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FIRST EXPERIENCE WITH OZONE USED IN FRUIT TREE STORAGE

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Abstract

In the present study we investigated the possibility to use air ozone in fruit tree storage. The variables were: rate of ozonization, length of exposure to ozonized air and storage temperature. We assessed the impact of ozone on the vitality of the trees or bench grafted rootstocks, any changes appearing after storage and the feasibility of this technique under real conditions. Supplementary research was done to test the impact of ozonization on the potential occurrence of microorganisms and pests on trees. Results showed that trees can withstand relatively high rates of ozonization except for peach which is the most sensitive fruit; all the ozonization treatments impaired peach vitality. Other species withstand three months of storage in 1 °C and one hour of exposure to ozone in fortnight intervals without a significant impact on their vitality. A higher (5 °C) storage temperature with or without ozonization decreased the vitality of trees. Although it has been proved that ozonization can suppress damage-causing microorganisms (bacteria and fungi) on trees, the fortnight frequency of ozonization did not prevent mould to develop. Results also indicated the potential effect of ozonization on the disinfestation of plant material from overwintering pests. The main operation restrictions of ozone exposure is the ability of ozone to decompose some materials and that it is harmful to human health.

Keywords: fruit tree, nursery, store, ozone, fungi, cold room, pest, disease

INTRODUCTION

When young fruit trees are lifted from the nursery they must be stored until planting. Previously the trees were stored outdoors with the roots buried in some substrate. Nowadays however more and more trees, as well as rootstocks and hand-grafted plants are stored in cold rooms, usually the only way of storage in big nurseries. It makes handling the plants easier and it is less labour- and space-demanding. Moreover, it avoids potential damages caused by severe frosts and allows handling the plants throughout the storage season. The most common way is storage in cold rooms with temperatures just above 0 °C and high air humidity (Webster and Wertheim, 2005). Cold rooms are equipped with humidifiers keeping air humidity close to 100% or

built in a way that reduces losses of air humidity. These conditions allow to store the trees for many months, nevertheless, there is still danger of losses caused by fungi and bacteria which can develop and propagate in these conditions (Traquair and White, 1992). To reduce pathogen development the trees are sometimes treated with pesticides in the nursery or just before they are placed into the cold room. Despite these treatments diseases still develop, especially in the inner parts of storing pallets where ventilation is poor. Nursery men are looking for a solution to this problem with regard to potential losses but also due to the restricted use of pesticides. The problems are similar when storing fruits and vegetables. A possible solution is the use of air ozone or ozonized water. It is a method which has been increasingly studied in the past years (Palou *et al.*, 2001; Palou *et al.*, 2002; Minas *et al.*, 2010; Carletti *et al.*, 2013; Antos *et al.*, 2018). Ozonization was also tested as insect control, for example on grain (McDonough *et al.*, 2011, Hansen *et al.*, 2012, Jian *et al.*, 2013, Isikber *et al.*, 2015) or dates (Al-Ahmadi *et al.*, 2009). The main advantage is that ozone exposure does not leave any residues and could reduce the use of pesticides. Nonetheless, higher concentrations of ozone are injurious to life tissues and to humans. Before its introduction to praxis it is necessary to learn how to use it effectively and safely. There is no evidence for the use of ozone in fruit nursery storage. The objective of this research was to find out whether stored trees can be exposed to ozone and to answer the following questions: Will ozonization have a negative impact on the vitality of trees? Will it be effective against surface microorganisms and will it suppress their development? Will ozonization suppress overwintering pests? Are there any operation problems with ozonization?

MATERIALS AND METHODS

Research was conducted at the Research and Breeding Institute of Pomology Holovousy Ltd., Czech Republic, in 2018. Two ozonization experiments were conducted. The first with nursery trees and the other with bench (hand) grafted rootstocks. The first experiment included research on the impact of ozonization on microorganisms and tests on the effect of ozonization on overwintering pests. The other experiment was focused on ozone exposure of bench grafted rootstocks.

Experiment 1
Ozonization of trees

Young one and two year old trees of 7 fruit species were used for the experiment with 10 different treatments of 50 trees each described in Tab. I.

The trees were lifted from the commercial nursery in November and stored in a cold room at a temperature of 1 °C until used for the treatments. The trees were not treated with a fungicide before storage. The trial was launched on 9/1/2018 when the treatment began. In the first treatment (Tab. II) the trees were stored outdoors in natural conditions with roots buried in the soil. The trees in the other treatments (2–10) were stored as follows: they were placed into big plastic foil bags which were closed (not hermetically) and placed into fruit palboxes to keep the trees in standing position and for easy handling. The main reason was to keep high humidity close to 100%; at the bottom of the bag was a shallow layer of water. The variables in treatments 2–10 were the following: storage temperature was either 1 °C or 5 °C, the trees were ozonized by the air either

I: *Overview of the species, cultivars, rootstocks, age and number of trees per treatment used for the trial*

Species	Cultivar	Rootstock	Age of trees	Trees per treatment
Apple (*Malus domestica* Borkh.)	'Galaval'	M9	Two year old	10
Pear (*Pyrus communis* L.)	'Beurré Bosc'	MA (+interstem)	Two year old	5
Sweet cherry (*Prunus avium* L.)	'Kordia'	Gisela 5	One year old	10
Sour cherry (*Prunus cerasus* L.)	'Fanal'	mazzard	One year old	5
Plum (*Prunus domestica* L.)	'Toptaste'	St. Jul. A	One year old	5
Peach (*Prunus persica* (L.) Batsch)	'Benedicte'	St. Jul. A	One year old	5
Apricot (*Prunus armeniaca* L.)	'Anegat'	St. Jul. A	One year old	10

in a whole cold room (lower concentrations) or ozonized directly into closed bags (higher concentrations) and the duration of ozonization in hours differed. The descriptions of individual treatments are summarized in Tab. II. The trees were stored in various conditions from 9/1/2018 to 9/4/2018 and then planted outdoors. The last ozonization was done on 23/3/2018. Plants were exposed to ozone once a fortnight in two ways, i.e. ozonization of the whole cold room (volume 320 m^3) when the bags with trees were temporarily opened to reach a lower concentration of ozone and ozonization of the trees in closed bags of a volume of ca 1 m^3 where the final concentrations of ozone were higher. The ozonizer Annihilator 10000 (manufactured by Ozontech, s.r.o., Zlín, Czech Republic) for generation of ozonized air was used. The declared production is 10 g of ozone per hour. The maximal output was 10 l/min of ozonized air and concentration of 12 840 ppm. Since the made concentrations were higher than the available sensors allowed to measure, only theoretical concentrations were calculated. The exact concentrations of ozone were not measured.

When the trees of treatments 3–5 and 7 were ozonized they were transported to the cold room where the temperature was 1 °C and the bags with the trees were opened to allow exchange with the air in the ozonized cold room. The cold room was ozonized by blowing ozonized air right in front of the cooling evaporator with the fan close to the roof of the room. The fan was turned on for the whole time of ozonization to circulate and mix the air in the cooling room. The ozonizer started at the same time as when the bags were opened and ozonization ran the whole time. It means that the concentration of ozone increased during the treatment from zero to the final concentration and was not constant. Theoretical concentrations are given in Tab. III. When the bag was taken out of the ozonized cold room it was left open to air out for an hour and then closed and put back to the cold room with ambient atmosphere. Treatments 8-10 were also transported to the separate room at 1 °C but the ozonized air was blown directly from the ozonizer into the closed bags. Before the start of ozonization we placed a small but strong electric fan into the bags which was turned on periodically to mix the air in the bag. The concentration increased during the treatment. The bags were opened immediately after ozonization for one hour to air out and then closed again and put back to the cold room into ambient atmosphere.

At the end of the treatment period (9/4) the trees were taken out of the bags and visually assessed for the presence of fungi or other characteristics possibly induced by the treatments. The colour (green or brown) of the small wounds made on twigs was assessed visually to check the viability of inner tissues and the presence of callus tissues was assessed too. Then the trees were planted

II: *Overview of the used treatments*

Nr.	Treatment	Description
1.	O	The trees were placed outdoors (O) in the basement and the roots were buried in the soil, the old traditional way of storage.
2.	C1°	The trees were stored in cold room (C) at 1 °C in ambient atmosphere. Standard storing set as the control.
3.	C1°2H	The trees were stored as C1° but once a fortnight placed for 2 hours (H) in the cold room which was ozonized and the bag was opened.
4.	C1°4H	The trees were stored as C1° but once a fortnight placed for 4 hours in the cold room which was ozonized and the bag was opened.
5.	C1°8H	The trees were stored as C1° but once a fortnight placed for 8 hours in the cold room which was ozonized and the bag was opened.
6.	C5°	The trees were stored in the cold room at 5 °C in ambient atmosphere.
7.	C5°8H	The trees were stored as C5° but once a fortnight placed for 8 hours in the cold room (1 °C) which was ozonized and the bag was opened.
8.	D1H	The trees were stored as C1° but once a fortnight the closed bag was directly (D) ozonized by the air from the ozonizer for 1 hour.
9.	D6H	The trees were stored as C1° but once a fortnight the closed bag was directly ozonized by the air from the ozonizer for 6 hours.
10.	D12H	The trees were stored as C1° but once a fortnight the closed bag was directly ozonized by the air from the ozonizer for 12 hours.

III: *Theoretical concentrations of ozone in the box or bag in various treatments at the end of ozonization*

Treatment	Concentration of ozone (mg/kg)
C1°2H (box)	21.7
C1°4H (box)	32.9
C1°8H + C5°8H (box)	41.7
D1H (bag)	3 726.2
D6H (bag)	9 379.4
D12H (bag)	9 948.6

outdoors and pruned as usual after planting and drip irrigated as needed. At the end of the season we assessed the growth of the trees and sorted them into three classes: Normal – when the growth of the tree had no significant problems, Poor – when some parts of the tree died or did not sprout or the growth was otherwise unacceptable, Dead – trees that did not grow. The proportions of the trees (all species together) in each class out of the total amount of trees were compared among the treatments using the Pearson $\chi 2$ test. Since the number of trees of each species in each treatment was low (5 or 10) the information value of the statistical analysis was very low and was inapplicable.

Microbiological analysis

Twigs of fruit trees from the control treatment C1° were selected for microbiological analysis to test the impact of ozonization on fungi and bacteria present on the trees. Samples of twigs from all the fruit species were taken twice, at the beginning and in mid-term of the experiment. These samples were not surface-sterilized, thus our analyses included both epiphytes and endophytes. Twigs of fruit trees were collected and then cut into segments (approx. 10 × 10 mm). The twig segments (10 g) were placed into Erlenmeyer flasks with 50 ml sterile dH_2O and shaken for 1 hour; this was repeated 3 times. The suspension of twig segments was diluted 100 and 1000 times (10^{-2} and 10^{-3}) and 100 µl of the suspension was plated on potato dextrose agar with chloramphenicol (PDA + chloramphenicol; Himedia) and Mueller-Hinton agar (MHA; Himedia). The plates were then placed into the cold storage room (1 °C, RH 95 %) with ozone-enriched atmosphere for 2, 4 and 8 hours at the same time as treatments 3–5 were ozonized. After ozone exposure the plates were incubated at 22 °C for 48 hours and colony-forming units (CFU) were counted. Single colonies of fungi and bacteria were transferred to fresh PDA and MHA and were identified using the molecular method.

Total fungal and bacterial DNA were extracted from pure cultures using the Exgene Plant SV mini kit (GeneAll, Seoul, Korea), according to the manufacturer's instructions. For 16S rRNA analysis, the following primers were used: UniBact16S-F (5´-ATCATGGCTCAGATTGAACGCT-3´) and UniBact16S-R (5´ - CTGCTGCCTCCCGTAGGAGT-3´). For ITS analysis the following primers were used: UniFungiITS-F (5´ - GGTTTCCGTAGGTGAACCTGC-3´) and UniFungiITS-R: (5´ - ACCAAGAGATCCGTTGTTGAAAG - 3´). The PCR reaction was conducted in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) and contained 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.25 µM of the appropriate primer set for detection of fungi or bacteria, and 1U Mol*Taq*DNA polymerase (Molzym, Bremen, Germany.) in a 10x PCR buffer. For amplification of 16S rRNA and ITS gene the following conditions were used: 5 min at 95 °C, and 39 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s 72 °C, followed by final extension for 5 min at 72 °C and 8 °C hold. The PCR products were purified using ExpinTM Combo GP kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions. Sequencing reactions were performed with the Big Dye version 3.1 (Thermo Fisher Scientific) on a genetic analyzer GA3500 (Thermo Fisher Scientific). All sequences were edited using program MEGA 6 (Kumar *et al.*, 2004) and identified by using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/; Altschul *et al.*, 1990).

The data were analysed using an analysis of variance (ANOVA), the group means were compared by Tukey´s HSD test. All analyses were performed with XLStat2006 (Addinsoft, New York, USA).

Overwintering pests

During January 2018, 2–3 year-old apple branches were taken from the experimental orchards of RBIP Holovousy which were naturally infested with Green Apple Aphids (*Aphis pomi*; de Geer, 1773), Red Spider Mites (*Panonychus ulmi*;

Koch, 1836) and San Jose Scales (*Quadraspidiotus perniciosus*; Comstock, 1881) in the phase of overwintering developmental stages. Green Apple Aphids and Red Spider Mites overwinter in the egg stages. The overwintering stage of San Jose Scales is described as nymphs of the first stage in the black circle stage. Infested branches from the orchards were placed in the bag of treatment D12H and together with the trees exposed for 12 hours. At the end of ozonization the branches with the pests were transferred to the laboratory where they were divided into segments.

These segments were then placed on filter paper in Petri dishes. Each segment was bounded with glue to avoid leakage of the hatched individuals; the hatched individuals were counted. Four segments were placed in each Petri dish. For each treatment four dishes with four segments were evaluated, i.e. four replicates. Petri dishes with the tested materials were transferred to a laboratory with controlled temperature of about 23 °C.

In total, three treatments were evaluated (Green Apple Aphids, Red Spider Mites, San Jose Scales) and compared with the untreated control 14 days after the treatment. Live and dead eggs of the Red Spider Mite and Green Apple Aphid were counted. As living individuals were identified hatched mites and aphids. Unhatched eggs were evaluated as dead. For San Jose Scales the hatched larvae and males were counted. The resulting efficiency was determined according to the Abbott's formula (Abbott, 1925).

Experiment 2
Ozonization of bench grafted rootstocks

Bench grafted rootstocks of 2 species were used as experimental material, i.e. apple rootstock M9 grafted with the variety 'Red Jonaprince' and plum rootstocks St. Julien A grafted with the variety 'Valor'. The rootstocks were stored in a commercial nursery cold room at a temperature of around 1 °C before grafting. After they were bench grafted they were divided into five equal groups for future treatment. Each group comprised 60 apple and 60 plum bench grafts. Furthermore, half of the rootstocks in each treatment and species was bounded by Medel film (manufactured by Aglisco, Ltd., Japan) and only a 1 cm tip of the grafts was waxed. The other half was bound by rubber band (Fleicoband, 180 × 6 mm) and the whole graft and upper part of the rootstock was waxed. A mixture of red wax (Avion spol. s.r.o., Židlochovice, Czech Republic) and wax Rebwachs WF (Stahler Agrochemie GmbH + Co, Germany) in a 2 : 1 ratio was used. Both ways of binding are common in commercial nurseries and the objective was to test if the ozonized air caused degradation of the material. The main tested variable was the way the grafted rootstocks were treated at the beginning of storage. As a control served the treatment where the rootstocks were slightly moistened with clean water and put into the tight bag for storage. In the other treatment (CHEM) the rootstocks were soaked in the fungicide solution of Topsin M 500 SC 0,07% + Merpan 80 WG 0,2%, corresponding with common nursery procedure and put into the bag for storage. The other three treatments (10M, 30M, and 60M) differed in the length of exposure. The rootstocks were slightly moistened with clean water and then tightly (but not hermetically) wrapped in plastic foil bags of an approximate volume of 0.08 m^3. The pipe from the ozonizer was inserted directly into the bag. The rootstocks were exposed for 10, 30 or 60 minutes. The same ozonizer with the same concentrations of emitted air as in experiment 1 was used for ozonization. The pipe hole was closed with a tape at the end of ozonization, and the bags were not opened to air out. From the time of grafting (28/2/2018) to the time of planting they were all stored in the same conditions (1 °C and high air humidity). The exposures were conducted on 7/3/2018. Before planting the rootstocks were visually assessed for any changes induced by various treatments. After planting they were assessed for the growth parameters in June. We assessed the number of normally growing plants, the number of plants where the graft was not growing and the number of plants where the graft had fallen out. We did not assess the actual concentration of ozone in the bags during treatment.

RESULTS

Experiment 1
Ozonization of trees

Results of visual control of trees after storage are summarized in Tab. IV. Browning of peach twig tissues was monitored in all the ozonized treatments except C1°2H. The browning was light except for D6H, D12H where it was heavy. There was little mould on roots of all the cold-room stored trees. In treatments D6H and D12H the mould was well developed but only on the bigger pieces of soil and not on the roots. Callus tissues were seen near the buds of pear in treatments ozonized in the whole box and on two treatments stored in bags but not ozonized. There were also calluses on

apples of treatment C1°. Trees of both treatments stored at 5 °C were already sprouting at the end of storage. The roots of sweet cherry and pear in treatment D12H looked bleached but there were no brown tissues.

Assessment of growth is summarized in Tab. V. The proportion of normally growing trees was higher in all treatments stored in the cold room at 1 °C except D6H and D12H. The proportion of normally growing trees was lower in O and C5° but did not differ significantly from the control C1°. The proportion of normally growing trees was significantly lower in C5°8H, D6H and D12H in comparison to the control C1°. The proportion of dead trees was significantly higher in treatments D12H, D6H and O than in all the other treatments. The proportion of poorly growing trees was higher in D12H, D6H and C5°8H. Tab. VI summarizes the proportion of normally growing trees for each species and gives a detailed survey of the main species influenced by various treatments. If we compare the species we see that the lowest proportion of normally growing trees was peach (28 %) and the highest was pear (84 %). The other species averaged between 66 % to 79 %. Treatments D6H and D12H had the lowest proportion of normally growing trees. The exception was apple and sour cherry trees

IV: *Visual assessment of the colour of tissues, fungi (mould) development on the surface of the trees and occurrence of callus tissues after storage*

Treatment	Brown twig tissue	Fungi (mould)	Callus tissues	Note
O	none	none	none	-
C1°	none	only little on roots	on pear and apple	-
C1°2H	none	only little on roots	slightly on pear	-
C1°4H + C1°8H	slightly on peach	only little on roots	slightly on pear	-
C5°	none	only little on roots	slightly on pear	already sprouting
C5°8H	slightly on peach	only little on roots	slightly on pear	already sprouting
D1H	slightly on peach	only little on roots	none	-
D6H	on peach	only on pieces of soil on roots more than D1H	none	-
D12H	on peach	only on pieces of soil on roots, more than D6H	none	root tissues of sour cherry and pear looked bleached

V: *Percentage of trees in three classes of growth after planting. It includes all the trees of all species together in each treatment*

Treatment	Growth		
	Normal	Poor	Dead
O	78 bc	6 b	16 c
C1°	90 ab	10b	0 d
C1°2H	90 ab	8 b	2 d
C1°4H	88 ab	10 b	2 d
C1°8H	94 a	6 b	0 d
C5°	78 bc	18 ab	4 d
C5°8H	68 c	30 a	4 d
D1H	90 ab	8 b	2 d
D6H	26 d	32 a	42 b
D12H	4 e	28 a	68 a

Different letters represent significant differences among the treatments using Pearson $\chi 2$ test at the statistical significance level 0.05 for single class separately.

VI: *Percentage of normally growing trees in various treatments for all species separately*

Treatment	Apple	Pear	Plum	Apricot	Peach	Sweet cherry	Sour cherry
O	20	100	100	100	60	90	100
C1°	100	100	100	100	80	100	20
C1°2H	90	100	100	100	20	100	100
C1°4H	100	100	100	100	0	100	80
C1°8H	100	100	100	100	40	100	100
C5°	80	100	60	80	40	100	60
C5°8H	60	100	100	80	20	50	80
D1H	100	100	100	100	20	90	100
D6H	80	20	0	30	0	0	20
D12H	0	20	20	0	0	0	0
Average	73	84	78	79	28	73	66

in treatments O and C1° respectively, with only 20 % of normally growing trees. Normal growth of peach trees was higher only in C1° (80 %) and O (60 %) but in the other treatments the proportion was lower than 40 %.

Microbiological analysis

The isolated bacterial and fungal taxa belonged to different genera and included common and potentially pathogenic species. Nine bacterial genera were obtained from samples of fruit twig suspensions. The 16S rRNA gene sequence analysis of the isolated bacterial colonies showed identity with the genera *Acidovorax*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Herbaspirillum*, *Massilia*, *Pseudomonas* and *Sphingomonas*. More than 20 fungal genera were obtained. The ITS gene sequence analysis of the isolated fungal colonies showed the most frequent genera such as *Cladosporium* sp., *Didymella* sp., *Penicillium* sp., *Fusarium* sp., *Boeremia* sp., *Alternaria* sp., *Trichoderma* sp., *Aureobasidium* sp., *Epicoccum* sp., *Mucor* sp., *Rhizopus* sp., *Aspergilus* sp., *Phoma* sp., *Cadophora* sp., *Mortierella* sp., *Truncatella* sp., *Clonostachys* sp. and *Coniothyrium* sp.

Analysis of variance ($F_{1,19} = 63$, $P < 0{,}0000$) showed significant differences between the two variables (ozone treatment: $F = 22$; $P<0{,}0000$; microorganisms: $F = 6$, $P < 0{,}02$). The CFU of microorganisms was the highest in the untreated control. In general, the CFU of microorganisms decreased depending on the length of exposure to ozone. The CFU of bacteria was higher than the CFU of fungi and yeast in the untreated control and in the ozone treatment (2 h exposure to ozone). The CFU of bacteria was lower than the CFU of fungi and yeast in the ozone treatment (4 and 8 h exposure to ozone, respectively) (Tab. VII).

VII: *Effect of ozone treatment on the number of bacteria, fungi and yeast CFU*

Treatment		Microorganisms		
		Bacteria (CFU x 10^6/g)	**Fungi and Yeast (CFU x 10^6/g)**	**Average [a]**
Control		10.53 ± 0.45 Aa	3.91 ± 0.99 Ab	7.22
Ozone treated	2 h	2.26 ± 1.16 Ba	1.31 ± 0.47 Bb	1.78
	4 h	0.76 ± 0.44 Bb	1.25 ± 0.44 Ba	1.00
	8 h	0.41 ± 0.14 Bb	0.74 ± 0.41 Ba	0.57

CFU of bacteria, fungi and yeast were counted after 48 h at 25 °C. Data represent the mean of three replicates ± standard deviation. For each attribute the mean values with the same lowercase letters among microorganisms in the same row are not significantly different at a 0,05 statistical significance level of probability (Tukey HSD test) and with the same uppercase letters among treatments in the same column are not significantly different.
[a] Mean of row data

Overwintering pests

The results of the experiment are summarized in Fig. 1. The efficiency on eggs of Green Apple Aphids is 99 %. Only one individual hatched from the treated eggs, whereas 117 individuals hatched in the untreated control. In contrast to Green Apple Aphids the efficiency on the Red Spider Mite was 0 %. The number of hatched nymphs of the Red Spider Mite exceeded the number of nymphs in the untreated control. A 100 % reduction of San Jose Scale males was monitored, but the effectiveness of ozone on the nymphs was only 42 %.

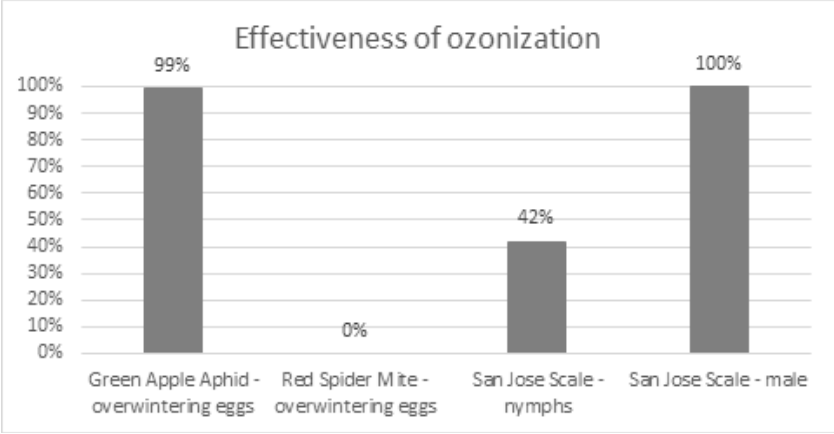
Experiment 2

There were significant differences in the proportions of normally growing bench grafts in apple and plum (Tabs. VIII and IX). In both species the proportion was the lowest in treatments combining 60 minutes and 30 minutes of ozonization bound by rubber band. In apple the 10 minute ozonization of rubber-bound grafts also resulted in a significantly lower number of normally growing grafts (20%). In the other treatments the proportion of normally growing grafts was always higher than 90 % in apple and similar to the untreated control. The proportion of fallen grafts in the ozonized rubber-bound apple bench grafts was significantly higher than in the other treatments. The proportion of plum plants with fallen grafts did not differ significantly. The proportion was the highest (10%) in the rubber-bound bench grafts exposed to ozone for 60 minutes. There were no significant differences in growth between the chemically treated bench-grafts and the control.

DISCUSSION

Visually the impact of ozone exposure on peach tissues was negative with the exception of C1°2H. Further growth of peach was also impaired even in the treatment with no brown tissues. It is obvious that peach is the most sensitive species and the limit (exposure, concentration) that the peach can withstand will be lower than our weakest ozonization treatment. We saw some callus tissues on pear and in one case also on apple. It was in cold room storage without ozonization and in cold room ozonization (C-treatments). It could be ascribed to higher ethylene concentrations in the bags as reported by Maas *et al.* (2008). They tested storage of pear in various conditions and various ethylene concentrations and proved that a higher concentration of ethylene leads to callus tissues and bark lesions which, based on the amount and development, had a negative impact on growth. We did not prove that the occurrence of callus tissues complicated subsequent growth. The trees in our experiment were ventilated at least once a fortnight but longer storage without ventilation could increase the ethylene concentrations. Maas *et al.* (2008) suggested to use the 1-MCP ethylene antagonist as a possible solution. The ozone is able to decompose organic substances and could possibly have a positive influence on the reduction of ethylene concentrations in storage. Nevertheless, for this we have no evidence and it will have to be proved by future research. Concerning the impact of exposures on growth we can see that there were no problems with storage at 1 °C and exposure ozone in the cold room except for peach trees which were negatively affected by all the ozonization treatments. Even the direct (into bag) ozonization for one hour did not negatively affect the growth of all the species except for peach. It is obvious that with the exception of peach all the species can withstand a wide range of concentrations, exposures and frequencies without decreasing the vitality. However, treatments D6H and D12H created conditions that the trees cannot withstand. This information is important because to a greater extent it allows further testing of lower concentrations in semi-operation experiments with higher confidence that the tree will not be affected. The use of a higher temperature (5 °C) for 3 months either with or without ozone exposure impaired subsequent growth of trees and cannot be recommended. We reported a worse vitality of apple trees stored outdoors but for this we have no clear explanation.

In previous research the ozone concentrations for fruit storage were usually lower (Palou *et al.*, 2002; Minas *et al.*, 2010; Antos *et al.*, 2018) and their effectiveness in terms of microorganism development differed. Our results also proved that ozone is able to suppress the occurrence of microorganisms on the trees. In their study Palou *et al.* (2001) confirmed the synergistic effect of a combination of gaseous ozone and cold storage resulting in the reduction of sporulation and growth of *Penicillium* sp., which we also isolated in our experiment. However this effect was observed only during exposure to ozone; when the infected fruit was transferred to a normal environment, sporulation was restored. A similar finding was confirmed for other pathogens causing fruit rot diseases such as *Monilinia fructicola*, *Botrytis cinerea*, *Mucor piriformis* or *Penicillium expansum*



1: *Results of ozone effectiveness on selected overwintering pests of fruit crops*

VIII: *Growth of bench grafts of the variety ´Red Jonaprince´/M9 in various treatments*

Treatment	Binding material	Normal growth	Not growing grafts	Fallen grafts
60M	rubber	0.0 c	6.7 b	93.3 a
30M	rubber	10.0bc	13.3 ab	76.7 a
10M	rubber	20.0b	33.3 a	46.7b
10M	Medel film	90.0a	6.7bc	3.3c
60M	Medel film	93.3 a	0.0c	6.7c
CHEM	Medel film	93.3a	6.7 bc	0.0c
Control	Medel film	93.3 a	6.7 bc	0.0 c
CHEM	rubber	96.6 a	3.3 bc	0.0 c
Control	rubber	96.6 a	3.3 bc	0.0 c
30M	Medel film	100.0 a	0.0 c	0.0 c

Different letters represent significant differences among the treatments using Pearson χ2 test at the statistical significance level 0.05 for single class separately.

IX: *Growth of bench grafts of the variety 'Valor'/St. Julien A in various treatments*

Treatment	Binding material	Normal growth	Not growing grafts	Fallen grafts
60M	rubber	36.7 c	53.3 ab	10.0 a
30M	rubber	40.0 c	60.0 a	0.0 a
60M	Medelfilm	66.7 b	33.3 bc	0.0 a
10M	rubber	86.7 ab	13.3 cd	0.0 a
CHEM	Medelfilm	86.7 ab	13.3 cd	0.0 a
Control	rubber	90.0 a	10.0 d	0.0 a
10M	Medelfilm	93.3 a	3.3 d	3.3 a
Control	Medel film	93.3 a	6.7 d	0.0 a
30M	Medel film	96.7 a	3.3 d	0.0 a
CHEM	rubber	96.7 a	3.3 d	0.0 a

Different letters represent significant differences among the treatments using Pearson χ2 test at the statistical significance level 0.05 for single class separately.

(Palou *et al.*, 2002). We used periodical treatment with concentrations higher than used previously for fruits. It seems that the longer interval between treatments is not optimal because mould developed. Even though no mould was visible on the upper parts of the trees there was always some mould on roots or on the soil particles. We actually discovered mould 14 days after the last ozonization. The problem of ozone exposure to disinfect the planting material is the complicated approach of ozone to bigger soil particles and plant organs, more particularly to the roots when covered with soil. Direct gas contact is necessary to eliminate the pathogens. The sensitivity of pathogens to ozone may vary depending on the fungal species, spore sensitivity, spore morphology, substrate, humidity and rates of ozone. It was also found out that if the fungus structures are located inside the infected fruit, they are protected and able to survive ozone exposure (Palou *et al.*, 2001). This means that if the fruit trees are already infected by a disease, which had penetrated into the plant tissue or the soil particles are bigger, ozone exposure cannot sufficiently protect the tree against the spreading of the infection. Although pure cultures of microbial pathogens can be eliminated by exposure to low rates of gas ozone, it does not mean that viruses or permanent structures of pathogens such as spores and cysts can be eradicated too (Carletti *et al.*, 2013). Based on the above mentioned, we think that lower concentrations and more frequent or longer or permanent exposures will be more effective and also better applicable with affordable control sensors. Nevertheless, permanent ozonization with lower concentrations must be tested before its introduction to fruit tree storage. Since ozone can decompose some fungicides (Antos *et al.*, 2018) the combination of fungicide treatment before storage and ozone exposure during storage may be contra-productive.

We used low concentrations of ozone in preliminary tests against overwintering pests but without success (data not published). Based on that it was decided to test higher rates and expose the infested branches to the highest rates of ozone of treatment D12H. We partially confirmed the results of previous works (Al Ahmadi *et al.*, 2009; McDonough *et al.*, 2011; Hansen *et al.*, 2012; Jian *et al.*, 2013; Isekbera *et al.*, 2015) that ozonization could be used to reduce some pests in our case the overwintering stages. It is obvious that higher the concentrations which we used and which decreased the vitality in several expositions cannot be applied in storage in cold rooms. Yet it could be utilized as a single treatment for smaller amounts of propagating material, e.g. for sanitation of grafts which are often transported worldwide. More detailed and better monitored research will be necessary to determine how the individual overwintering pests can be effectively treated.

As regards the use of ozonization for bench grafted rootstocks we have proved that 30 minutes of direct (into bag) ozonization of Medel film-bound plants did not decrease their vitality and represents an optimal solution, though the vitality of the same bench grafts treated for 60 minutes did somewhat decreased. The use of the rubber band is impossible because it is decomposed by ozone even when covered by grafting wax. There were no other crucial complications connected with the use of ozone in fruit tree storage. The only issue is the safe use of ozone because in higher concentrations it is harmful to human health. The safe rate depends on the length of exposure and concentration and it is stipulated by the law of each country.

CONCLUSION

Trees can withstand relatively high rates of ozone exposure except for peach trees which are the most sensitive. Ozonization can control microorganisms (bacteria and fungi) causing potential damage, nevertheless, the fortnight frequency of ozonization did not prevent the development of mould. Longer, more often or permanent exposure to lower concentrations and exactly known concentrations seems to be prospective and applicable against microorganism development and possibly a better option for storage of trees. Ozone exposure has the potential to disinfest plant material from overwintering pests but it will be necessary to focus on a more precise determination of the concentration of ozone that does not damage the plant material but at the same time will be effective in reducing various pests. The main operation restrictions of ozonization are health and safety issues of operation and the ability of ozone to decompose some materials. The use of ozonization seems to be a promising method in fruit tree storage.

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