croatia
Source: FAOSTAT, 2022

The main center of strawberry cultivation in Croatia is the area around the capital Zagreb, the so-called "Zagreb ring", and strawberries from the Vrgorac area are also known (Maretić and Duralija, 2014).

Croatia still manages to meet only a third of its own strawberry needs. Croatia has favorable climatic conditions for cultivation, a large number of unemployed and nearby neighboring countries are looking for this type of fruit, so it would be understandable that Croatia grown more strawberries. According to the FAO (2022) data in Table 2.2., the lowest value of import was in 2011 ($1,992,000) and also in 2011 Croatia exported the largest amount of strawberries ($717 000). So, the most favorable situation in Croatia in terms of imports and exports was in 2011 (Figure 2.6.).

Table 2.2. Import and export of strawberries from 2011 to 2020

YEAR	IMPORT QUANTITY IN TONNES	EXPORT QUANTITY IN TONNES	IMPORT VALUE (x $1000)	EXPORT VALUE (x $1000)
2011	959	217	1992	717
2012	1282	179	2451	596
2013	1058	186	2152	582
2014	1343	216	2833	533
2015	1362	146	2509	274
2016	1519	36	2900	85
2017	1484	49	3131	150
2018	1205	111	2951	172
2019	1895	7	4201	19
2020	1415	54	3551	173

Source: FAOSTAT, 2022

In 2020, Croatia imported the largest quantities of strawberries from Germany (374 tonnes) and Greece (324 tonnes). It exported the most to Slovenia, namely 51 tonnes (FAOSTAT, 2022). Croatia exports very little, the amount of imports is drastically higher than the amount of exports as shown in the Figure 2.7.

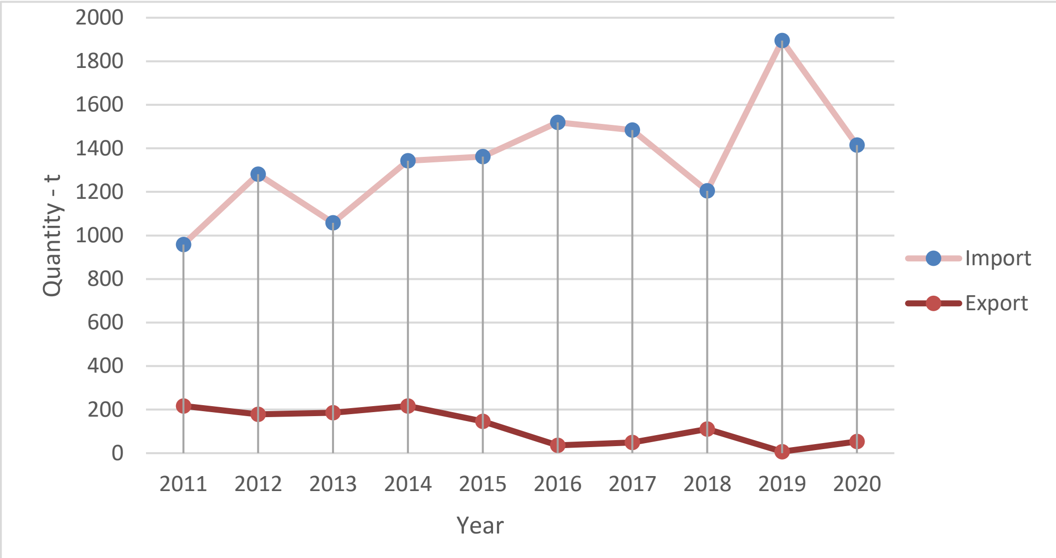

Figure 2.7. The value of strawberry imports and exports in Croatia from 2011 to 2020
Source: FAOSTAT, 2022

2.1.2. The method of growing strawberries

Strawberries can be grown outdoors and indoors. Planting strawberries in the open air implies planting strawberries with a system of cultivation in strips (Figure 2.9.). It is very important to prepare the soil for planting (choice of location, terrain planning, meliorative fertilizing and deep plowing of the soil). They are planted in August. The first year after planting gives the highest quality nature of fruits. They can be planted manually or using machines. The gap in the row is 25 to 30 cm. This gap provides a composition of 37,000 to 44,500 seedlings per 1 ha (Croatian Institute for Agricultural Advisory Service, 2021).

Figure 2.9. Planting strawberries in the open area

Source: https://gospodarski.hr/rubrike/vocarstvo-rubrike/prilog-broja-isplativ-uzgoj-jagoda/ - 02.09.2022

In breeding, it is important to provide enough water, apply protection and adequate fertilizing. It is also important to ensure the shading of plants during strong insolation and high temperatures. Harvesting should be carried out 2-3 times a week. The soil for growing strawberries should be well drained, deep, sandy, rich in humus, pH reactions 5.5-7. The preculture should not be tomatoes, peppers, potatoes, and other strawberry varieties (Duralija, 2015).

On indoors, strawberry is grown in greenhouses and hydroponics. The most intensive way to grow strawberries is to grow in greenhouses for early and off-season production, when the price of strawberries is 3-4 times higher. In a protected area, the temperature of the soil and the air is higher by 6-12 °C than in the open field. In addition, growing strawberries in a protected area implies higher yields (even up to 40-50%), so this system of cultivation can be extremely profitable, even though the investments are quite large. Hydroponic cultivation (Figure 2.10.) implies cultivation in a protected area with precise dosage of the necessary nutrients and water in accordance with the phenophase of the plant (Bogović, 2011).

Figure 2.10. Hydroponic cultivation of strawberries

Source: https://www.savjetodavna.hr/2019/02/06/hidroponska-proizvodnja-jagoda/- 02.09.2022

Several systems of hydroponic cultivation are distinguished by the construction of the object, placement of plants, planting intervals, selection of varieties and types of seedlings (www.savjetodavna.hr). The biggest advantage of growing strawberries indoors is that it is possible to control the factors of cultivation. Growing strawberries on the substrates is also increasingly represented.

2.2. Pests and diseases in strawberry production

Strawberries are very susceptible to diseases and pests. Some of the most significant pests of strawberries are: *Caenorhinus germanicus* Hrbst*, Anthonomus rubi* Hrbst.*, Aphis forbesi* Weed*, **Drosophila suzukii*** **Matsumura**, *Tarsonemus pallidus* Banks, *Tetranychus urticae* Koch*.* Of the numerous pest diseases that attack strawberries, the most common are viral diseases: ***Botrytis cinerea*** Pers*, Phytophthora fragariae* Mont*, Mycosphaerella fragariae* Tul.*, Phomopsis obscurans* Sutton. In bold are marked the most important pest and disease in Croatian strawberry production.

2.2.1. Grey mold

Gray mold or rot of fruits (Figure 2.11.). is the most common and important disease on strawberries. The causative agent of the disease is the fungus *Botrytis cinerea* (Miličević, 2015). Ripe fruits are the most commonly infected, but sometimes there may be an infection of green fruits, flower stalks, flowers and leaves. The initial symptoms are the appearance of a light brown spot on which a cobweb coating, grayish-brown color occurs later, representing the mycelium of the fungus consisting of very branched conidiophores with conidia. In case of stronger infection and favorable climatic conditions (high humidity and lower temperatures), and inadequate protection, the disease can completely destroy the fruits of strawberries. Suppression is carried out by fungicides (botriticids) of which in our country licensed are preparations based on pyrimetanil, fenhexamide, pyraclostrobin and boskalide, as well as fludioxonell and ciprodinyl (Miličević, 2015).

Symptoms of gray mold are characterized by a change in tissue color with a wet and necrotic appearance of lesions and grayish mycelium, consisting of single-celled conidia and branched conidiophores over the affected areas (Miličević, 2015).

Figure 2.11. Grey mold on strawberries
Source: https://strawberryscout.ca.uky.edu/botrytisgraymold - 21.08.2022.

The disease can be observed in strawberries that are still in the field/greenhouse or in the postharvest during storage, transportation, and commercialization. In all cases, the disease can affect both qualitative and quantitative attributes (Filippi et al., 2021). *Botrytis cinerea* initially colonizes dead or senescent tissues, which serve as an energy source for the subsequent establishment and colonization of healthy tissues. Humidity is the most important factor affecting the appearance and intensity of the appearance of gray strawberry mold. Although the disease develops at temperatures below 0°C, the optimum temperatures for the development of the disease are 15-25°C (Grabke, et al.,2014).

2.2.2. Spotted wing drosophila

The species *Drosophila suzukii* (Figure 2.12) is a polyphagous pest of the family Drosophilidae originating from Southeast Asia, which causes greatness of damage in the cultivation of strawberries (Pajač Živkovic et al., 2018). It is prone to the attack of healthy fruits unlike other acetic flies that prefer rotten fruits (Berry, 2012).

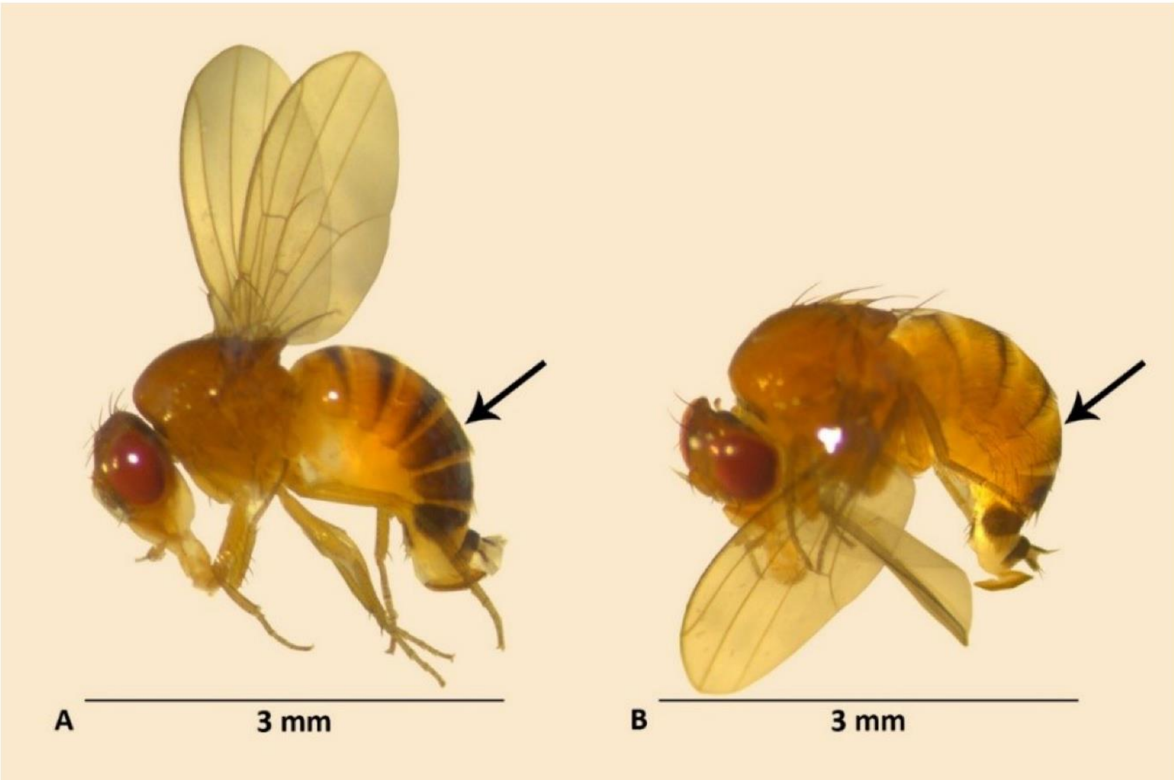

Figure 2.12. *Drosophila suzukii*
Source: Panel et al., 2018

According to the European Organization for Plant Protection (EPPO), the species is considered potentially harmful in fruit production in all European and Mediterranean countries (Pajač and Barić, 2010). The adult form of the acetic fly of the fruit, *D. suzukii*, is yellow brown in color, has red eyes, and the body dimension ranges from 2.25 to 4 mm, while the wingspan is from 6 to 8 mm. On abdomen segments there are dark brown stripes. Males of the species *D. suzukii* differ from other males of the genus *Drosophila* by the characteristic black spot on the tip of the wings and arrangement of sex combs on the front legs that are visible as two black stripes (Walsh et al., 2011). The greatest harm is done by larvae feeding on the meat of the fruit, and the affected fruits can also be susceptible to secondary infections of pathogens such as fungi, yeasts, and bacteria, which can cause even faster fruit decay and further losses in production (Pajač and Barić, 2010). The fruits attacked by this pest are not suitable for consumption and their market value is declining. Despite its exceptional ability to adapt to various climatic conditions, rapid reproduction and a wide range of hosts, the pest spreads rapidly and causes economic damage in fruit production around the world (De Ros et al., 2015).

D. suzukii was registered in Croatia for the first time at the end of 2010 in Istria on raspberries, peaches, and vines (Mastek Milek, 2015).

2.3. Ozoning

Ozone (O_3) is a gas composed of three oxygen atoms that occur naturally in small quantities in the atmosphere where its maximum concentration does not exceed 0.001%. Ozone has a half-life duration of 20-50 minutes before decomposition on an oxygen molecule (O_2). Decomposition into an oxygen molecule releases one oxygen atom that is very reactive and reacts with the cell membranes of the organism by disrupting their normal cellular function (Lemić et al., 2019b). Artificially, ozone is most easily generated by electric discharge in the air (coronal discharge) (Virić Gašparić and Lemić, 2020).

The mechanism of action of ozone on insects and pests is not fully known.

The main advantages of using ozone over chemical preparations are that there are no eye chemicals, it is possible to neutralize it with thermally activated carbon, and it is short-lived.

Ozone is used in some parts of agriculture. Potential applications include reducing odors in the poultry industry, removing the smell of lagoon waste from pig breeding, and reducing pathogens in the storage of grapes, potatoes, and onions (Lemić et al., 2019a).

Little research has been conducted on combating pathogens using ozone, some of them are:

1) Boonkorn et al. (2012) examined the effects of ozone on green mold growth (*Penicillium digitatum*) and found that exposure to ozone (200 µL L−1) for 4 and 6 hours reduces the growth of fungi on fruit bark.
2) Sharpe et al. (2009) examined the effect of ozone on *Botrytis cinerea* and *Sclerotinia sclerotiorum*. The viability of *B. cinerea* dispute was reduced by more than 99.5% ($P < 0.01$), and the height of the air mycelium was reduced from 4.7 mm in control to less than 1 mm after exposure to an ozone of 450 or 600 ppb in 48 h to 20 °C. The sporulation of *B. cinere*a was also significantly inhibited by ozone treatments.

However, ozone did not have a significant impact on the mycelial growth of *S. sclerotiorum* in vitro.

3) Modesti et al. (2019) used ozoned water to vine plants and partially suppressed populations of microorganisms, in particular pathogenic fungi.
4) Allen et al. (2003) with a dose of ozone of 0.16 mg/g achieved 96% effectiveness in inactivating mushroom spores on barley in just five minutes.
5) Greishop et al. (2020) found that ozone at a concentration of 0.8 ppm does not act on diseases and pests in orchards, but consider that concentrations between 2 and 5 ppm on nozzles could provide some level of protection.

Research on the effectiveness of ozone in controlling pests of animal origin is more numerous. Some of them are:

1) Lemić et al. (2019b) investigated the ozonation effectiveness of six different species of insects that were at different stages of development. The results revealed the harmful effects of ozone on insects. The greatest effectiveness of ozonation was established in adult insects, and very small or none on the larvae.
2) Xinyi et al. (2017) concluded that an ozone concentration of 0.42 g/m^3 effectively killed adults of phosphine-susceptible and -resistant strains of *T. castaneum, O. surinamensis, S. oryzae* and *S. zeamais.*
3) McDonough et al. (2011) found that most of the ozone tolerant phases of *T. castaneum* was pupae and eggs, which required treatment of 180 min at 1800 ppm of ozone to achieve 100% mortality. Eggs of *P. punctele* also required 180 minutes at 1800 ppm of ozone to achieve 100% mortality. Ozone treatments of 1800 ppm for 120 min and 1800 ppm for 60 minutes were needed to kill all *S. zeamais* adults and *S. oryzae* adults, respectively.
4) Dong et al. (2022) found that ozone concentration of 700 ppm after 24 h treatment causes complete mortality of *Rhyzopertha dominica* and *Tribolium castaneum*.
5) Tian (2015) concluded that *Blattella germanica* eggs are the most ozone-tolerant life stage of *Blatella* and that 100% mortality can only be achieved after 24 h of exposure to 480 ppm of ozone.

3. Materials and methods of research

The investigation was conducted on strawberry fruit in the laboratory of the Department of Agricultural Zoology, Faculty of Agriculture in Zagreb. During the strawberry season, the effect of ozone on postharvest pests was tested. Ripe strawberries from commercial sources were treated with different ozone concentrations (up to 6 ppm) and in different treatment durations (up to 1 hour). Research was done between 5th July and 21st July 2022. The laboratory temperature ranged from 21,1°C up to 38,6°C, and humidity was between 16% and 41% throughout the study period.

3.1. Experimental design

For research, strawberries from local cultivation were purchased.

In the experiment, 400 strawberries were ozoned, arranged in 40 plastic containers with 10 fruits each (Figure 3.1). Variants in the experiment were ozone time exposure of 10, 30, and 60 minutes and control variant (without ozone). Each variant was treated in four repetitions. The number of days of ozonation, the time and distribution of strawberries were different. For the control variant, 4 containers with 10 strawberries each were put in plastic bags with many little holes, and were not treated at all. The containers in which the strawberries were ozoned were plastic containers without a lid so that the ozone could flow freely through the chamber.

Figure 3.1. Strawberries prepared for ozonation

3.2. Ozonator

For ozonation, the ozonator of Steril Systems model OZ75 (Steril Systems, Germany) was used. The oz75 ozone generator is suitable for single-dose disinfection and odors removal in large rooms where there are no staff and is specially designed to meet the needs of the food industry (www.sterilsystems.com). Ozone output is 7500 mg/h. This power output generates 0.05 pm of ozone in a liter of air. The output quantity was not changed.

Figure 3.2. Ozonator

3.3. Ozonation

Ozonation was carried out in the greenhouse room. The room in which the volume survey was carried out was 16 m^3. The room was filled with ozone for 10 minutes before the strawberries were introduced.

In the first day of ozonation on July 05, 2022, the relative humidity of the air was 39%, and the temperature was 21.1°C. All strawberries (36 containers) except the ones in control were ozoned. After filling the room with ozone, strawberries were placed in the room. The first part of strawberries was extracted from the ozoned greenhouse after 10 minutes. That is, four containers with 10 strawberries each that were intended for ozonation for one, two or three days (12 containers in total) were taken out after being ozoned for 10 minutes. The second part of the strawberries was extracted after 30 minutes, also 12 containers that were scheduled for ozonation for 30 min during one, two or three days. The remaining 12 containers were taken out after 60 minutes. Strawberries that were intended for ozonation for a day were stored in plastic bags with holes (Figure 3.3.). They were stored in Analytical laboratory of the Department of Soil Amelioration (MELILAB) on 26°C. That was four containers treated for 10 minutes, four containers treated for 30 minutes, and four containers treated for 60 minutes with 10 strawberries in each, which means 12 containers with 120 strawberries in total. The remaining 24 containers were also taken to analytical laboratory by the next day.

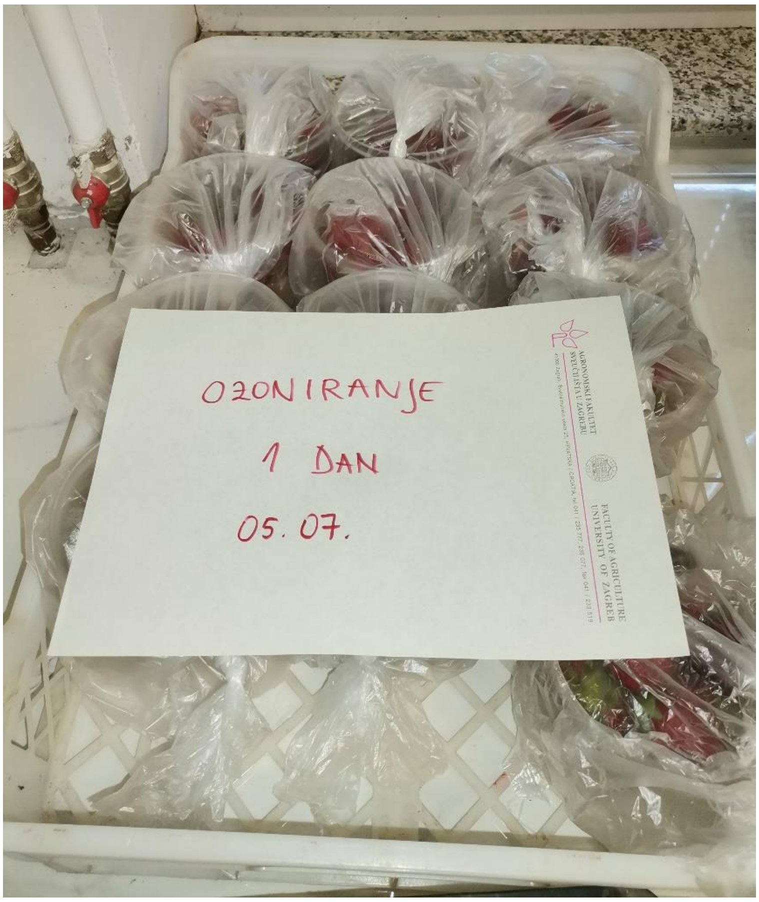

Figure 3.3. Ozonated strawberries on the first day

On the second day 24 remaining containers were ozonated. Twelve of them were intended to be ozonated for two days and 12 to be ozonated for three days. The relative humidity of the air in room in which strawberries were treated was 16%, and the temperature was 38.6°C. Like the day before, strawberry containers were taken out of the greenhouse after being ozonated for 10, 30 and 60 minutes. The first 8 containers intended to be ozonated for 10 minutes during two and three days were taken out after 10 minutes. The second 8 containers were taken out after 30 minutes and the last 8 containers were taken out after 60 minutes. Twelve containers with 10 strawberries in each were then stored in plastic bags. They were stored in Analytical laboratory of the Department of Soil Amelioration (MELILAB) on 26°C.

On the third day last ozonation on strawberries was done. The relative humidity of the air in the room was 41%, the temperature was 25.8°C. First, four containers were taken out of the greenhouse after being ozonated for 10 minutes, the second four after 30 minutes and the last four after an hour. After three days of ozonation they were all in tied plastic bags with fine holes punctured and placed in MELILAB at 26°C until the end of the experiment. During all three days, strawberries were ozonated with the same ozone output of 7500 mg/h.

Before reading the data, strawberries were sorted by the degree of damage according to the Fillipi et al. (2021). Strawberries were classified by the degree of damage of 8%, 28%, 46%, 77%, 82% and 100% (Figure 3.4.)

Figure 3.4. Degrees of damage on strawberries according to Fillipi et al. (2021)

3.3.1. Readings

Efficacy of ozone for all repetitions of all variants was recorded four, seven and fourteen days after the third day of ozonation (Figure 3.5.). The first inspection of strawberries was carried out on July 11, 2022, the second one on July 14, 2022 and the third on July 21, 2022.

Figures 3.5. Sorting strawberries by category (photo: Helena Virić Gašparić)

3.4. Data analysis

Results were monitored over a two-week period and the efficacy of ozone was evaluated according to Abbot. The efficacy of all ozone variants was subjected to analysis of variance (ANOVA) to determine the difference in the effect of ozone on strawberry pests at different concentrations and treatment durations. Statistical data processing (ANOVA, Tukey's HSD test) was performed using ARM 2021® GDM software (GDM Solutions, 2022).

4. Results

The first inspection of strawberries (Figure 4.1) was carried out on July 11, 2022, after which strawberries were classified by the degree of damage of 8%, 28%, 46%, 77%, 82% and 100%.

Figure 4.1. Strawberries appearance on the first inspection

The appearance of strawberries after the 2nd reading can be seen on Figure 4.2.

Figure 4.2. Appearance of strawberries on the second reading

At the third reading, the strawberries were completely covered with mold (Figure 4.3).

Figure 4.3. Strawberry appearance on the third reading

After placing strawberries in previously mentioned categories, the percent of damage was calculated. Percentage of damage after first reading is represented in Figure 4.4.

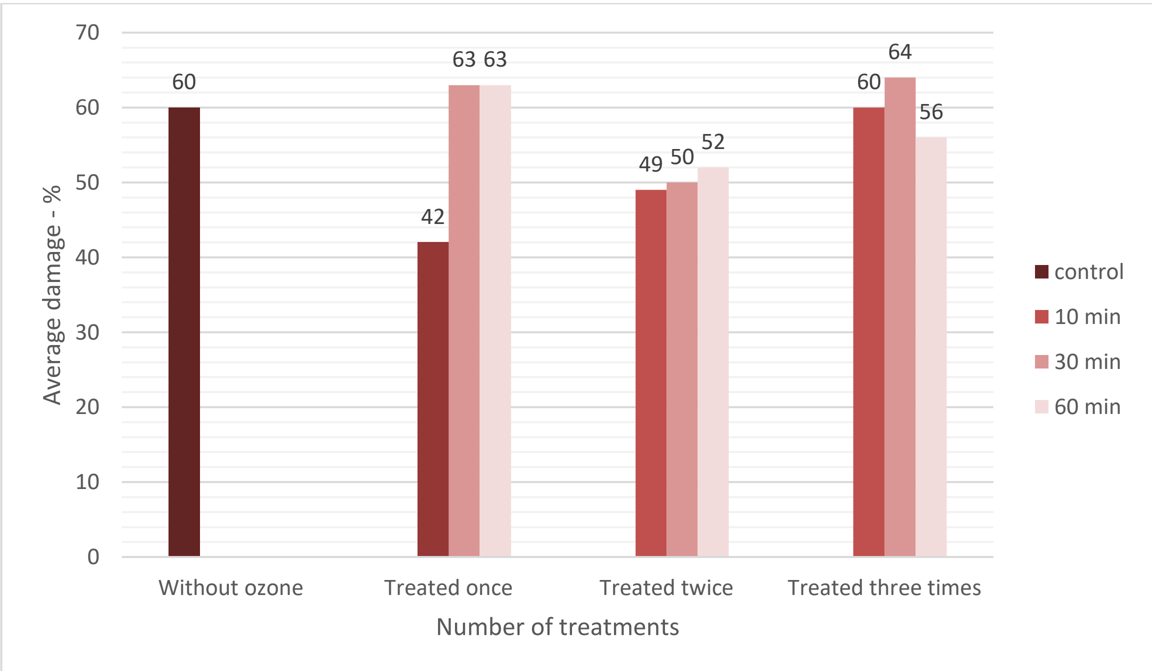

Figure 4.4. Average damages of *Botrytis cinarea* on Strawberries after the first reading

Percentages of damage after second reading are represented in Figure 4.5.

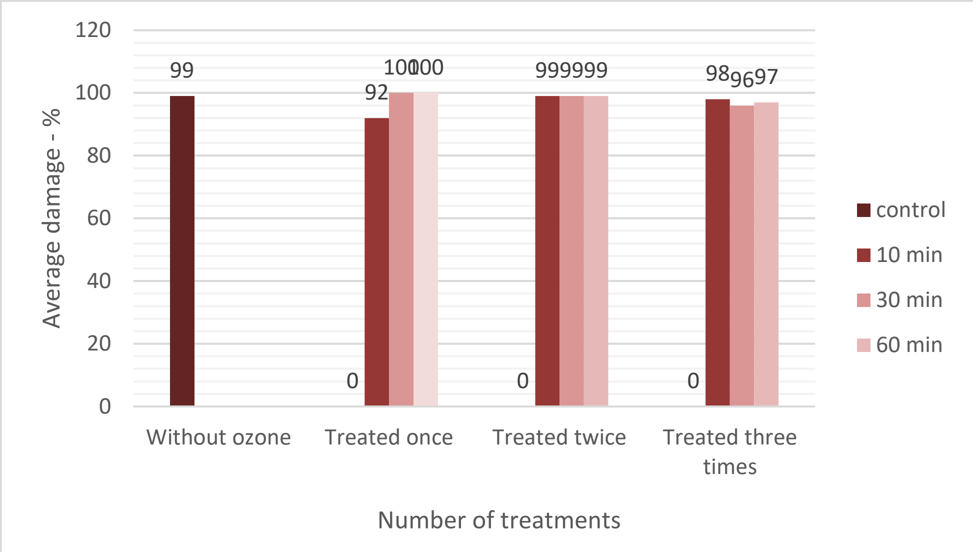

Figure 4.5. Average damages of *Botrytis cinarea* on Strawberries after the second reading

After third reading on July 21 the 100% of damage was noted. During this reading four *Drosophila suzukii* species were found. One was found in the container with strawberries treated for 10 minutes during one day, one in container with strawberries treated for 10 minutes during two days and two in container with strawberries treated for 60 minutes during two days.

After three days of reading the results had been summarized (Table 4.1.). The duration of ozonation did not significantly affect the effectiveness. After 14 days, all strawberries were infected and there was the appearance of flies. Although there were some variations in damages between variants and repetitions, after statistical analysis no significant differences were found between variants in all reading periods.

Table 4.1. Percentages of damages of *Botrytis cinarea* on Strawberries during three days

			DAMAGE %		
VARIANT	DURATION	REPETITION	1st reading July, 11	2nd reading July, 14	3rd reading July, 21
Control		1	51.67	100.00	100.00
		2	36.67	100.00	100.00
		3	56.67	96.67	100.00
		4	96.67	100.00	100.00
Treated once	10	1	31.67	75.00	100.00
	10	2	75.00	100.00	100.00
	10	3	28.33	95.00	100.00
	10	4	31.67	98.33	100.00
Treated once	30	1	43.33	100.00	100.00
	30	2	41.67	100.00	100.00
	30	3	75.00	100.00	100.00
	30	4	91.67	100.00	100.00
Treated once	60	1	36.67	100.00	100.00
	60	2	26.67	100.00	100.00
	60	3	88.33	100.00	100.00
	60	4	100.00	100.00	100.00
Treated twice	10	1	38.33	100.00	100.00
	10	2	40.00	95.00	100.00
	10	3	38.33	100.00	100.00
	10	4	80.00	100.00	100.00
Treated twice	30	1	90.00	100.00	100.00
	30	2	43.33	98.33	100.00
	30	3	33.33	98.33	100.00
	30	4	31.67	100.00	100.00
Treated twice	60	1	98.33	100.00	100.00
	60	2	35.00	100.00	100.00
	60	3	31.67	98.33	100.00
	60	4	41.67	98.33	100.00
Treated three times	10	1	76.67	100.00	100.00
	10	2	51.67	100.00	100.00
	10	3	53.33	100.00	100.00
	10	4	60.00	93.33	100.00
Treated three times	30	1	38.33	88.33	100.00
	30	2	90.00	100.00	100.00
	30	3	40.00	95.00	100.00
	30	4	88.33	100.00	100.00
Treated three times	60	1	41.67	90.00	100.00
	60	2	45.00	100.00	100.00
	60	3	81.67	100.00	100.00
	60	4	56.67	100.00	100.00
Tukey's HSD			ns	ns	ns

5. Discussion

The study was carried out with the main aim of determining the effectiveness of ozone in combating strawberry pests and determining the duration of ozonation needed to cause mortality of pathogens. This study did not show that ozone has a negative effect on gray mold. It is believed that this is precisely because the strawberries were stored in conditions favorable for the development of gray mold. Ozonation was not carried out under ideal conditions, and for more precise results it is necessary to repeat it.

The most common way to create ozone in industry and households is to drain the electric air. It is used air. It is a relatively cheaper method and can get 3 to 6% ozone (Law and Kiss, 1991). In contact with air, it is broken down into two atomic oxygen (O2), in 20 to 50 minutes (Msayleb, 2015). In 1982. ozone was categorized as generally safe for use by the U.S. Environmental Protection Agency (EPA), and since 2001 the U.S. Food and Drug Administration (FDA) has recognized it as a secondary direct food additive and it is used in the meat industry (Sopher et al., 2002).

The texture of strawberries, in addition to organoleptic properties and biological value, is one of the main attributes that determines the suitability of the fruit for consumption and can also be an initial marker that illustrates the microbiological and biochemical changes that occur in the fruit during storage. Fruits that are too soft are characterized by a high sensitivity to mechanical damage, which contributes to the increased multiplication of microorganisms that cause spoilage and rotting of fruits, thereby significantly shortening the period of safe storage. Moreover, consumers negatively perceive fruits that are too soft and discourage buying and consumption (Contigiani et al., 2020). Because of that it is very important to extend the life of stored strawberries.

In agriculture, the application of ozone is partially neglected due to the lack of knowledge about the specifics of ozone chemistry and its potential to combat pests. However, it is used as an alternative in cereal warehouses to control pests (Sopher et, al., 2002). In practice, it is used in several ways, some of them are using a dorsal sprayer, an atomizer, an ozonator etc.

The application of ozone is an alternative to traditional insecticides and fungicides as a biofumigant against several pests, microorganisms, and mycotoxins, and is certainly a method that has a future in plant protection, but the question is whether it also acts on the mold *B. cinarea.* Since there are not many answers to the question of whether ozone works on strawberries, new ozoning techniques must be developed. Yasse et al. (2012) argues that the use of high concentrations in a short period of time is essential and could result in a more lasting response of the fruit. Piechowiak et al. (2022) realized that the process of ozonation significantly prevented the loss of texture of the fruit during storage at room temperature. The positive effect of ozone was directly related to the inhibition of the activity of enzymes involved in the degradation of the walls of fruit cells.

Similarly to our results, Perez et al. (1999) also found that ozone had no effect on the amount of mold after three days of strawberry storage. The appearance of strawberries was maintained at acceptable levels until the 8th day of storage while their taste was maintained at acceptable levels until the 12th day of storage. In this experiment, after six days of storage, there were still containers without the development of gray mold with acceptable fruits for consumption, but after nine days of storage, gray mold was present in each bowl and no fruit was acceptable for consumption. Based on the general appearance, which is the dominant criterion for determining the selection of products as consumable, the application of heat treatment, ozone at 0.5 ppm and ozone at 0.5 ppm plus heat treatment contributed to the extension of the strawberry shelf life to 10 days, while the shelf for unprocessed strawberries was eight days. On the contrary, the application of ozone to 1.0 ppm, 1.5 ppm, 1.0 ppm plus heat treatment and 1.5 ppm plus heat treatment reduced the shelf life of the product to six days (Perez et al., 1999). From this we can conclude that lower concentrations of ozone have better influence on the strawberry shelf life. However, ozonation cannot prevent the appearance of a moldy smell on the last day of storage.

According to Nadas et al. (2003) *Botrytis cinerea* in the presence of ozone grew more slowly. Strawberry fruits (*Fragaria × ananassa* cv. *Camarosa*) were stored for three days at 2°C in the air with or without ozone of 1.5 µL/L, and then transferred to room temperature. Visible mycelial growth developed faster on fruits previously stored in the air. The ozone-enriched refrigerated fresher of naturally infected 'Camarosa' fruit reduced the incidence of decay, weight loss and softening of the fruit, but resulted in a reversible loss of fruit aroma. Here we can again conclude that the temperature was crucial. According to Hilderbrand et al. (2001), the effectiveness of ozone was able to significantly reduce the CFU of fungi and bacteria, but ozone did not affect *Botrytis cinarea*. Contigiani et al. (2020) managed to make ozone treatments delay the onset of *B. cinarea* infection by four days. In that experiment the strawberries were stored at 5±1°C all the time, which differs from our experimental conditions. Research by Piechowiak et al. (2022) showed that the process of cyclic ozonation of strawberry fruits with gas at a concentration of 10 and 100 ppm contributes to the improvement of texture parameters during storage at room temperature. Moreover, fruits ozonated with lower concentration gas have been shown to have better mechanical parameters as in the studies shown so far. Zhou et al. (2014) indicate that one of the reasons for the deterioration of the strength of the fruit is the loss of antioxidant properties and the appearance of oxidative stress in the plant cell.

After all, today it has been verified that the use of ozone to extend the shelf life of various agricultural products is a promising system, not only because of its high efficiency, but also because of its low cost and ease of use. Strawberry fruits are very perishable and need special attention but unfortunately in this study the use of ozone has not proven to be effective. As a potential problem, it is considered that the purchased strawberries were not of superior quality and completely fresh, the application of non-satisfactory ozone concentrations, the wrong storage temperature and storage conditions. Even a small extension of the shelf life of this perishable product can have a positive impact on its commercial value.

6. Conclusions

1. The ozonation process did not prevent the loss of texture of the fruit during storage at room temperature.
2. Ozone in this experiment did not have a detrimental effect on combating strawberry pests.
3. For the species *Botrytis cinarea*, further studies should be carried out with altered doses of ozone and ozonation times. Storage temperature should be reduced in order to be able to see the efficiency of ozone.
4. No differences have been proven with regard to the time of exposure of ozone or the amount of ozone applied.
5. It is necessary to conduct a new study, with controlled atmosphere during ozonation and subsequent fruit storage.

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