ISSN on-line 1592-1573
ISSN print 0394-6169

AHS

Advances in Horticultural Science

Vol. 37 - n. 3, 2023

FUP
FIRENZE
UNIVERSITY
PRESS

Advances in Horticultural Science
Published by **Firenze University Press** - University of Florence, Italy
Via Cittadella, 7 - 50144 Florence - Italy
http://www.fupress.com/ahs

Direttore Responsabile: **Francesco Ferrini**, University of Florence, Italy.

ADVANCES IN HORTICULTURAL SCIENCE

Formerly Rivista dell'Ortoflorofrutticoltura Italiana
founded in 1876 and issued by University of Florence, Italy

Advances in Horticultural Science is covered in the following indexing and abstracting services:

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Advances in Horticultural Science is published by the Department of Agri-Food Production and Environmental Sciences, University of Florence, Viale delle Idee, 30, 50019 Sesto Fiorentino (FI), Italy
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Adv. Hort. Sci., 2023 37(3): 243-253 DOI: 10.36253/ahsc-13895

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In vitro propagation and microtuberization of potato (*Solanum tuberosum* L.) Spunta variety in Lebanon

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Citation:
DALLEH M., BORJAC J., YOUNES G., CHOUEIRI E., CHEHADE A., ELBITAR A., 2023 - *In vitro propagation and microtuberization of potato (Solanum tuberosum L.) spunta variety in Lebanon.* - Adv. Hort. Sci., 37(3): 243-253.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 25 October 2022
Accepted for publication 30 August 2023

Key words: *In vitro* culture, microtubers, shootlets proliferation, *Solanum tuberosum* L., Spunta.

Abstract: One of the factors that causes low productivity of potatoes in Lebanon is the limited availability of certified seeds. The aim of this study was to establish a rapid protocol for *in vitro* propagation and microtuberization of potato (*Solanum tuberosum* L.) of Spunta variety. Meristems culture associated to thermotherapy (one month/37°C) constituted the first step. The highest percentage of reactive meristem (92%) was observed on MS medium devoid of growth regulators while MS medium containing Kin 0.4 mg.l^{-1}, GA3 0.5 mg.l^{-1} and IBA 0.5 mg.l^{-1} yielded the highest average number of shootlets (7.8) in the seventh subculture. The lowest number of days obtained for microtuber formation was 10 and the highest average number of microtuber (1.49) was obtained with shootlets incubated under C2 culture conditions (16-h day/8-h night for initial 7 days at 25±2°C; for remaining period: continuous dark at 17±2°C). Contrary the highest microtubers average length (10.75 mm), average width (7.41 mm) and average weight (646.26 mg) were produced under C1 culture conditions (16-h day/8-h night at 25±2°C). Medium supplemented with 5 mg.l^{-1} BAP and 6% sucrose presented the highest average number of microtubers of 2.36 and 1.94 respectively. Type and concentration of cytokines and sucrose concentration did not have significant effect on the average length, width and weight of microtubers produced.

1. Introduction

Potato (*Solanum tuberosum* L.) is the most important non-cereal food crop in the world (Bamberg *et al.,* 2016; Nikitin *et al.,* 2018). It is cultivated in about 150 countries (Basera *et al.*, 2018). Potato cultivation is considered as strategic as it occupies the world fourth place in production

after Maize, Wheat, and Rice (FAO, 2019).

In potato production, the quality of seed potatoes planted is an important determinant of the final yield and quality (Roy, 2014). When farmers use their farm-saved seed potatoes for several cropping cycles without renewing the seed lot from a reliable source, seed-borne diseases accumulate and cause severe yield and quality losses (Fuglie, 2007). Potato propagation takes place primarily asexually, through tubers and microtubers (Zhang *et al*., 2006).

Potato microtubers are minute tubers produced through *in vitro* culture technique yielding disease-free and high-quality seeds that can be preserved for a long time (Badoni and Chauhan, 2009). Extensive physiological research has revealed that tuberization is controlled by several factors, such as hormonal combination, ratio of photoperiod**,** nutrient compositions among others (Naresh *et al.,* 2011). Many researchers used different growth regulators for *in vitro* induction of microtubers in potato (Hossain, 2005). Cytokinins, such as benzylaminopurine (BAP), and kinetin (Kin) are among these growth regulators (Al-Safadi *et al.,* 2000; Sarkar *et al.,* 2006; Aksenove *et al.,* 2009). In addition, sucrose, the cheap and safe disaccharide, is considered as a superior agent and a critical stimulus for inducing microtubers (Hussain *et al.,* 2006; Nistor *et al.,* 2010).

In Lebanon, potato production is important for food security as well as a source of revenue in rural areas. It is a strategic crop for Lebanese agriculture, covering about 19,000 ha in the Bekaa plain (MOA, 2012) and with production of approximately 300,000 tons per year constituting the greatest field crop tonnage in Lebanon. It is cultivated mainly in the Bekaa valley (central-eastern Lebanon, 900-1000 m above the sea level, 70% of total area) and in Akkar plain (northern Lebanon, 25-30% of total area) (Choueiri *et al.,* 2017).

Lebanon does not produce certified potatoes' seeds, they are imported mainly from the European Union (EU) Member States. In the early years of the 21st century, Lebanon imported between 15,000 and 20,000 tons of potato seeds each year and relied heavily on this import with prices ranging between $750 and $1,000 per ton (Abou-Jawdah *et al.,* 2001). The absence of a seed certification program, the introduction and exchange of potato seeds of unknown sanitary status and the lack of phytosanitary measures resulted in increased incidence and severity of potatoes' diseases (Abou-Jawdah *et al.,* 2001). To prevent the further spread of these diseases and to avoid the introduction of new pathogens, it is important to reinforce the seed certification scheme that mandates using and trading of only certified potato seeds. Such a scheme will help the Lebanese farmers to improve their production, get involved in a certified seed production program and reduce the incidence of diseases, and thus economical losses. The implementation of a potato seed production program in Lebanon is possible; especially that Lebanon is characterized by a wide range of microclimates favorable to produce them (Abou-Jawdah *et al.,* 2001). In a report published by the International Potato Center (IPC) in 1975, areas in Lebanon including the Laklouk, Daher el Beidar, and Northern Bekaa were listed among the qualified areas for potato seeds production.

Hence, the present study was initiated with the objective to determine optimum concentration of sucrose, the effect of two cytokinins (BAP and Kin) on the microtuberization capacity of the potato cultivar Spunta under different incubation conditions.

2. Materials and Methods

Plant material

The experiment was conducted at the Lebanese Agricultural Research Institute (LARI), Department of Plant Biotechnology. The mother plants used in this study were of Spunta variety. Thermotherapy combined with meristem culture has been successfully established for efficient eradication of the potatoes' viruses. About 10 tubers were subjected to a temperature of 37°C for 50 days, under a photoperiod of 16 hours/day. At the end of the thermal treatment, the sprouts from the treated tubers were separated in portions of 3 cm and surface sterilized using 70% ethanol for 1 min followed by 5% (v/v) sodium hypochlorite containing two drops of Tween-20 for 10 minutes. Finally, the explants sources were washed 4 times with sterile distilled water (10 min each wash) before being used for the collection of meristems.

In vitro propagation

Meristems have been cut with the first pair of leaf primordial and cultivated in 9 cm Petri dishes on 3 MS basal medium (Murashige and Skoog, 1962). P1 medium lacked any additional growth regulators. P2 medium contained Kinetin (Kin), Gibberellic acid (GA3) and indole-3 butyric acid (IBA) at concentra-

tions of 0.2 mg.l^{-1}, 0.5 mg.l^{-1} and 0.5 mg.l^{-1} respectively. P3 medium was supplemented with Kin at 0.4 mg.l^{-1}, GA3 at 0.5 mg.l^{-1} and IBA at 0.5 mgl^{-1}. In addition, to all media, a vitamin mixture consisting of nicotinic acid (5 mg.l^{-1}), ascorbic acid (20 mg.l^{-1}), pyridoxin (5 mg.l^{-1}), thiamin (10 mg.l^{-1}) and myo-inositol (100 mg.l^{-1}) was added. The pH of all media was adjusted to 5.7 prior to the addition of agar (0.7%). The media were autoclaved at 121°C for 20 min. Each medium was prepared in ten replicas with 5 meristems per replica for testing. The *in vitro* cultivated 150 meristems were then shifted to a culture growth room at 25±2°C with 16 h photoperiod under white light intensity of 3000 lux. Thirty days later, survival rate of meristems was recorded. The regenerated shootlets from the meristems were fragmented to obtain uninodal cuttings and transferred onto fresh medium. Subcultures of 30 days interval were re-conducted seven times using the same 3 initiation media i.e., P1, P2 and P3, under the same culture conditions described above with a set of 60 shootlets per medium (10 repetitions of 6 shootlets). The newly regenerated shootlets per explant were recorded by the end of each subculture to calculate the multiplication rate as follows:

Multiplication rate= Number of new shootlets/Number of initial shootlets

Sanitary control

To check the sanitary status of the shootlets that arose from the thermotherapy-treated meristems, the Tissue-Blot Immunoassay (TBIA), a reliable, routine and cost-efficient serological test that allow processing of large numbers of individual plant samples serological test and that is commonly used to detect plant viruses in field and vegetable crops (Makkouk and Kumari, 1996) was performed to detect six main potato viruses PVA, PVX, PVY, PVM, PVS and PLRV. In addition, all samples were tested by the double-antibody sandwich enzyme-linked immunosorbant assay (DAS - ELISA, Loewe, Germany) using specific antibodies (Clark and Adams, 1977). All shootlets resulting from the meristems culture were subjected to the sanitary control. Each shootlet was numbered and divided into 2 parts, the first part was sent to the plant protection laboratory at LARI to be analyzed, while the second part of each plant remained in the culture medium pending the sanitary control results.

Pre-tuberization

Shootlets from the fifth subculture were aseptically transferred into culture-tubes containing 10 ml of pre-tuberization medium (P1). Cultures were grown for 20 days in culture growth room at 25±2°C and 16 h photoperiod under white light intensity of 3000 lux.

Tuberization

Eight MS Liquid media with two sucrose concentrations 6% and 8% respectively, supplemented with 2 cytokinins, Kin (0, 2 and 4 mg.l^{-1}) or BAP (0 and 5 mg.l^{-1}) or their combinations were used as an inducing medium for microtuber production. Ten ml of each liquid medium were added to the tubes containing the pre-tuberization MS solid medium after 20 days of culture. For each culture medium, two culture conditions were studied. In the first culture condition, the incubation was in culture growth room for 16-h day, 8-h night at 25±2°C and 3000 lux for 60 days. In the second condition, an initial incubation at 16-h day, 8-h night at 25±2°C and 3000 lux, for 7 days followed by continuous dark at 19±2°C to 60 days. Conditions are summarized in Table 1. For each culture medium and condition, 32 replicas were prepared. Microtubers produced were harvested at day 60. Data were recorded on days to microtubers formation, average number, average length, average width and average weight of microtubers.

Statistical analysis

Means ± standard deviations were recorded for each step of the propagation protocol and analyzed by using standard analysis of variance (ANOVA). Duncan's multiple range test was used to show differences among the treatments' means. All statistical analyses were performed using SAS for Windows (SAS Institute Inc., 1995).

3. Results

In vitro propagation

The survival rate of meristems was recorded after 30 days. The highest percentage of reactive meristem was formed on P1 medium that is devoid of growth regulators with an average of 920±4.8%, whereas a significant decrease of surviving reactive explants of 74±5.8% and 58±5.0% ($p<0.05$) was obtained on P2 and P3 media respectively as shown in figure 1 and 2 (A-C). No significant changes in percent of reactive meristems was observed between P2 and P3 media.

The effect of growth regulators added in the culture medium is summarized in Table 2. Medium P3

Table 1 - Culture media and culture conditions tested for *in vitro* microtubers production of potato

Medium	Medium composition	Culture condition C1
M1	20 ml of MS solid medium with 6% sucrose + 0 Hormones	16-h day, 8-h night at 25±2°C
M2	20 ml of solid MS medium with 8% sucrose + 0 Hormones	16-h day, 8-h night at 25±2°C
M3	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M1 medium + 5 mg.l^{-1} BAP	16-h day, 8-h night at 25±2°C
M4	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M2 medium + 5 mg.l^{-1}BAP	16-h day, 8-h night at 25±2°C
M5	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M1 medium + 4 mg.l^{-1} Kin	16-h day, 8-h night at 25±2°C
M6	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M2 medium + 4 mg.l^{-1}Kin	16-h day, 8-h night at 25±2°C
M7	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M1 medium + 5 mg.l^{-1}BAP + 2 mg.l^{-1}Kin	16-h day, 8-h night at 25±2°C
M8	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M2 medium + 5 mg.l^{-1}BAP + 2 mg.l^{-1}Kin	16-h day, 8-h night at 25±2°C
Medium	*Medium composition*	*Culture Condition C2*
M1	20 ml of MS solid medium with 6% sucrose + 0 Hormones	For initial 7 days, 16-h day, 8-h night at 25±2°C, for remaining period continuous dark at 19±2°C
M2	20 ml of solid MS medium with 8% sucrose + 0 Hormones	For initial 7 days, 16-h day, 8-h night at 25±2°C, for remaining period continuous dark at 19±2°C
M3	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M1 medium + 5 mg.l^{-1} BAP	For initial 7 days, 16-h day, 8-h night at 25±2°C, for remaining period continuous dark at 19±2°C
M4	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M2 medium + 5 mg.l^{-1} BAP	For initial 7 days, 16-h day, 8-h night at 25±2°C, for remaining period continuous dark at 19±2°C
M5	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M1 medium + 4 mg.l^{-1} Kin	For initial 7 days, 16-h day, 8-h night at 25±2°C, for remaining period continuous dark at 19±2°C
M6	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M2 medium + 4 mg.l^{-1} Kin	For initial 7 days, 16-h day, 8-h night at 25±2°C, for remaining period continuous dark at 19±2°C
M7	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M1 medium + 5 mg.l^{-1}BAP + 2 mg.l^{-1}Kin	For initial 7 days, 16-h day, 8-h night at 25±2°C, for remaining period continuous dark at 19±2°C
M8	MS solid medium, after 20 days of culture, addition of 20 ml of liquid M2 medium + 5 mg.l^{-1}BAP + 2 mg.l^{-1}Kin	For initial 7 days, 16-h day, 8-h night at 25±2°C, for remaining period continuous dark at 19±2°C

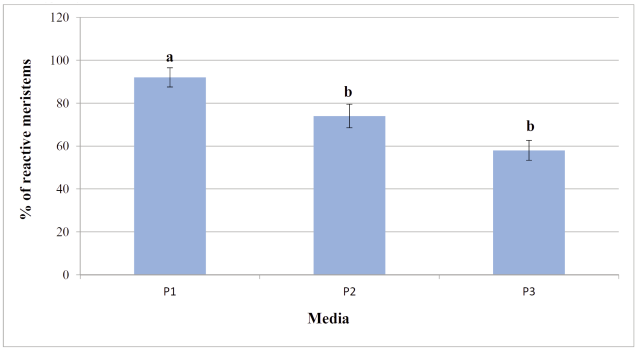

Fig. 1 - Effect of culture medium on the percentage of reactive meristem. Means with the same letter are not significantly different according to Duncan's.

presented the highest multiplication rate of 7.8±1.2 in the seventh subculture. Whereas in medium P2, the best multiplication rate obtained was 6.9±0.7 in the sixth subculture. Illustrations related to shootlets proliferation are presented in figure 2 (D and E).

It is important to note that all shootlets obtained in the meristems' cultures coupled with thermotherapy were free of the tested PVA, PVX, PVY, PVM, PVS and PLRV viruses.

In vitro microtuber formation

In vitro tuberization was obtained after propaga-

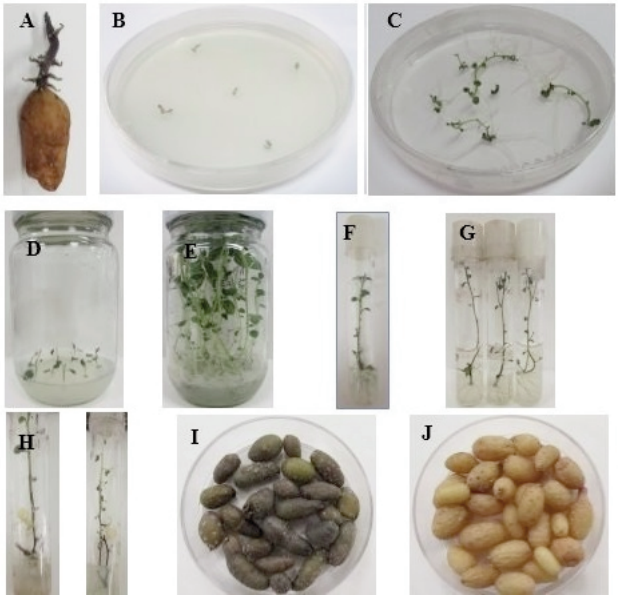

Fig. 2 - Represent the stages of the *in vitro* propagation and microtubers production of a representative under the three pre-tuberization conditions. A= Sprout of the treated tuber. B= Meristem initiation. C= Shootlets elongation. D= Uniodal cutting explants. E= Shootlet proliferation. F= Shootlet on pretuberization medium. G and H = Shootlets on tuberization medium. I= Microtubers produced under culture conditions C1. J= Microtubers produced under culture conditions C2.

Table 2 - Effect of culture media on the average number of shootlets per explant as recorded along the 7 subcultures

Media	Average number of shootlets per explant						
	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Subculture 5	Subculture 6	Subculture 7
P1	0.70±0.3	1.17±0.2	3.87±0.4	4.07±0.5	5.08±0.5	5.4±0.2	4.00±0.6
P2	1.0±0.0	0.9±0.5	2.63±0.7	6.2±0.7	6.07±1.1	6.9±0.7	5.40±0.4
P3	1.2±0.6	1.5±1.0	5.57±0.7	7.33±0.9	6.00±0.2	7.4±0.9	7.80±1.2

P1= medium without any additional growth regulators
P2= medium contained Kinetin (0.2 mg·l^{-1}), Gibberellic acid (0.5 mg·l^{-1}) and indole-3 butyric acid (0.5 mg·l^{-1}).
P3= medium was supplemented with Kinetin at 0.4 mg·l^{-1}, Gibberellic acid at 0.5 mg·l^{-1} and indole-3 butyric acid at 0.5 mg·l^{-1}

tion of the shoots (Fig. 2 E-H). The effect of BAP, Kin, or their combination in the presence of sucrose (6% or 8%) under both culture conditions were studied for microtubers' formation and development. Table 3 summarizes the results of microtubers' formation under all treatments.

Both the cytokinin type and its concentration significantly ($p<0.0001$) affected the average number (AN) of microtubers produced. Media containing BAP alone or in combination with Kin was superior to other media. The highest AN of microtubers per shootlet, i.e. 2.36 and 2.22, were produced when shootlets were propagated on M3 and M7 media with 5 mg.l^{-1}BAP or 5 mg.l^{-1}BAP + 2 mg.l^{-1}Kin respectively under C2 conditions. On the other hand, no statistically significant effect was observed when varying the type nor the concentration of the cytokines on the average length (AL) and average width (AW) or the average weight AWe of microtubers ($p=0.12$, $p=0.44$ and $p=0.39$ respectively) as shown in Table 3.

There is a linear relation between size and weight of microtuber implying that each factor that influenced the microtuber weight directly influenced microtuber size.

Effect of culture condition on microtuber formation

Results showed that the effect of culture condition was highly significant with p value <0.0001 for AN, AL,

Table 3 - Effect of cytokinin type and concentration on the average number (AN), average length (AL), average width (AW) and average weight (Awe) of microtubers produced

Culture media	Culture conditions	Average number of microtubers	Average length of microtubers (mm)	Average width of microtubers (mm)	Average weight (mg)
M1	C1	0.22±0.42 h	8.07 cd	5.05 e	375.3 d
M2	C1	0.78±0.70 fg	11.14 ab	7.28 abc	622.2 abc
M3	C1	1.53±0.62 cd	12.00 a	7.34 abc	673.1 ab
M4	C1	1.44±0.91 cde	10.07 abc	6.90 abcd	593.4 abcd
M5	C1	1.00±0.71 efg	11.8 a	7.96 a	734.5 a
M6	C1	0.94±0.82 fg	10.78 ab	7.23 abc	621.9 abc
M7	C1	1.58±0.95 cd	11.07 ab	7.43 ab	635.4 abc
M8	C1	1.25±0.85 def	11.1 ab	7.75 ab	643.3 abc
M1	C2	0.87±0.50 fg	9.45 bcd	6.8 abcd	535.4 abcd
M2	C2	0.55±0.80 gh	7.24 d	5.00 e	369.1d
M3	C2	2.36 a±1.05	8.89 bcd	5.64 de	473.5 bcd
M4	C2	1.56±0.98 cd	8.84 bcd	5.91 cde	561.5 abcd
M5	C2	1.00±0.65 efg	9.12 bcd	5.86 cde	487 bcd
M6	C2	1.58±1.05 cd	7.85 cd	5.34 e	406.3 cd
M7	C2	2.22±1.15 ab	7.85 cd	5.08 e	416.3 cd
M8	C2	1.80±1.16 bc	9.78 abc	6.26 bcde	600.7 abcd

Means followed by different letters in each column are significantly different for P≤0.05.

AW and AWe of microtubers (Table 4). AN was 1.49 for shootlets incubated under C2 condition and 1.09 for shooltlets cultivated under C1 condition. Values of AL, AW and AWe are 10.75 mm, 7.41 mm and 646.26 mg for microtubers produced under C1 condition and 8.63 mm, 5.65 mm and 481.23 mg for microtubers obtained under C2 condition respectively.

Under C1 culture condition, most of the cultures produced green microtuber, and this could be due to synthesis of the alkaloid solanine (Hoque, 2010). On the other hand, brown-colored tuber was observed in C2 culture condition (Fig. 2, I and J).

Effect of sucrose concentration on microtuber formation

The effect of sucrose on microtuber production is presented in Table 5. Results revealed a highly significant effect of sucrose concentration on the AN of microtubers produced. The media added with 5 mg.l^{-1} BAP or 5 mg.l^{-1} BAP + 2 mg.l^{-1} Kin, both supplemented with 6% of sucrose, presented the highest AN of microtubers, 1.95 and 1.9 respectively. On the other hand, sucrose concentration had no effect on AL, AW and the AWe of microtubers (Table 5).

Time-dependent effect of growth regulators, sucrose concentration and culture condition on microtuber formation

Microtuber formations was followed in a time dependent manner (Fig. 3). Shootlets incubated under C1 conditions in media supplemented with sucrose (6% or 8%) devoid of any growth regulators, i.e. M1 and M2, needed 60 days for microtubers formation as compared to C2 conditions that needed 40 and 45 days respectively. When incubated in the presence of the diverse cytokinins (M3 till M8) under C1 conditions, microtubers' formation was evident at day 30. Shootlets incubated in M3 and M7 media, supplemented with 5 mg.l^{-1} BAP and 5 mg.l^{-1} BAP + 2 mg.l^{-1} Kin respectively, showed the fastest microtubers' formation at day 10. Other cytokinin combinations (M4, M5 and M8) required between 15 to 20 days. This confirm that M3 and M7 combination were the best for microtubers' formation.

Table 4 - Effect of culture condition on the average number (AN), average length (AL), average width (AW) and average weight (Awe) of microtubers produced

Culture conditions	Average number of microtubers	Average length of microtubers (mm)	Average width of microtubers (mm)	Average weight (mg)	Colour of microtuber
C1	1.09 b	10.75 a	7.41 a	646.26 a	Green
C2	1.49 a	8.63 b	5.65 b	481.23 b	Brown

C1 culture conditions= 16-h day, 8-h night (25±2°C),
C2 culture conditions= For Initial 7 days, 16-h day, 8-h night (25±2°C), For remaining period: continuous dark (19±2°C)
Means followed by different letters in each column are significantly different for P≤0.05.

Table 5 - Effect of culture condition on the average number (AN), average length (AL), average width (AW) and average weight (Awe) of microtubers produced

Culture media	Sucrose concentration	Average number of microtubers	Average length of microtubers (mm)	Average width of microtubers (mm)	Average weight (mg)
M1	6%	0.55 d	8.76 a	5.93 a	455.35 a
M2	8%	0.67 d	9.19 a	6.14 a	495.65 a
M3	6%	1.95 a	10.45 a	6.49 a	573.3 a
M4	8%	1.5 b	9.46 a	6.41 a	577.45 a
M5	6%	1 c	10.46 a	6.91 a	610.75 a
M6	8%	1.26 bc	9.32 a	6.29 a	514.1 a
M7	6%	1.9 a	9.46 a	6.26 a	525.85 a
M8	8%	1.53 b	10.44 a	7.01 a	622 a

C1 culture conditions= 16-h day, 8-h night (25±2°C),
C2 culture conditions= For Initial 7 days, 16-h day, 8-h night (25±2°C), For remaining period, continuous dark (19±2°C)
Means followed by different letters in each column are significantly different for P≤0.05.

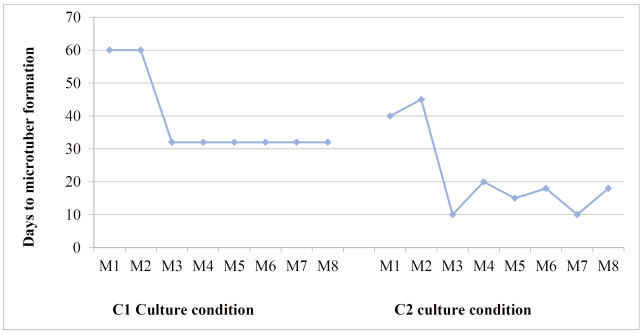

Fig. 3 - Days to microtuber formation under different conditions.

4. Discussion and Conclusions

Accumulation of seed-borne diseases is a major cause of low yield and quality losses in agriculture. Potato is a strategic crop for many populations. Producing disease-free potato microtubers is vital to improve its production. This study aimed to determine the best conditions and agents (sucrose and cytokinins) on the microtuberization capacity of the potato cultivar Spunta. As meristem tips are free from viruses, elimination and generation of virus free plants were shown by many researchers through meristem culture to obtain disease free potato plantlets (Jha and Ghosh, 2005; Bhuiyan, 2013).

Our findings provide evidence on the importance of having media devoid of growth regulators on the formation of reactive meristem in order to obtain the highest percentage. These results are in contradiction with others where Al-Taleb *et al.* (2011) indicated that the medium supplemented with 0.5 mg.l^{-1} of IBA was the best for shoots development and where Hajare *et al.* (2021) showed that best shoot initiation was obtained on MS medium supplemented with 1.5 mg.l^{-1} BAP + 3.0 mg.l^{-1} NAA for Gudiene variety, whereas 1.0 mg.l^{-1} BAP and 2.0 mg.l^{-1} NAA produced more shoots in Belete variety. According to a study done by Kanwal *et al*. (2006) who investigate the *in-vitro* micropropagation of potato cultivar Kuroda, the increase of added BAP concentrations in MS medium induced an increase in the rate of shoot formation.

According to Salem and Hassanein (2017) who studied the effect of genotype on multiplication and micro-tuberization of potato *in vitro,* the highest number of microshoot was formed by cv. Hermes after cultivation in a medium supplemented with 1 mg.l^{-1} BAP + 0.5 mg.l^{-1} GA_3, whereas the least one was formed by cv. Spunta implying that the number of obtained microshoots is cultivar-dependent.

At the level of the multiplication phase, our results showed that the media P3 containing Kin 0.4 mg.l^{-1}, GA3 0.5 mg.l^{-1} and IBA 0.5 mgl^{-1} caused the highest number of shootlets/plant. Similar results were obtained by Emaraa *et al.* (2017) who supplemented their media with Kin (0.2 mg.l^{-1}) and naphtaleneacetic acid (NAA, 0.2 mg.l^{-1}) and obtained maximum number of shootlets/plant. Among the different cytokinins used by researchers when studying micropropagation of plants, Kin stands among the best to promote shoot formation and shoot length (Van-Staden *et al.,* 2008; Hoque, 2010). El Dessoky *et al.* (2016) also showed that the highest percentage of shoot initiation and multiplication were observed on MS medium containing Kin (0.1 mg.l^{-1}) and GA3 (5 mg.l^{-1}).

Concerning microtuber formation, this study revealed that the addition of BAP into the culture medium was more effective in inducing *in vitro* tuberization in comparison to kinetin. The highest average number (AN) of microtubers was produced when propagated shootlets were subcultured on M3 medium containing 5 mg.l^{-1} BAP. These results agree with others (Aksenove *et al.,* 2009; Hoque, 2010; Sota *et al.*, 2020) who also showed that BAP has greater potential for microtuberization than kinetin and had an effect on reduction of total sugar and subsequently have increased starch content (Sarkar *et al.,* 2006).

Furthermore, Liljana *et al.* (2012) observed that tuberization occurred only in the presence of BAP (2 mg.l^{-1}) + IAA (1 mg.l^{-1}). Wang and Hu (1982) and Badoni and Chauan (2010) also reported that the optimum condition for *in vitro* tuberization of virus-free potatoes were in a medium containing BAP at 10 mg.l^{-1}. Vural *et al.* (2018) used BAP (2.5 mg.l^{-1}) with NAA (0.5 mg.l^{-1}) in their *in vitro* micropropagation of potato and found that these cytokinins had a positive effect on micro tuber formation and can be recommended to use them commercially in mass production.

On the other hand, these cytokinins showed no significant effect on the average length (AL), average width (AW) and average weight (AWe) of microtubers. Our results contradict those of Aryakia and Hamidoghli (2010) who showed that BAP and Kin induced changes in microtuber size and weight in the *in vitro* microtuberization of two potato cultivars, Arinda and Diamant.

Prat (2004) reported that Kin played a significant role in creating sink during plant growth, and through

regulating the expression of a gene involved in the partition of assimilates towards the stolon as observed in potato. Our study reports that kin exerted no significant effect on growth, diameter and weight of microtuber and this is in agreement with Kefi *et al.* (2000) and Kanwal *et al.* (2006).

In general, a linear relation between size and weight of microtuber exists (Liu and Xie, 2001). Any factor that influences microtuber weight will directly influence microtuber size. The microtubers parameters AN, AL, AW, and AWe obtained differed based on the culture condition. Growth condition C2 proved to be better for microtuber formation, whereas condition C1 was better for AL, AW, AWe. Under C1 culture condition, most of the culture produced green microtubers and this could be due to the synthesis of the alkaloid solanine that has fungicidal and pesticidal properties and that is considered as one of the plant's natural defenses. Hoque has also shown that when potato tubers are exposed to light, they turn green and increase glycoalkaloid production (Hoque, 2010). On the other hand, culture condition C2 yielded brown-colored tubers (Fig. 1, I and J). These results are in agreement with those of Salem and Hassanein (2017) who showed that dark conditions were better for microtuber formation compared to a 16-h photoperiod. Similarly, Sakha *et al.* (2004) also reported that microtuber formation frequency is higher under dark than light conditions. In addition, García and Bolaños (2017) showed that under the light condition with a photoperiod of 16-hour light and 8-hour dark, the number of microtubers was lower, but their biomass was higher and greener. On the other hand, under the dark condition, the number of microtubers was greater, with a lower biomass average and cream color. Garner and Blacke (1989) Al-Hussaini *et al*. (2015), showed in periods of total darkness, the microtuber weight is reduced. Other study also concluded that the light condition of 8 hours may be of benefit to the *in vitro* tuberization and increased the size and uniformity of microtubers compared to the *in vitro* tuberization under dark conditions (Pruski *et al*., 2002). Thus, an appropriate combination of light and dark conditions with short days can synchronize and accelerate the initiation and development of microtubers as well as increase their numbers.

Finally, concerning the effect of sucrose on microtuber formation, lower sucrose concentration (6%) resulted in an increase in the average number of the produced microtubers. Whereas sucrose presence had no effect on the length, width and weight (Table 5). Our results agree with those of Fufa and Diro (2014) with respect to the effect of 6% sucrose on the AN of the microtuber. Aslam *et al.* (2011) also found that a medium containing 6% sucrose was optimal in terms of minimum time of induction, average tuber number and weight of microtubers per single nodal explant in cultivar Desiree. Imani *et al.* (2010) also reported that MS medium supplemented with 6% of sucrose yielded the maximum number and the highest AL and Aw of microtubers. In plant tissue culture, most plant requires an exogenous carbohydrates source because of the limited photosynthesis that occurred *in vitro* (Lian *et al.,* 2014). Tuberization is known to be regulated by carbohydrates availability such as sucrose which is the transported form of sugar required for starch synthesis (Abelenda *et al.,* 2019). Sucrose is essential for the *in vitro* tuberization as an energy source and a signal for microtuber formation (Donnelly *et al*., 2003; Fufa and Diro, 2013; MotallebiAzar *et al.,* 2013).

In this study, to obtain microtubers of a sufficient weight, potato microshoots were cultured for 7 days in 16-h photoperiod followed by 50 days in dark. This type of treatment was referred to as light/dark treatment (Table 1). It increased microtuberization by enhancing tuberonic acid synthesis that was shown to play an important role in *in vitro* tuber formation (Alisdair and Willmitzer, 2001).

In conclusion, the best combination for rapid microtuberization obtained in our study was 6% sucrose, 5 $mg.l^{-1}$ BAP for Spunta potato cultivar cultured under C2 culture condition was within 10 days. Hossain *et al.* (2015) who investigated the effect of sucrose, growth regulators and potato varieties ('Diamant' and 'Cardinal') on rapid microtuberization found that the best combination was 9% sucrose at 5 $mg.l^{-1}$ of 6-benzyl aminopurine cultivar within 6-8 days while Zakaria *et al.* (2008) who studied the optimum level of Benzyl Adenine (BA) and Chloro Choline Chloride (CCC) to obtain large-size microtubers of the potato cultivar Diamant showed that early microtuber induction occur by using 500 mg/l of CCC within 15.9 days or by using 10 mg/l of BA within 13.3 days.

In the present study, the effect of two growth regulators, Kinetin and BAP, and the presence of sucrose and different photoperiodic conditions on the performance of microtubers in the potato cultivar Spunta was evaluated. Microtubers were produced from *in vitro* grown plantlets regenerated in MS medium supplemented with kin 0.4 $mg.l^{-1}$, GA3 0.5 $mg.l^{-1}$ and IBA

0.5 mg.l$^{-1.}$ The highest number of microtuber was obtained in M3 medium during C2 condition supplemented with 60 g.l^{-1} sucrose whereas C1 condition was better for AL, AW, and AWe of microtubers. The results indicated that microtuber induction of potato was highly dependent on sucrose, growth regulator and photoperiod conditions interaction. Production of large microtubers is important for successful utilization of microtubers in seed potato production. Hence, this study proposes an economical and reproducible method to obtain larger microtubers under laboratory conditions and it could be experimented to produce seed potato from *in vitro* grown tubers.

Acknowledgements

This study is part of the research activities of the Tissue Culture Unit, department of Plant Biotechnology funded by the Lebanese Agricultural Research Institute (LARI). Authors would like to thank Dr. Michel AFRAM, President Director General of LARI for his valuable support.

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Adv. Hort. Sci., 2023 37(3): 255-269 DOI: 10.36253/ahsc-14247

AHS
Advances in Horticultural Science

Effect of some chemical and natural preservative solutions on vase life, water relations and some chemical composition of *Dianthus caryophyllus* L. cut flowers

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Citation:
SARHAN A.M.Z., HEIKAL A.A.M., SAADAWY F.M., NOOR EL-DEEN T.M., ABD ELKAREEM K.M., 2023 - *Effect of some chemical and natural preservative solutions on vase life, water relations and some chemical composition of Dianthus caryophyllus L. cut flowers.* - Adv. Hort. Sci., 37(3): 255-269.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 24 July 2022
Accepted for publication 19 May 2023

Key words: $AgNO_3$, amino-oxyacetic acid, boric acid, carnation, cv. Turbo, 8-hydroxyquinoline sulfate, holding solutions, pulsing, silver thiosulfate.

Abstract: To investigate the effect of some pulsing and holding solutions on the quality of carnation cv. Turbo cut flowers, a laboratory experiment was conducted in the Agricultural Research Center and Cairo University, Egypt during 2020 and 2021 seasons. In this regard distilled water, silver thiosulfate (STS) at 0.4 ppm + sucrose 10% (PS1) and $AgNO_3$ at 10.0 ppm + sucrose 10% (PS2) were employed as a pulsing solution for 15 min while distilled water, sucrose 4% (HS1), boric acid (BoA) at 200 ppm + sucrose 4% (HS2), 8-hydroxyquinoline sulfate (8-HQS) at 300 ppm + sucrose 4% (HS3), Amino-oxyacetic acid (AOA) at 250 ppm + sucrose 4% (HS4), 8-HQS + AOA + sucrose 4% (HS5), BoA + 8-HQS + sucrose 4% (HS6), BoA + AOA + sucrose 4% (HS7), BoA + 8-HQS + AOA + sucrose 4% (HS8), rosemary extract at 25% + sucrose 2% (HS9) and thyme extract at 25% + sucrose 2% (HS10) were used as holding solutions. Regarding pulsing solutions, PS2 and PS1 exhibited a positive effect on all studied traits, while the mastery was to HS8 concerning the effect of holding solutions. Pulsing cut carnations in a solution containing PS2 followed by holding in HS8 resulted in the highest values in terms of vase life, water balance, chlorophyll a, carotenoids and total sugars, while the highest water uptake and loss and chlorophyll b were obtained by pulsing in PS1 followed by holding in HS8. It is recommended to pulse carnation cv. Turbo cut flowers in $AgNO_3$ at 10.0 ppm + sucrose 10% solution for 15 min followed by holding in BoA + 8-HQS + AOA + sucrose 4% for getting the longest vase life, enhancing water uptake and maintaining water balance. Additionally, this preservative solution effectively reduces chlorophyll degradation and preserves the content of carbohydrates throughout the postharvest period.

1. Introduction

Carnation (*Dianthus caryophyllus* L.; fam. Caryophyllaceae) is native to Southern Europe and the Mediterranean region and is a half-hardy perennial flowering plant with a wide range of colours. Each stem of the carnation forms a terminal flower; hence inflorescence is generally a terminal cyme. The flowering shoots can be single or multiple (stem sprays) (Ponnuswami and Sowmeya, 2015). Moreover, the exceptional keeping qualities of carnations make them an excellent choice for cut flowers. They possess remarkable longevity, the ability to withstand long-distance transportation, and an exceptional capacity to rehydrate even after prolonged shipping (Panwar *et al*., 2022).

There is a wide range of techniques applied to extend flower preservation, including the use of flower preservatives, inhibitors of ethylene action, growth regulators, calcium and the control of temperature and flower dehydration (Finger and Barbosa, 2006). Pulsing solutions are used basically to provide sugars (sucrose or glucose at 2-20%) and silver compounds (STS) (Armitage and Laushman, 2003). Pulsing solutions are used on freshly harvested flowers that are in a bud stage where a short period (or pulse) in a high-sugar solution will extend the vase life or open buds. Sugar is the main ingredient while STS is used to reduce ethylene sensitivity (Jones, 2001). To promote water uptake, the cut flower's stems are placed in a holding solution which contains an acidifier for hydration, a biocide of bacterial control and an energy/food source, which is typically sugar. The flowers usually stay in these solutions for one to several days as they are transported to local distributors and retailers and are used for retail displays (Dole and Faust, 2021).

A lot of chemicals are used in formulations of pulsing or/and holding solutions e.g. STS, $AgNO_3$, 8-HQS, AOA and boric acid. Ebrahimzadeh *et al.* (2008) concluded that STS is used to inhibit harmful effects of ethylene and prolong vase life in many ornamentals including carnation, $AgNO_3$ inhibits ethylene synthesis and action and is used as an antimicrobial agent, 8-HQS is an antimicrobial additive in preservatives and is ethylene synthesis inhibitor, AOA inhibits the biosynthesis of ethylene, and boric acid used as anti-ethylene synthesis. Various authors have demonstrated the beneficial effects of the aforementioned chemicals, integrated either individually or in combinations into preservative solutions, on carnation cut flowers cvs. Dolce Vita, Amstel, Monte Lisa, Aliceo, and Paola (Wawrzynczak and Goszczynska, 2003), cvs. Kristina, Aleda, Master, and Vienna (Krishnappa and Reddy, 2004), cvs. Nelson, Dream, and Delphi (Lopez *et al.*, 2008), cv. Optima (Karimi *et al*., 2012), cv. Charmant (Darwish *et al*., 2014), cv. Felice (Madhuri *et al*., 2016), and cv. Mirella (Adam and Eldeeb, 2021).

Certain natural materials e.g. plant extracts and essential oils are used instead of chemicals in cut flowers' preservative solutions due to the harmful effects of such chemicals (particularly those based on silver compounds) on human health. A lot of studies were carried out to investigate the effect of these natural materials. In this regard, Hashemabadi *et al.* (2021) on *Dianthus caryophyllus* L. cv. Yellow Candy stated that both vase life and solution uptake were increased by using dill essential oil in the solution compared to distilled water. Numerous studies have provided evidence of the impact of employing natural substances in preserving solutions for carnation cut flowers. For example, investigations have been conducted on the utilization of dill, clove, and coriander oils on cvs. Farida and Madam Collate (Shanan *et al*., 2010); extracts of lupin and clove and juice of the lemon on cv. Domingo (El-Ashwah, 2011); essential oils of dill, geranium and caraway on cv. Yellow Candy (Rad, 2018) and clove essential oil on cv. Cinderella (Eldeeb and Adam, 2021). Such materials were used in these reported studies either individually or in combination with other post-harvest chemicals.

Therefore, the present study was carried out to investigate the effect of different pulsing (mainly STS, $AgNO_3$ or distilled water) and holding (boric acid, 8-HQS, AOA, rosemary and thyme water extracts) solutions on vase life, water relations, pigments content and total sugars of carnation cut flowers with the possibility to get the longest vase live with the best quality.

2. Materials and Methods

A laboratory experiment was carried out in the Post-harvest Lab., Ornamental Plants and Landscape Gardening Res. Dept., Horticulture Res. Inst., Agricultural Research Center, Giza, Egypt and Ornamental Horticulture Dept., Faculty of Agriculture, Cairo University, Egypt during 2020 and 2021 seasons with the aim to study the effect of some pulsing and holding solutions (containing some natural extracts) on the postharvest quality of carna-

tion cut flowers.

Plant materials

Fresh cut flowers of carnation (*Dianthus caryophyllus* L.) cv. Turbo, at the paintbrush stage with red color were obtained from a local commercial greenhouse farm in Giza, Egypt, in the first week of January each season. Flowers' stem lengths were adjusted to 60 cm with 3.0-3.5 cm flower diameter, while fresh weight ranged from 18 to 20 g. Immediately, after transferring to the laboratory under dry conditions, about 5 cm of stem bases were recut under water and 4 pairs of leaves were left on each stem and then rapidly precooled by placing them in cold water for three hours.

Experiment treatments

Pulsing solutions

These precooled cut flowers were distributed in 500 ml jars (3 flowers/jar) and then equally divided into three groups, each one containing 300 ml from one of the following applied pulsing solutions:

1. Distilled water
2. STS at 0.4 ppm + sucrose at 10% (PS1)
3. $AgNO_3$ at 10.0 ppm + sucrose at 10% (PS2)

STS (silver thiosulfate; $1AgNO_3.4Na_2S_2O_3{\cdot}5H_2O$) solution was prepared according to Gorin *et al*. (1985). In this regard, both $AgNO_3$ (0.079 g) and $Na_2S_2O_3{\cdot}5H_2O$ (0.462) were dissolved separately in 500 ml deionized water, then $AgNO_3$ solution was poured into $Na_2S_2O_3{\cdot}5H_2O$ solution with continuous stirring. While the $AgNO_3$ solution was prepared by dissolving 0.01 g of $AgNO_3$ in deionized distilled water to prepare a 10-ppm concentration.

Holding solutions

Each group of the pulsed cut flowers were divided into 11 subgroups (3 flowers/500 ml jar containing 300 ml of different holding solutions) and kept under lab conditions (light intensity at 1000 lux supplied by fluorescent lamps, the average temperature at 18-20°C and relative humidity at 50-55%) as follows:

1. Control (distilled water)
2. Sucrose at 4% (HS1)
3. Boric acid at 200 ppm (BoA) + sucrose at 4% (HS2)
4. 8-hydroxyquinoline sulfate at 300 ppm (8-HQS) + sucrose at 4% (HS3)
5. Amino-oxyacetic acid at 250 ppm (AOA) + sucrose at 4% (HS4)
6. 8-HQS + AOA + sucrose at 4% (HS5)
7. BoA + 8-HQS + sucrose at 4% (HS6)
8. BoA + AOA + sucrose at 4% (HS7)
9. BoA + 8-HQS + AOA + sucrose at 4% (HS8)
10. Rosemary extract at 25% (RE) + sucrose at 2% (HS9)
11. Thyme extract at 25% (TE) + sucrose at 2% (HS10)

Extracts preparation

Both thyme (*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.) extracts were prepared by water extraction. In this concern, 250 g of the dry herb was extracted in 250 ml of distilled water with boiling at 100°C and stirring for 30 minutes. After that, the solution was filtered using filter paper, and the remaining solution was completed to 1000 ml with distilled water. Afterwards, 250 ml of each solution was poured into 1000 ml of distilled water to get a 25% concentration.

Experiment design

This experiment was laid out as a complete randomized design (CRD) in a factorial experiment. Factor (A) was represented by 3 levels of pulsing solutions, while factor (B) was represented by 11 levels of holding solutions. Thus, a total of 33 treatments were utilized. Each treatment consisted of 3 replicates, with each replicate containing 3 jars. Within each jar, there were 3 flowers, resulting in a total of 27 flowers per treatment.

Data collection

Vase life

Vase life was determined as the number of days to the beginning of flowers wilting.

Water relations

The total water uptake (g/flower) was calculated by subtracting the weight of water at the end of the experiment from the initial weight.

The total water loss (g/flower) was determined by measuring the difference between the weight of jars with spikes and solution at the beginning of the experiment and the weight of jars with spikes and solution at the end of the experiment.

The total water balance (g/flower) was obtained by subtracting the total water loss from the total water uptake.

Determination of pigments and sugars

At the end of flower longevity, pigment and sugar contents were measured on the attached leaves.

Contents of chlorophylls a, b and carotenoids were determined colourimetrically in fresh leaves according to the method described by Wellburn and Lichtenthaler (1984).

Total sugars in the dry leaves were determined

colourimetrically according to Dubois *et al.* (1956).

Statistical analysis

The obtained data were statistically analyzed as a factorial experiment using MSTAT Computer Program (MSTAT Development Team, 1989). Duncan's multiple range test (Duncan, 1955) was used to compare the means between various treatments.

3. Results and Discussion

Vase life

The data reported in Table 1 clearly show that PS2 significantly increased the vase life of carnation cut flowers to 18.4 and 18.7 days in the first and second seasons, respectively. Conversely, pulsing the cut flowers in distilled water solution recorded only 16.3 and 16.5 days in both seasons, respectively.

Preserved carnation cut flowers in different holding solutions showed a significant influence on vase life (days) as presented in Table 1. The highest number of days was recorded by holding in HS8 (24.3 and 24.6 days) in the first and second seasons, respectively. The lowest values were obtained by holding the cut flowers in HS9 (12.6 and 12.9 days), HS10 (12.7 and 13.0 days) and distilled water (12.8 and 12.6 days) in both seasons, respectively.

Comparing to the control treatment (DW), it was evident that all combined treatments involving both pulsing and holding solutions led to a substantial improvement in the vase life of cut carnation flowers. The key technique employed to achieve this outcome involved pulsing the flowers in a PS2, along with

Table 1 - Effect of pulsing and holding solutions and their interaction on vase life (days) of *Dianthus caryophyllus* cv. Turbo cut flowers during 2020 and 2021 seasons

Holding solutions (B)	Pulsing solutions (A)			
	PS1	PS2	DW	Mean (B)
	First season (2020)			
Control (DW)	13.67 o-q	14.18 op	10.67 t	12.84 f
HS1	15.69 l-n	15.98 lm	14.69 no	15.45 e
HS2	18.04 ij	18.02 ij	16.71 kl	17.59 d
HS3	21.44 de	23.11 bc	17.73 jk	20.76 b
HS4	19.09 hi	22.13 c-e	16.38 lm	19.20 c
HS5	17.60 jk	18.00 ij	15.60 mn	17.07 d
HS6	21.87 de	21.24 ef	20.33 fg	21.15 b
HS7	20.11 gh	19.36 gh	19.42 gh	19.63 c
HS8	23.98 b	26.67 a	22.40 cd	24.35 a
HS9	12.67 qr	13.29 pq	11.84 rs	12.60 f
HS10	13.09 pq	11.04 st	14.00 op	12.71 f
Mean (A)	17.93 b	18.46 a	16.34 c	
	Second season (2021)			
Control (DW)	13.00 p-r	14.23 n-p	10.83 s	12.69 f
HS1	15.45 l-n	16.00 k-m	14.90 m-o	15.45 e
HS2	18.23 g-i	18.10 g-i	17.13 i-k	17.82 d
HS3	21.68 de	23.63 bc	17.80 h-j	21.04 b
HS4	19.51 fg	22.20 c-e	16.50 j-l	19.40 c
HS5	17.93 h-j	18.87 gh	16.33 k-m	17.71 d
HS6	22.30 cd	21.50 de	20.80 ef	21.53 b
HS7	20.80 ef	19.53 fg	19.50 fg	19.94 c
HS8	24.57 b	27.00 a	22.23 c-e	24.60 a
HS9	13.43 p	13.60 op	11.83 q-s	12.96 f
HS10	13.23 pq	11.63 rs	14.33 n-p	13.07 f
Mean (A)	18.19 b	18.75 a	16.56 c	

PS1= STS at 0.4 ppm + sucrose 10%, PS2= AgNO3 at 10.0 ppm + sucrose 10%, HS10 sucrose 4%, HS2= boric acid at 200 ppm + sucrose 4%, HS3= 8-HQS at 300 ppm + sucrose 4%, HS4= AOA at 250 ppm + sucrose 4%, HS5= 8-HQS + AOA + sucrose 4%, HS6= boric acid + 8-HQS + sucrose 4%, HS70= boric acid + AOA + sucrose 4%, HS8= boric acid + 8-HQS + AOA + sucrose 4%, HS9= rosemary extract at 25% + sucrose 2%, HS10= thyme extract at 25% + sucrose 2%.

holding them in HS8. This combination resulted in vase lives of 26.6 and 27.0 days in both seasons, respectively.

The lowest number of days of vase life in both seasons was recorded by pulsing and holding in distilled water only (10.6 and 10.8 days), pulsing in PS2 + holding in HS10 solution (11.0 and 11.6 days) and pulsing in distilled water and holding in HS9 solutions (11.8 and 11.8 days), respectively.

The aforementioned findings were consistent with the results obtained in previous studies on carnation e.g. Serrano *et al.* (2001), Lopez *et al.* (2008), Hashemabadi (2014), Liu *et al.* (2018) and Gocan *et al.* (2021). Similar outcomes were observed on roses cut flowers (Elgimabi, 2014; Kumar *et al.*, 2017), gerbera (Bhanushree and Rao, 2015; Jafarpour *et al.*, 2015), hydrangeas (Kazaz *et al.*, 2020; Suntipabvivattana *et al.*, 2020), Cymbidium (Usha *et al.*, 2014). Kabari and Soleimandarabi (2019) focused on Alstroemeria cut flowers, while Ichimura *et al.* (2009) examined cut *Eustoma*, *Delphinium*, and snapdragon flowers.

In this regard, Darwish *et al.* (2014) reported that using a solution of 300 ppm 8-HQS + 40 g/l sucrose and 0.4 mM STS + 50 g/l sucrose significantly increased vase life of carnation cut flowers cv. Felice. Also, Badawy *et al.* (2016) revealed that $AgNO_3$ showed the longest vase life of *Chrysanthemum* cut flowers cv. Royal Accent.

Water relations

Water uptake

Data reported in Table 2 show that PS1 solution significantly enhanced water uptake of carnation cut flowers resulting in the highest values in both seasons, (48.2 and 49.9 g/flower in 2020 and 2021,

Table 2 - Effect of pulsing and holding solutions and their interaction on water uptake (g/flower) of *Dianthus caryophyllus* cv. Turbo cut flowers during 2020 and 2021 seasons

Holding solutions (B)	Pulsing solutions (A)			
	PS1	PS2	DW	Mean (B)
	First season (2020)			
Control (DW)	36.52 mn	36.55 mn	32.06 p	35.04 g
HS1	47.74 h-j	36.20 mn	34.01 op	39.32 f
HS2	57.35 d	50.23 fg	46.81 ij	51.46 c
HS3	52.18 ef	48.95 gh	48.85 g-i	49.99 d
HS4	50.57 fg	40.68 l	40.96 kl	44.07 e
HS5	59.70 c	57.10 d	54.11 e	56.97 b
HS6	56.49 d	46.68 j	46.54 j	49.91 d
HS7	46.71 j	42.77 k	45.90 j	45.12 e
HS8	67.61 a	65.47 b	60.15 c	64.41 a
HS9	29.31 q	37.80 m	37.53 m	34.88 g
HS10	26.79 r	34.56 no	29.95 q	30.43 h
Mean (A)	48.27 a	45.18 b	43.35 c	
	Second season (2021)			
Control (DW)	38.42 mn	38.46 mn	34.10 p	36.99 g
HS1	49.42 h-j	38.23 mn	36.11 o	41.25 f
HS2	58.75 d	51.85 fg	48.53 ij	53.04 c
HS3	53.74 ef	50.60 gh	50.50 g-i	51.61 d
HS4	52.17 fg	42.58 l	42.85 kl	45.87 e
HS5	61.03 c	58.51 d	55.61 e	58.39 b
HS6	57.92 d	48.41 j	48.27 j	51.53 d
HS7	48.43 j	44.60 k	47.64 j	46.89 e
HS8	68.71 a	66.63 b	61.47 c	65.60 a
HS9	31.55 q	39.78 m	39.53 m	36.95 g
HS10	29.11 r	36.64 no	32.17 pq	32.64 h
Mean (A)	49.93 a	46.94 b	45.16 c	

PS1= STS at 0.4 ppm + sucrose 10%, PS2= AgNO3 at 10.0 ppm + sucrose 10%, HS10 sucrose 4%, HS2= boric acid at 200 ppm + sucrose 4%, HS3= 8-HQS at 300 ppm + sucrose 4%, HS4= AOA at 250 ppm + sucrose 4%, HS5= 8-HQS + AOA + sucrose 4%, HS6= boric acid + 8-HQS + sucrose 4%, HS70= boric acid + AOA + sucrose 4%, HS8= boric acid + 8-HQS + AOA + sucrose 4%, HS9= rosemary extract at 25% + sucrose 2%, HS10= thyme extract at 25% + sucrose 2%.

respectively). The lowest significant values were recorded when the cut flowers were pulsed in a distilled water solution. (43.3 and 45.1 g/flower in 2020 and 2021, respectively).

All applied holding solutions positively affect the water uptake of cut carnations as shown in Table 2. The holding solution containing HS8 resulted in the highest water uptake (64.4 and 65.6 days in 2020 and 2021, respectively). On the other hand, holding in solutions containing either rosemary or thyme extracts adversely affected water uptake of carnation cut flowers (34.8 and 36.9 g/flower for rosemary and 30.4 and 32.6 g/flower for thyme extract in 2020 and 2021, respectively). Furthermore, it is noteworthy that when the flowers were held in a distilled water solution alone, it exhibited a similar effect to holding them in a solution containing rosemary extract, with no significant difference observed (35.0 and 36.9 g/flower in the first and second seasons, respectively.

With regard to the interaction between pulsing and holding solutions, it can be observed that pulsing in PS1 solution followed by holding in HS8 recorded the highest significant values in both seasons (67.6 and 68.7 g/flower, respectively in 2020 and 2021). Water uptake of carnation cut flowers was decreased by all applied pulsing solutions when combined with holding solutions containing either rosemary, thyme or only distilled water. The lowest values in this regard were obtained by pulsing in PS1 in addition to holding in HS10 solutions (26.7 and 29.1 g/flower in 2020 and 2021, respectively).

Water loss

As shown in Table 3, the greatest water loss was observed when the flowers were pulsed in PS1 in both seasons. The water loss values recorded were

Table 3 - Effect of pulsing and holding solutions and their interaction on water loss (g/flower) of *Dianthus caryophyllus* cv. Turbo cut flowers during 2020 and 2021 seasons

Holding solutions (B)	Pulsing solutions (A)			
	PS1	PS2	DW	Mean (B)
	First season (2020)			
Control (DW)	38.23 ij	41.25 f-h	37.46 jk	38.98 e
HS1	49.26 d	33.77 l-n	33.21 mn	38.75 e
HS2	54.14 bc	46.64 e	42.28 f-h	47.69 b
HS3	47.32 de	42.61 fg	43.50 f	44.48 c
HS4	46.57 e	35.72 kl	37.80 jk	40.03 de
HS5	59.81 a	53.39 c	53.53 c	55.58 a
HS6	51.81 c	42.04 f-h	39.90 h-j	44.58 c
HS7	42.71 fg	38.23 ij	40.46 g-i	40.46 d
HS8	60.64 a	56.01 b	51.78 c	56.15 a
HS9	35.58 k-m	43.42 f	42.50 fg	40.50 d
HS10	29.50 o	41.67 f-h	32.84 n	34.67 f
Mean (A)	46.87 a	43.16 b	41.39 c	
	Second season (2021)			
Control (DW)	39.40 l-n	43.28 hi	42.09 i-k	41.59 d
HS1	50.21 c-e	31.40 t	33.73 rs	38.45 e
HS2	50.79 cd	43.83 hi	40.89 j-l	45.17 c
HS3	42.20 ij	40.25 j-m	42.19 ij	41.55 d
HS4	44.50 h	33.28 r-t	35.40 p-r	37.73 e
HS5	58.60 a	52.08 c	55.00 b	55.22 a
HS6	48.63 d-f	37.05 o-q	37.57 n-p	41.08 d
HS7	40.89 j-l	36.97 o-q	38.37 m-o	38.74 e
HS8	54.72 b	46.74 fg	47.01 fg	49.49 b
HS9	39.95 k-m	48.30 ef	45.33 gh	44.53 c
HS10	32.58 st	46.85 fg	35.17 qr	38.20 e
Mean (A)	45.68 a	41.82 b	41.16 b	

PS1= STS at 0.4 ppm + sucrose 10%, PS2= AgNO3 at 10.0 ppm + sucrose 10%, HS10 sucrose 4%, HS2= boric acid at 200 ppm + sucrose 4%, HS3= 8-HQS at 300 ppm + sucrose 4%, HS4= AOA at 250 ppm + sucrose 4%, HS5= 8-HQS + AOA + sucrose 4%, HS6= boric acid + 8-HQS + sucrose 4%, HS70= boric acid + AOA + sucrose 4%, HS8= boric acid + 8-HQS + AOA + sucrose 4%, HS9= rosemary extract at 25% + sucrose 2%, HS10= thyme extract at 25% + sucrose 2%.

46.9 and 45.7 g/flower, respectively in 2020 and 2021. The lowest values were recorded when the flowers were pulsed in a distilled water solution (41.4 and 41.2 g/flower in 2020 and 2021, respectively). There was no significant difference between pulsing the flowers in a solution of PS2 (41.8 g/flower) and using distilled water in the second season only.

Regarding the effect of holding solution, it can be said that holding in HS8 or HS5 solutions resulted in the highest water loss in both seasons. Only in the first season, there was no significant difference between them, while in the second one, HS5 was significantly higher than HS8. In the first and second seasons, the results were 56.1 and 49.4 g/flower for HS8 and 55.5 and 55.2 g/flower for HS5 respectively.

As for the effect of interaction, the highest water loss was obtained by pulsing in PS1 then holding in either HS8 (60.6 and 54.7 g/flower) or HS5 (59.8 and 58.6 g/flower in 2020 and 2021, respectively). However, in the first season, there was no significant difference between these two combined treatments. In contrast, in the second season, when combined with a holding solution containing HS5, it resulted in a significantly higher water loss compared to the combination of HS8. The lowest significant water loss was obtained by pulsing in PS1 followed by holding in HS10 in the first season (29.5 g/flower) and PS2 followed by HS1 only in the second one (31.4 g/flower).

Water balance

Regarding the impact of pulsing solutions on the water balance of carnation cut flowers, the data presented in Table 4 revealed that PS2 exhibited superiority in this regard, recording the highest values of

Table 4 - Effect of pulsing and holding solutions and their interaction on water balance (g/flower) of *Dianthus caryophyllus* cv. Turbo cut flowers during 2020 and 2021 seasons

Holding solutions (B)	Pulsing solutions (A)			
	PS1	PS2	DW	Mean (B)
	First season (2020)			
Control (DW)	-1.71 m	-4.69 n	-5.40 no	-3.93 f
HS1	-1.52 lm	2.43 j	0.79 k	0.57 e
HS2	3.21 h-j	3.59 g-j	4.53 f-i	3.78 d
HS3	4.87 e-g	6.34 c-e	5.35 d-f	5.52 b
HS4	4.00 f-i	4.95 e-g	3.17 ij	4.04 cd
HS5	-0.11 kl	3.71 g-j	0.58 k	1.39 e
HS6	4.68 f-h	4.65 f-i	6.64 cd	5.32 b
HS7	4.00 f-i	4.54 f-i	5.44 d-f	4.66 bc
HS8	6.97 bc	9.45 a	8.37 ab	8.26 a
HS9	-6.27 op	-5.62 n-p	-4.97 no	-5.62 g
HS10	-2.70 m	-7.11 p	-2.89 m	-4.23 f
Mean (A)	1.40 b	2.02 a	1.96 a	
	Second season (2021)			
Control (DW)	-0.98 m	-4.82 o	-7.99 q	-4.60 e
HS1	-0.79 m	6.83 ij	2.38 k	2.81 d
HS2	7.96 gh	8.01 gh	7.63 gh	7.87 c
HS3	11.54 c	10.35 e	8.31 g	10.07 b
HS4	7.67 gh	9.29 f	7.46 hi	8.14 c
HS5	2.44 k	6.43 j	0.61 l	3.16 d
HS6	9.29 f	11.35 cd	10.70 de	10.45 b
HS7	7.54 h	7.63 gh	9.27 f	8.15 c
HS8	13.98 b	19.89 a	14.46 b	16.11 a
HS9	-8.40 q	-8.52 q	-5.80 p	-7.57 g
HS10	-3.46 n	-10.20 r	-2.99 n	-5.55 f
Mean (A)	4.25 b	5.11 a	4.00 c	

PS1= STS at 0.4 ppm + sucrose 10%, PS2= AgNO3 at 10.0 ppm + sucrose 10%, HS10 sucrose 4%, HS2= boric acid at 200 ppm + sucrose 4%, HS3= 8-HQS at 300 ppm + sucrose 4%, HS4= AOA at 250 ppm + sucrose 4%, HS5= 8-HQS + AOA + sucrose 4%, HS6= boric acid + 8-HQS + sucrose 4%, HS70= boric acid + AOA + sucrose 4%, HS8= boric acid + 8-HQS + AOA + sucrose 4%, HS9= rosemary extract at 25% + sucrose 2%, HS10= thyme extract at 25% + sucrose 2%.

2.0 g/flower and 5.1 g/flower in 2020 and 2021, respectively. Pulsing solution containing distilled water occupied the second position without significant differences in the first season only (1.9 g/flower).

Regarding the effect of holding solutions, HS8 resulted in the highest significant positive values in both seasons (8.2 and 16.1 g/flower in 2020 and 2021, respectively). The lowest values were obtained when using HS9, which resulted in negative values of -5.6 g/flower and -7.5 g/flower in the first and second seasons, respectively.

A significant interaction between pulsing and holding solutions was observed. Pulsing in PS2 then holding in HS8 seemed to be the most effective treatment resulting in the highest positive values (9.4 and 19.8, in 2020 and 2021, respectively. The lowest values, on the other hand, were obtained when using any pulsing solution followed by HS9, HS10, or HS1. Specifically, pulsing the flowers in PS2 followed by holding them in HS10 resulted in the lowest values in this regard, measuring -7.1 g/flower and -10.2 g/flower in the first and second seasons, respectively.

In this regard, Bhanushree and Rao (2015) conducted a research on *Gerbera jamesonii* cv. Lomborgini and reported that the application of $AgNO_3$ at 20 ppm resulted in an increase in water uptake and water loss. Khella *et al.* (2018) reported that water uptake of *Limonium sinuatum* cv. Girlie Wings cut flowers was enhanced by STS at 500 mg/l for 1/2 h or by STS at 500 mg/l for 1/4 h followed by $AgNO_3$ at 500 mg/l for 1/2 h. In a study conducted by Kazaz *et al.* (2020) on cut hydrangeas, it was found that the application of 8-HQS at 200 mg/l resulted in an improvement in solution uptake compared to the control group. These findings align with the results reported by Elgimabi (2014) on *Rosa damascena* cv. Trigintipetala and Usha *et al.* (2014) on *Cymbidium hybrid* cv. Red Princess.

Determination of pigments and sugars

Pigments content

The pulsing solution containing PS1 recorded the highest values of chlorophyll a (0.349 and 0.354 mg/g f.w.), chlorophyll b (0.234 and 0.241 mg/g f.w.) and carotenoids (0.184 and 0.182 mg/g f.w.) in both seasons, respectively. The lowest values for chlorophyll a, chlorophyll b, and carotenoids were observed when using distilled water, with recorded values of 0.293 and 0.297, 0.218 and 0.214, and 0.133 and 0.143 in the first and second seasons, respectively (Tables 5, 6, and 7).

Concerning the effect of holding solutions, HS8 resulted in the highest values of chlorophyll a (0.626 and 0.641 mg/g f.w.), chlorophyll b (0.435 and 0.434 mg/g f.w.) and carotenoids (0.236 and 0.247 mg/g f.w.) in both seasons, respectively. It could be noticed that the lowest values in terms of chlorophyll a (0.167 and 0.171 mg/g f.w.) and carotenoids (0.110 and 0.116 mg/g f.w.) in both 2020 and 2021seasons were obtained by using a holding solution containing HS9, while HS10 produced the lowest values in case of chlorophyll b (0.105 and 0.116 mg/g f.w. in 2020 and 2021 respectively). In general, all holding solution formulations involving HS9, HS10 or distilled water resulted in the lowest values for the measured parameters.

Combined treatment of PS2 in addition to HS8 resulted in the highest significant values in terms of chlorophyll a (0.642 and 0.655 mg/g f.w.) and carotenoids (0.238 and 0.255 mg/g f.w.) and occupied the second rank in case of chlorophyll b with values of 0.468 and 0.466 in 2020 and 2021, respectively. On the other hand, the highest values of chlorophyll b were obtained when using PS1 in combination with HS8 (0.481 and 0.485 mg/g f.w. in 2020 and 2021, respectively).

PS1 combined with HS9 gave the lowest values in terms of chlorophyll a, while pulsing and holding in distilled water resulted in the lowest values of chlorophyll b (0.052 and 0.059 mg/g f.w.) and carotenoids (0.062 and 0.060 mg/g f.w.) in both seasons.

In a similar context, Badawy *et al.* (2016) conducted a study on Chrysanthemum cut flowers cv. Royal Accent and reported that $AgNO_3$ exhibited the least decrease in chlorophyll content. Khella *et al.* (2018) reported that STS at 500 mg/l for 1/2 h or STS at 500 mg/l for 1/4 h followed by $AgNO_3$ 500 mg/l for 1/2 h enhanced pigments content of *Limonium sinuatum* cv. Girlie Wings cut flowers. The same results were reported by Elgimabi (2014) on *Rosa damascena* cv. Trigintipetala.

Total sugars content

The data presented in Table 8 clearly show that pulsing cut carnations in PS1 resulted in the highest percentage of sugars, with recorded values of 28.0% and 28.5% in 2020 and 2021, respectively. Following closely in the second position, a solution of PS2 showed a percentage of 27.7% in 2020 (not being significantly different from the highest) and 28.0% in

Table 5 - Effect of pulsing and holding solutions and their interaction on chlorophyll a (mg/g f.w.) of *Dianthus caryophyllus* cv. Turbo cut flowers during 2020 and 2021 seasons

Holding solutions (B)	Pulsing solutions (A)			
	PS1	PS2	DW	Mean (B)
	First season (2020)			
Control (DW)	0.250 t	0.281 r	0.143 z	0.225 h
HS1	0.267 s	0.323 o	0.247 t	0.279 g
HS2	0.353 l	0.383 k	0.314 p	0.350 e
HS3	0.480 d	0.463 e	0.327 no	0.423 b
HS4	0.395 j	0.440 f	0.288 q	0.374 d
HS5	0.329 mn	0.398 j	0.187 w	0.305 f
HS6	0.416 h	0.429 g	0.430 g	0.425 b
HS7	0.408 i	0.410 i	0.333 m	0.383 c
HS8	0.636 b	0.642 a	0.601 c	0.626 a
HS9	0.120 [	0.212 u	0.170 y	0.167 j
HS10	0.186 w	0.196 v	0.178 x	0.187 i
Mean (A)	0.349 b	0.380 a	0.293 c	
	Second season (2021)			
Control (DW)	0.255 r	0.279 p	0.139 y	0.224 h
HS1	0.264 q	0.334 m	0.243 s	0.280 g
HS2	0.355 l	0.403 k	0.330 m	0.363 e
HS3	0.483 d	0.454 e	0.334 m	0.424 b
HS4	0.406 jk	0.458 e	0.295 o	0.386 c
HS5	0.329 m	0.417 gh	0.190 v	0.312 f
HS6	0.420 g	0.413 hi	0.440 f	0.424 b
HS7	0.405 jk	0.408 ij	0.323 n	0.379 d
HS8	0.650 b	0.655 a	0.618 c	0.641 a
HS9	0.131 z	0.213 t	0.169 x	0.171 j
HS10	0.197 u	0.199 u	0.184 w	0.193 i
Mean (A)	0.354 b	0.385 a	0.297 c	

PS1= STS at 0.4 ppm + sucrose 10%, PS2= AgNO3 at 10.0 ppm + sucrose 10%, HS10 sucrose 4%, HS2= boric acid at 200 ppm + sucrose 4%, HS3= 8-HQS at 300 ppm + sucrose 4%, HS4= AOA at 250 ppm + sucrose 4%, HS5= 8-HQS + AOA + sucrose 4%, HS6= boric acid + 8-HQS + sucrose 4%, HS70= boric acid + AOA + sucrose 4%, HS8= boric acid + 8-HQS + AOA + sucrose 4%, HS9= rosemary extract at 25% + sucrose 2%, HS10= thyme extract at 25% + sucrose 2%.

2021 (being significantly different from the highest). Conversely, the lowest values were observed when using distilled water, with percentages of 25.8% and 26.0% in 2020 and 2021, respectively.

When considering the influence of holding solutions, it was evident that HS8 outperformed other treatments in terms of total sugars, resulting in the highest values in both seasons (47.5% and 48.3% in 2020 and 2021, respectively). Conversely, HS9 exhibited a negative effect, leading to the lowest sugar content (11.4% and 11.7%).

Regarding the combined treatments, PS2 in addition to HS8 produced the highest values, recording 52.0% and 54.6% in 2020 and 2021, respectively. On the other hand, when distilled water was combined with HS9, the lowest values were observed, with percentages of 10.5% and 11.1% in 2020 and 2021, respectively.

These results align with the findings of Badawy *et al.* (2016), who reported that $AgNO_3$ exhibited the highest total carbohydrate content in Chrysanthemum cut flowers cv. Royal Accent. Additionally, Khella *et al.* (2018) demonstrated that STS at 500 mg/l for 1/2 h enhanced the total carbohydrate percentage of *Limonium sinuatum* cv. Girlie Wings cut flowers, followed by the treatment with STS at 500 mg/l for 1/4 h and $AgNO_3$ at 500 mg/l for 1/2 h. Similarly, Chore *et al.* (2020) investigated *Gladiolus grandiflorus* L. cv. Fado and found a significant increase in total soluble sugars in spikes pulsed with 600 ppm 8-HQS + 5% sucrose compared to the control. These results are consistent with the findings

Table 6 - Effect of pulsing and holding solutions and their interaction on chlorophyll b (mg/g f.w.) of *Dianthus caryophyllus* cv. Turbo cut flowers during 2020 and 2021 seasons

Holding solutions (B)	Pulsing solutions (A)			
	PS1	PS2	DW	Mean (B)
	First season (2020)			
Control (DW)	0.105 x	0.174 r	0.052 \	0.110 j
HS1	0.156 t	0.184 q	0.154 t	0.165 h
HS2	0.206 p	0.251 n	0.260 m	0.239 f
HS3	0.396 d	0.423 c	0.294 l	0.371 b
HS4	0.217 o	0.357 ef	0.222 o	0.266 e
HS5	0.185 q	0.300 k	0.143 u	0.209 g
HS6	0.362 e	0.316 i	0.338 g	0.339 c
HS7	0.294 l	0.309 j	0.332 h	0.312 d
HS8	0.481 a	0.468 b	0.356 f	0.435 a
HS9	0.079 [	0.163 s	0.119 w	0.120 i
HS10	0.088 z	0.098 y	0.128 v	0.105 k
Mean (A)	0.234 b	0.277 a	0.218 c	
	Second season (2021)			
Control (DW)	0.127 n-p	0.181 j-m	0.059 q	0.122 h
HS1	0.172 k-n	0.181 j-m	0.168 k-n	0.174 g
HS2	0.208 i-k	0.260 gh	0.240 g-i	0.236 e
HS3	0.398 bc	0.437 ab	0.285 fg	0.373 b
HS4	0.230 h-j	0.368 cd	0.215 h-k	0.271 d
HS5	0.188 j-l	0.284 fg	0.144 l-o	0.205 f
HS6	0.367 cd	0.324 d-f	0.324 d-f	0.338 c
HS7	0.291 e-g	0.337 de	0.316 d-f	0.315 c
HS8	0.485 a	0.466 a	0.350 cd	0.434 a
HS9	0.088 pq	0.171 k-n	0.124 n-p	0.128 h
HS10	0.095 o-q	0.130 m-p	0.123 n-p	0.116 h
Mean (A)	0.241 b	0.285 a	0.214 c	

PS1= STS at 0.4 ppm + sucrose 10%, PS2= AgNO3 at 10.0 ppm + sucrose 10%, HS10 sucrose 4%, HS2= boric acid at 200 ppm + sucrose 4%, HS3= 8-HQS at 300 ppm + sucrose 4%, HS4= AOA at 250 ppm + sucrose 4%, HS5= 8-HQS + AOA + sucrose 4%, HS6= boric acid + 8-HQS + sucrose 4%, HS70= boric acid + AOA + sucrose 4%, HS8= boric acid + 8-HQS + AOA + sucrose 4%, HS9= rosemary extract at 25% + sucrose 2%, HS10= thyme extract at 25% + sucrose 2%.

of Elgimabi (2014) on *Rosa damascena* cv. Trigintipetala and Bhanushree and Rao (2015) on *Gerbera jamesonii* cv. Lomborgini.

The present study exhibited the beneficial role of certain chemicals used in pulsing or holding solutions of carnation cut flowers either individually or in combinations. The application of these chemicals resulted in a significant increase in the vase life of cut flowers, more than doubling its duration. Furthermore, these treatments also had a positive impact on water relations and chemical composition, including chlorophylls a, b, carotenoids, and total sugars. It is well known that the vase life of cut carnation is considered one of the most vital traits for florists (Panwar *et al.*, 2022). Finger and Barbosa (2006) summarized the factors affecting the longevity of cut flowers as (1) their tender nature, (2) a lot of stresses leading to water uptake reduction, stored carbohydrates exhaustion and respiration increment (3) the harmful effects of ethylene. Otherwise, the vase life of cut flowers is affected basically by ethylene which enhances the senescence of many cut flowers as well as microorganisms which reduce the amount of water uptake by causing a vascular blockage (Zencirkiran, 2010). Halevy (1987) reported that carnation cut flowers are highly sensitive to ethylene either endogenously produced or exogenously applied. Carnation is known to be highly susceptible to the buildup of microorganisms in the vase solution or at the cut ends of the flower stems. This accumulation can result in blockage of the vascular system and ultimately reduce the vase life of the flowers (Van Doorn

Table 7 - Effect of pulsing and holding solutions and their interaction on carotenoids (mg/g f.w.) of Dianthus caryophyllus cv. Turbo cut flowers during 2020 and 2021 seasons

Holding solutions (B)	Pulsing solutions (A)			
	PS1	PS2	D.W.	Mean (B)
	First season (2020)			
Control (DW)	0.139 n	0.179 k	0.062 t	0.127 i
HS1	0.179 k	0.207 i	0.123 pq	0.170 h
HS2	0.189 j	0.211 hi	0.131 o	0.177 f
HS3	0.230 de	0.237 ab	0.133 o	0.200 c
HS4	0.210 hi	0.232 b-d	0.125 p	0.189 e
HS5	0.188 j	0.214 h	0.119 q	0.174 g
HS6	0.234 a-d	0.226 ef	0.232 cd	0.231 b
HS7	0.222 fg	0.219 g	0.148 m	0.196 d
HS8	0.236 a-c	0.238 a	0.234 a-d	0.236 a
HS9	0.091 s	0.172 l	0.065 t	0.110 j
HS10	0.110 r	0.135 no	0.086 s	0.110 j
Mean (A)	0.184 b	0.207 a	0.133 c	
	Second season (2021)			
Control (DW)	0.129 n	0.201 i	0.060 r	0.130 h
HS1	0.182 j	0.207 h	0.127 n	0.172 g
HS2	0.200 i	0.218 g	0.148 l	0.189 e
HS3	0.216 g	0.230 de	0.142 m	0.196 c
HS4	0.199 i	0.235 c	0.143 m	0.192 d
HS5	0.182 j	0.235 cd	0.131 n	0.182 f
HS6	0.225 f	0.216 g	0.246 b	0.229 b
HS7	0.228 ef	0.209 h	0.158 k	0.198 c
HS8	0.236 c	0.255 a	0.249 b	0.247 a
HS9	0.089 p	0.179 j	0.079 q	0.116 i
HS10	0.118 o	0.141 m	0.093 p	0.118 i
Mean (A)	0.182 b	0.212 a	0.143 c	

PS1= STS at 0.4 ppm + sucrose 10%, PS2= AgNO3 at 10.0 ppm + sucrose 10%, HS10 sucrose 4%, HS2= boric acid at 200 ppm + sucrose 4%, HS3= 8-HQS at 300 ppm + sucrose 4%, HS4= AOA at 250 ppm + sucrose 4%, HS5= 8-HQS + AOA + sucrose 4%, HS6= boric acid + 8-HQS + sucrose 4%, HS70= boric acid + AOA + sucrose 4%, HS8= boric acid + 8-HQS + AOA + sucrose 4%, HS9= rosemary extract at 25% + sucrose 2%, HS10= thyme extract at 25% + sucrose 2%.

et al., 1991). Hence, eliminating ethylene production and microorganism accumulation is a vital procedure to prolong the vase life of cut carnations. All solutions containing sucrose showed a great influence compared to control (distilled water only) or solutions with only 2.0% sucrose. This observation aligns with the well-known fact that sucrose, as a source of sugar, plays a crucial role in preservative solutions. The use of sucrose in pulsing solution or as a constituent of vase solution may extend the vase life of the flowers by improving the water balance, stimulating flower opening or by delaying the senescence due to lower synthesis of ethylene, as observed in cut carnation (Finger and Barbosa, 2006).

This study clearly highlights the significance of using integrated silver thiosulfate (STS) in post-harvest solutions. In this regard, treatment with silver thiosulfate complex (STS) delayed the senescence of attached and detached petals of *Dianthus caryophyllus* cv. Barbara (Ichimura and Niki, 2014). STS is highly mobile in the xylem of carnation flowers and may become a practical treatment for carnation flowers (Reid and Kofranek, 1980). Hashemabadi (2014) revealed that STS treatment extended the longevity of cut carnation 'Tempo' flowers by reducing oxidative stress, improving the antioxidant system, reducing bacterial populations and delaying flowering. Chemicals such as STS, are also effective at the receptor level and prevent the binding of ethylene (Ebrahimzadeh *et al.*, 2008).

Using $AgNO_3$ in a pulsing solution of carnation cut flowers showed a great influence. In this regard,

Table 8 - Effect of pulsing and holding solutions and their interaction on total sugars (%) of Dianthus caryophyllus cv. Turbo cut flowers during 2020 and 2021 seasons

Holding solutions (B)	Pulsing solutions (A)			
	PS1	PS2	DW	Mean (B)
	First season (2020)			
Control (DW)	20.43 o	18.89 p	9.83 t	16.38 i
HS1	23.29 n	22.69 n	23.30 n	23.09 h
HS2	29.19 jk	23.43 n	29.95 j	27.52 f
HS3	40.87 d	39.72 de	30.28 ij	36.96 b
HS4	30.16 ij	33.40 g	27.68 l	30.41 e
HS5	25.79 m	28.75 kl	21.26 o	25.27 g
HS6	35.63 f	31.12 hi	39.01 e	35.25 c
HS7	31.72 h	30.00 ij	32.91 g	31.55 d
HS8	47.70 b	52.01 a	42.89 c	47.53 a
HS9	11.02 s	12.82 r	10.55 st	11.46 k
HS10	13.04 r	12.24 r	16.22 q	13.83 j
Mean (A)	28.08 a	27.73 a	25.81 b	
	Second season (2021)			
Control (DW)	21.39 o	19.20 p	9.98 u	16.86 h
HS1	24.71 lm	23.77 mn	22.75 no	23.74 g
HS2	31.08 hi	24.35 l-n	31.10 hi	28.84 e
HS3	42.73 c	40.24 de	31.64 g-i	38.20 b
HS4	29.13 jk	33.06 g	29.10 jk	30.43 d
HS5	25.64 l	27.55 k	21.87 o	25.02 f
HS6	34.95 f	31.76 g-i	38.94 e	35.22 c
HS7	30.38 ij	28.63 k	32.61 gh	30.54 d
HS8	48.48 b	54.67 a	41.86 cd	48.34 a
HS9	11.23 s-u	12.88 rs	11.10 tu	11.74 j
HS10	14.43 qr	12.06 st	16.01 q	14.17 i
Mean (A)	28.56 a	28.02 b	26.09 c	

PS1= STS at 0.4 ppm + sucrose 10%, PS2= AgNO3 at 10.0 ppm + sucrose 10%, HS10 sucrose 4%, HS2= boric acid at 200 ppm + sucrose 4%, HS3= 8-HQS at 300 ppm + sucrose 4%, HS4= AOA at 250 ppm + sucrose 4%, HS5= 8-HQS + AOA + sucrose 4%, HS6= boric acid + 8-HQS + sucrose 4%, HS70= boric acid + AOA + sucrose 4%, HS8= boric acid + 8-HQS + AOA + sucrose 4%, HS9= rosemary extract at 25% + sucrose 2%, HS10= thyme extract at 25% + sucrose 2%.

$AgNO_3$ is used as an antimicrobial, since the Ag^+ ion replaces the hydrogen cations (H^+) on surface proteins in the cell membranes of bacteria, which leads to loss of membrane integrity and causes cell death (Feng *et al*., 2000).

This study exhibited a positive influence of 8-HQS addition to the holding solution in extending vase life, enhancing water relations (uptake and balance) and chemical constituents of cut carnation flowers, this effect may be explained by the positive role of 8-HQS as a germicide agent. Preservative solutions of carnation cut flowers containing 8-HQS showed a strong inhibitory effect on fungi, yeasts and bacteria (El-Ashwah, 2011). Numerous authors have corroborated this fact, supporting the use of 8-hydroxyquinoline sulfate (8-HQS) in vase solutions to effectively reduce microbial counts. One such study by Madhuri *et al.* (2016) demonstrated that the addition of 8-HQS resulted in the lowest microbial counts in vase solutions. Kabari and Soleimandarabi (2019) on Alstroemeria cut flowers observed the lowest bacterial population in vase solution in the treatment of 200 8-HQS mg/l. In addition, 8-HQS is considered an ethylene synthesis inhibitor (Ebrahimzadeh *et al.*, 2008).

To explain the positive role of AOA in the present study, Son *et al.* (1995) reported that AOA appeared to inhibit the activities of arginine decarboxylase and ACC synthase. Ethylene production was significantly decreased by AOA at concentrations over 100 mg/l, the decline in ACC content was observed after using 100 or 150 mg/l AOA (Karimi *et al.*, 2012). Aminooxyacetic acid (AOA) inhibits the synthesis of ethylene by reducing the competitive and irreversible activity of 1-Aminocyclopropane-1-carboxylic acid (ACC)

synthase, reducing the amount of substrate for ACC oxidase, and therefore the conversion of ACC to ethylene (Finger and Barbosa, 2006).

Boric acid also showed a promising influence when it was integrated into the holding solution of cut carnation flowers, this could be explained by its role in preventing the early rise in ethylene production and considerably improving carnation vase life (Serrano *et al.*, 2001).

While many studies have highlighted the positive impact of natural extracts as a component in preservative solutions, such as El-Ashwah (2011) on carnation cv. Domingo, Khenizy *et al.* (2014) on *Gypsophila paniculata* L. "Perfecta,", Zaky *et al.* (2014) on carnation cv. America, and Hashemabadi *et al.* (2017) on carnation cv. White Liberty, our study did not observe a similar effect. The lack of satisfactory results in our study regarding the effectiveness of rosemary and thyme extracts may be attributed to the fact that these extracts were used in combination with sucrose at 2.0% only, without the addition of any germicidal agents to the solution. The presence of sugar in the solution without germicidal agents can promote the growth of microorganisms, since these extracts alone may not possess sufficient biocidal properties to effectively control microorganisms (Armitage and Laushman, 2003). Therefore, the outcomes of using natural extracts in our study were not as promising. In future research, it is recommended to consider incorporating germicidal agents along with these extracts, particularly when using extracts obtained through water extraction methods. This approach can help enhance the antimicrobial efficacy and overall performance of natural extracts as preservatives in cut flower solutions.Adam

In conclusion, it is highly recommended to pulse carnation cv. Turbo cut flowers in a solution containing $AgNO_3$ at 10.0 ppm + sucrose 10% for a duration of 15 minutes, followed by holding them in a solution of BoA + 8-HQS + AOA + Suc 4% to extend the vase life of the flowers, enhancing water uptake and maintaining water balance. Additionally, this preservative solution effectively reduces chlorophyll degradation and preserves the content of carbohydrates throughout the postharvest period.

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Adv. Hort. Sci., 2023 37(3): 271-280 DOI: 10.36253/ahsc-13827

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Pruning terms and techniques affect vigour and flower formation of Ukrainian sweet cherry cultivars

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OPEN ACCESS

Key words: canopy, flower, heading cut, pruning severity, shoot, stub, thinning cut.

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Citation:
BONDARENKO P., ALEKSEEVA O., SENIN V., KONDRATENKO P., 2023 - *Pruning terms and techniques affect vigour and flower formation of Ukrainian sweet cherry cultivars.* - Adv. Hort. Sci., 37(3): 271-280.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 30 September 2022
Accepted for publication 16 June 2023

Abstract: Excessive tree vigour and late entrance into full production, inherent to sweet cherry trees, are major challenges in the intensive cultivation of this crop. Possible ways to reduce the vigour and stimulate flower induction include shifting the term of pruning and reducing its severity. However, the reaction of the trees may differ depending on specific cultivar, soil and climatic conditions. Therefore, the aim of the study was to determine the effect of various techniques and terms of pruning on young sweet cherry trees in order to adapt the intensive cultivation technology to the arid conditions of southern Ukraine. The results showed a strong cultivar-specific reaction to various pruning treatments. Pruning young sweet cherry trees in late summer contributed to a reduction of trunk and canopy indices by 11-22% on one of the cultivars and an increase in the number of flowers per tree by 1.4-1.7 times on both cultivars, compared to dormant pruning. Low severity pruning reduced 1-year-old shoot length by 9-25% and increased the number of flowers by 1.5-2.5 times compared to more aggressive pruning. The effect of pruning treatments on tree vigour was more pronounced during the first and second year of their application.

1. Introduction

Sweet cherry (*Prunus avium* L.) is one of the most important stone fruit crops in the world, its annual gross production has increased by 1.4 times over the last 20 years and reached 2.6 million t in 2020 (FAO, 2020). Ukraine is one of the main sweet cherry producing countries, with annual production volume ranging between 60-85 thousand t, with 60% of industrial production concentrated in the south-eastern region (State Statistic Service of Ukraine, 2020). There is a well-developed extensive traditional

cultivation technology of the crop in the region that utilizes Mahaleb seedlings as rootstock, a central leader training system, and plant density of 200-350 trees ha^{-1} (Rulyev, 2003).

The main drawback of such cultivation technology is late entry of the trees into production and, as a result, late return of initial capital investment. Additionally, modern export markets for Ukrainian sweet cherry show increased requirements for the size and overall quality of the fruits. It is well known that the highest quality cherries are formed on young wood (Dolya, 2011; Claverie and Lauri, 2005). Trees with large canopies, characteristic for traditional cultivation technology, are not always able to ensure timely fruiting wood renewal, and thus, good fruit quality.

These factors contribute to the fact that sweet cherry cultivation technology in Ukrainian orchards is being intensified. Orchards with trees grafted on dwarfing rootstocks are not always commercially successful due to the low adaptivity of those rootstalks to the arid continental climate of southern Ukraine. Therefore, new orchards utilising interstems and rootstocks of medium and high vigour with a density of 667-1200 trees ha^{-1} to promote precocity may be a better alternative (Kishchak *et al.*, 2020; Bondarenko, 2018).

It should be noted that currently there are no well-developed pruning technologies for such orchards in Ukraine, and direct use of foreign techniques is ineffective and requires adaptation to the specific climate and soil conditions in the cultivation region. The main challenge when cultivating high density orchards is controlling the vigour and the size of tree canopies. Aggressive annual pruning in order to keep tree canopies within the limits of the planting scheme can cause strong vigour reaction, reduce flower bud initiation, and delay fruiting (Lang, 2005).

Different agronomic techniques can be used in order to reduce the vigour of sweet cherry trees. Application of growth regulators contributes to reduction in shoot length and increases the number of generative buds on a tree (Jacyna *et al.*, 2012; Elfving *et al.*, 2003). Root pruning can also be effective in reducing vigour (Pal and Mitre, 2016; Webster *et al.*, 1997). Another promising and easily applied method is shifting the term of tree pruning. In Ukraine, pruning in the second half of the growing season is being actively used in apple orchards (Melnyk and Mulienok, 2020; Chaploutskyy and Melnyk, 2015), and in the world - for sweet cherry as well (Blažková and Drahošová, 2012). In addition to potential decrease in vigour due to removing a part of assimilation area prematurely, pruning trees in August-September, compared to traditional pruning during dormancy, has an advantage that wounds from the cuts heal faster and trees are more resistant to pathogens, especially those of bacterial aetiology (Spotts *et al.*, 2010; Colhoun *et al.*, 2015). While scientific data on the degree of spread of bacterial diseases in Ukrainian orchards is insufficient (Patyka *et al.*, 2016), farmers report visual manifestations of bacterial diseases in most sweet cherry plantations (pers. comm.), underlining the importance of using agronomic measures, including pruning, to contain the infection. It is also indicated that summer pruning can reduce winter frost damage to generative organs for certain sweet cherry cultivars (Vaszily *et al.*, 2011).

Another challenge in intensive sweet cherry cultivation is the need of regular fruiting wood renewal in order to maintain yields and fruit quality. Inherently, sweet cherry has low regeneration and shoot-formation ability, further complicating this process for the farmer. Therefore, it is often recommended to avoid thinning cuts on 1-year-old wood and to instead use stub cutting to preserve a bigger number of growth points on the tree. Also, in order to stimulate spur formation, heading cuts can be avoided. That, however, reduces the number of new shoots in the subsequent season and worsens their position on a tree (Long *et al.*, 2015; Mika, 2006). In general, optimal techniques of pruning for intensive sweet cherry orchards in Ukraine are not yet fully determined.

The objective of this study was to determine the reaction of the young sweet cherry trees of different cultivars to various techniques and terms of pruning in order to adapt the intensive cultivation technology to the arid conditions of southern Ukraine.

2. Materials and Methods

Site description

The study was conducted in a commercial sweet cherry orchard located near the city of Melitopol, south-eastern Ukraine (46°80'N, 35°34'E, 38 m a.s.l.). The climate is moderately continental, the mean daily air temperature in January is -3.1°C and in July is +22.8°C; the average annual amount of precipitation is 475 mm. The soil of the experimental site is southern chernozem (black soil), with loam soil texture.

Experimental design

The orchard was planted in late October 2014 using 4.5×2 m planting scheme (1111 trees ha^{-1}) with 1-year-old maiden trees without lateral branches. Trees were grafted on Colt rootstock and trained as spindle canopies with a single central leader. Bud scoring and branch bending were applied to the trees during the first 2 years after planting, where necessary. Different pruning treatments were applied in the orchard starting from the 3rd leaf, and the results of the study in 2017, 2018 and 2019 present the reaction of the trees after 1, 2 and 3 pruning cycles, respectively. The orchard is drip irrigated. Plant protection and fertilisation were carried out in accordance with the recommendations for sweet cherry cultivation in the region.

'Krupnoplidna' and 'Melitopolska Chorna' cultivars were chosen for the study as they are the main cultivars in commercial orchards in southern Ukraine and have distinct growth habits, not similar to each other. Both cultivars are late ripening and were bred in Melitopol Fruit Growing Research Station named after M.F. Sydorenko, Melitopol, Ukraine (Quero-García *et al.*, 2017).

Two pruning terms were studied: during dormancy and in late summer. Dormant pruning is traditional for sweet cherry cultivation in Ukraine, and in the conditions of our experiment was performed in the second half of February in dry weather. Late summer pruning was performed between the 25th and 31st of August. While it is sometimes advised to prune trees earlier, even immediately after harvest (Ayala and Lang, 2017), our previous experience suggested that pruning as late as the 15th of August can cause regrowth, so the term was shifted to eliminate this risk.

Three different pruning severities with various techniques were studied:

- High severity (control). Traditional style of pruning for sweet cherry in Ukraine. Heading cuts are applied to most 1-year-old shoots (either by removing one third of the shoot length or pruning it back to 60 cm if removing one third of the shoot length still leaves more than 60 cm of length); thinning cuts are applied to undesirable 1-year-old shoots that grow straight up or down, overly thicken the canopy, intertwine with other shoots, or hinder tractor movement between the rows. On average, 45-50% of a tree's 1-year-old wood is removed by pruning.
- Medium severity. Heading cuts are applied only to strong 1-year-old shoots longer than 60 cm; stub cuts are applied to undesirable 1-year-old shoots, with stub length of 15-20 cm; thinning cuts are not used. On average, 35-40% of a tree's 1-year-old wood is removed by pruning.
- Low severity. Heading or thinning cuts are not used; stub cuts are applied to undesirable 1-year-old shoots, with stub length of 15-20 cm. On average, 25-30% of a tree's 1-year-old wood is removed by pruning.

In all variants of the experiment, the extension shoot of the central leader was headed to 80 cm every year. For the sake of the experiment, cuts on 2-year-old and older wood were avoided unless absolutely necessary. Pruning in all variants was done manually.

The following variants of the length of the stubs on annual shoots were studied: short stubs with 1-2 buds and long stubs with a length of 20 cm. Those lengths were chosen as the ones that are easy to apply to pruning in industrial orchards (20 cm is roughly the length of the pruning shears).

Every combination of cultivar, pruning term, pruning severity, and stub length was replicated 3 times with 3 trees in each replication. The experiment was arranged using randomized block design.

Measurements

Tree canopy parameters, trunk cross-sectional area (TCSA), and number and length of 1-year-old shoots were measured before pruning each year on the 15th of August.

Canopy volume was calculated as the volume of a cone using the following formula:

$$\text{Canopy volume} = 1/3\ (H-0.6)\ \pi\ \frac{(w_1 + w_2)^2}{4}$$

where H = height of the tree, m; 0.6 = distance between the ground and first lateral branch, m; W_1 = maximum width of the tree in the row, m; W_2 = maximum width of the tree across the row, m.

TCSA was measured 30 cm above the grafting point. All 1-year-old shoots longer than 10 cm were measured. Shoots shorter than 10 cm were considered spurs and not included into calculations of the number of 1-year-old shoots per tree and mean shoot length.

Number of flowers per tree was counted during full bloom (15th-25th of April during the years of the research). In the 3rd leaf (2017), both cultivars had only up to 20-30 flowers per tree, so the flowering

data for this year was excluded. Spring frosts during flowering (minimal air temperature in the orchard reached -4.3 C in April 2018 and -7.8 °C in April 2019) damaged up to 95% of pistils, which led to a poor fruit set and a very low number of fruits per tree. Thus, the data on yield and fruit quality was also excluded.

Data analysis

Statistical analysis of the results was conducted using the software Minitab 19 (Minitab Inc., State College, PA). Since the studied cultivars reacted differently to the treatments, a two-way analysis of variance was performed separately for each cultivar, with Tukey's range test with an accuracy of 0.05 carried out post hoc to determine the significant differences between the means. In addition, the cultivars were compared using a one-way analysis of variance with the same post hoc test. The exception was the reaction of trees to stub cutting, which was more uniform among the cultivars, and a single combined analysis of variance was performed. In order to determine the relationship between the indices, Pearson's correlation was used.

3. Results

Trunk cross-sectional area and canopy volume

The results of the experiment indicate different reactions by sweet cherry cultivars to pruning treatments. 'Krupnoplidna' trees inherently have a more spreading growth habit, wider crotch angles with a tendency to form round canopies, and better shoot formation ability at a young age. 'Melitopolska chorna' trees are more upright, with narrow crotch angles, and produce fewer shoots.

As a result, low pruning severity with no heading cuts allowed 'Krupnoplidna' trees to increase their canopy volume faster, exceeding the variant with high pruning severity by 35% in 2017 and 12-16% in 2018 and 2019 (Table 1). For 'Melitopolska Chorna' trees, pruning severity had no effect on canopy volume.

Pruning severity had a different effect on tree trunks depending on cultivar. 'Krupnoplidna' trees had higher TCSA and annual increase in TCSA when pruning severity was low, while for 'Melitopolska Chorna' those indices were the highest with aggressive high-severity pruning.

Table 1 - Influence of pruning term and severity on trunk cross-sectional area and canopy volume of sweet cherry trees

Variant	TCSA in 2019 (cm^2)	Annual increase in TCSA (cm^2)	Canopy volume (m^3)		
			2017	2018	2019
		'Krupnoplidna'			
Pruning severity					
High (c)	80.8 b	23.2 b	4.8 b	7.5 b	10.4 b
Medium	90.3 ab	26.2 ab	6.0 a	7.4 b	11.5 ab
Low	95.2 a	28.3 a	6.5 a	8.4 a	12.1 a
Pruning term					
Dormancy	93.3 a	26.6 a	5.9 a	8.7 a	11.6 a
Late summer	84.2 b	25.2 a	5.6 a	6.8 b	11.0 b
		'Melitopolska Chorna'			
Pruning severity					
High (c)	92.0 a	27.9 a	3.3 a	5.2 a	9.8 a
Medium	80.6 b	23.9 b	3.3 a	5.2 a	9.7 a
Low	76.8 b	22.2 b	3.4 a	5.4 a	9.8 a
Pruning term					
Dormancy	83.3 a	24.6 a	3.2 a	5.2 a	9.7 a
Late summer	83.0 a	24.8 a	3.4 a	5.4 a	9.7 a
Cultivar comparison					
Krupnoplidna	88.7 a	25.9 a	5.8 a	7.8 a	11.3 a
Melitopolska Chorna	83.2 a	24.7 a	3.3 b	5.3 b	9.7 b

TCSA = Trunk cross-sectional area.
Different letters within the same group indicate significant difference between the means according to Tukey's test ($p<0.05$).

Pruning 'Krupnoplidna' trees in late summer decreased tree vigour, with an 11% decrease in TCSA and lower canopy volume, most notably by 22% in 2018, compared to pruning during dormancy. These indices were not significantly different when comparing the effect of different pruning terms on 'Melitopolska Chorna' trees.

Due to growth habit differences, 'Melitopolska Chorna' trees had more compact canopies compared to 'Krupnoplidna', especially at a younger age: the difference of canopy volumes between the cultivars was 1.8 times in 2017, 1.5 times in 2018 and 1.2 times in 2019. Cultivars had no significant influence on TSCA of trees in the trial.

1-year-old shoot parameters

It was determined that low and medium pruning severity led to an 11-23% increase in the number of 1-year-old shoots per 'Krupnoplidna' tree in the first two years of the research (Table 2). After 3 cycles of pruning, however, this indicator levelled off among all variants. Pruning severity had no effect on the number of shoots on 'Melitopolska Chorna' trees.
Low pruning severity without heading or thinning cuts decreased the mean length of 1-year-old shoots by 9-25% on both studied cultivars, compared to traditional pruning techniques. For 'Krupnoplidna' trees, this effect appeared most strongly in the year following the first application of such pruning, while for 'Melitopolska Chorna' the shoot length decrease was more apparent starting from the second pruning cycle.

Pruning term had little effect on the number of shoots per tree, regardless of cultivar. The only statistically significant difference was observed for 'Melitopolska Chorna' in 2019, indicating that trees pruned during dormancy retained more vigour. Late summer pruning reduced the mean shoot length of 'Krupnoplidna' trees by 10-12% during the first two years of the research.

In general, the cultivar comparison highlights that 'Krupnoplidna' trees formed more new shoots per tree, especially in the first years after planting, whereas 'Melitopolska Chorna' trees formed more vigorous longer shoots.

Flower formation

Both the severity and term of pruning had a significant effect on flower formation in the orchard. In the case of 'Krupnoplidna', both low and medium prun-

Table 2 - Influence of pruning term and severity on shoot parameters of sweet cherry trees

Variant	Number of 1-year-old shoots per tree			Mean length of a 1-year-old shoot (cm)		
	2017	2018	2019	2017	2018	2019
			'Krupnoplidna'			
Pruning severity						
High (c)	62 b	131 b	239 a	77.7 a	51.1 a	51.4 a
Medium	73 a	146 ab	218 a	69.9 a	45.2 ab	44.3 b
Low	71 a	162 a	218 a	60.9 b	42.4 b	45.0 b
Pruning term						
Dormancy	66 a	136 b	232 a	73.2 a	49.1 a	46.6 a
Late summer	71 a	157 a	218 a	65.8 b	43.4 b	47.3 a
			'Melitopolska Chorna'			
Pruning severity						
High (c)	39 a	80 a	206 a	77.5 a	64.8 a	62.2 a
Medium	38 a	78 a	185 a	75.4 a	61.3 a	59.0 a
Low	36 a	78 a	180 a	70.2 b	48.9 b	49.4 b
Pruning term						
Dormancy	37 a	79 a	212 a	73.7 a	57.8 a	58.6 a
Late summer	38 a	79 a	169 b	75.0 a	58.8 a	55.2 a
Cultivar comparison						
Krupnoplidna	68 a	146 a	225 a	69.5 b	46.2 b	46.9 b
Melitopolska Chorna	38 b	79 b	190 b	74.3 a	58.3 a	56.9 a

Different letters within the same group indicate significant difference between the means according to Tukey's test ($p < 0.05$).

ing severity increased the number of flowers per tree on average by 1.5-1.7 times compared to high pruning severity (Fig. 1). For 'Melitopolska Chorna', only low pruning severity with no heading and thinning cuts affected the number of flowers per tree. The increase, however, was more significant - by 2.5 times on average.

Pruning the orchard in late summer promoted flower formation for both cultivars. Summer-pruned trees on average formed 1.4 and 1.7 times more flowers for 'Krupnoplidna' and 'Melitopolska Chorna' cultivars respectively, compared to dormant pruning. It should be noted that regardless of pruning treatments, based on the number of flowers per tree, 'Krupnoplidna' trees entered production during the 4th leaf (2018) while 'Melitopolska Chorna' trees still could not be considered bearing even in the 5th leaf (2019).

Stub length

Stub length of 1-year-old shoots significantly influenced the proportion of stubs that produced new shoots in the subsequent season. This index was 1.2 times higher on long stubs compared to short stubs (Table 3). Long stubs also formed 1.6 times more shoots per stub, which can be explained by a much higher number of buds on them compared to shorter stubs with only 1-2 buds. Stub length did not affect mean length of the new shoots on stubs, while pruning term did not significantly influence any of the studied parameters.

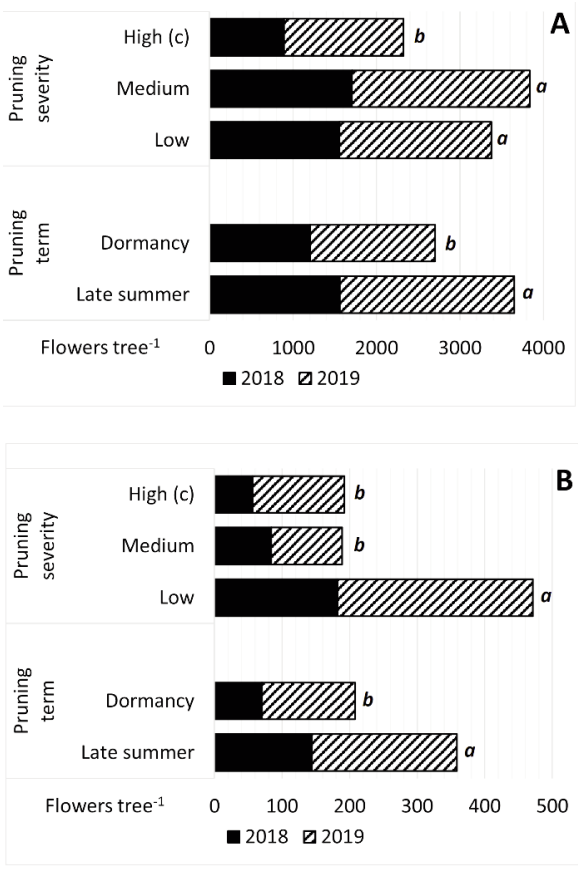

Fig. 1 - Influence of pruning term and severity on the number of flowers formed on sweet cherry trees of 'Krupnoplidna' (A) and 'Melitopolska Chorna' (B) cultivars. Note the difference of scale between the graphs. Different letters within the same group indicate significant difference between the means according to Tukey's test ($p<0.05$).

Table 3 - Reaction of sweet cherry trees to stub cutting

Variant	Proportion of stubs that produced shoots next year (%)	Number of 1-year old shoots per stub	Mean length of a 1-year-old shoot on a stub (cm)
Stub length			
Short (1-2 buds)	82 b	1.4 b	62.7 a
Long (20 cm)	96 a	2.2 b	67.7 a
Pruning term			
Dormancy	89 a	1.8 a	63.4 a
Late summer	90 a	1.8 a	67.0 a
Tree age			
3rd leaf (2017)	93 a	1.8 a	75.7 a
4th leaf (2018)	90 ab	1.8 a	58.1 b
5th leaf (2019)	85 b	1.8 a	61.8 b
Cultivar			
Krupnoplidna	85 b	1.9 a	62.3 b
Melitopolska Chorna	94 a	1.7 a	68.1 a

Different letters within the same group indicate significant difference between the means according to Tukey's test ($p<0.05$).

The proportion of stubs that produced new shoots decreased over the duration of the trial. As trees got older and more points of growth were formed throughout the tree canopy, new stubs were slightly less likely to form new growth. The number of shoots per stub, however, was not influenced by the tree age. Mean shoot length on the stubs followed the same tendencies as this index for the whole tree, being the highest in 2017 and decreasing by 18-23% in each subsequent year.

Stub cuts on 'Melitopolska Chorna' trees had a higher chance to produce new growth the next year, and those shoots were longer, compared to 'Krupnoplidna' trees.

4. Discussion and Conclusions

One of the main takeaways of our study is a strong cultivar-specific reaction to various pruning treatments. Most of the vigour indices were affected by pruning more significantly when it was applied to the trees of the cultivar 'Krupnoplidna', which is characterized by spreading round canopies and a higher ability to produce new shoots compared to the more compact upright canopies and fewer shoots formed per tree of 'Melitopolska Chorna' cultivar. This proves the importance of a cultivar-based approach to the choice of optimal training systems, plant density and pruning measures in intensive sweet cherry orchards (Long *et al.*, 2021).

The effect of different pruning severities on trunk growth was inconsistent among the studied cultivars. Other research on this topic shows similar results: exposure of trees to low severity pruning or no pruning at all can lead, depending on the cultivar studied, to TCSA decrease, increase, or no change in trunk parameters (Usenik *et al.*, 2008; Radomirska and Domozetova, 2017; Zec *et al.*, 2020). Summer pruning decreased TCSA and canopy volume for 'Krupnoplidna' trees, in comparison with dormant pruning. This can be explained by the decreased length of the shoots in this variant, leading to more compact canopies. A similar effect was observed in other studies for sweet cherry (Blažková and Drahošová, 2012), sour cherry (Gonda, 2006), peach (Ikinci *et al.*, 2014), but not plum (Sosna, 2010).

The number of shoots formed on the tree was largely influenced by cultivars and tree age. An initial increase in shoot formation observed on 'Krupnoplidna' trees in variants with low and medium severity appeared mainly in multiple new shoots forming near the terminal end of the previous-year shoots. These types of branches with long sections of spurs and new growth only at terminal points may be problematic from an agronomic point of view, as sweet cherry spurs become less productive and die relatively quickly with age, especially in suboptimal lighting conditions, resulting in blind wood (Ayala and Lang, 2017; Bondarenko and Alekseeva, 2020). Renewing such branches by stub cutting can also be ineffective, particularly in the lower zones of the canopy and when trees are older (Stan, 2015; Hansen and Black, 2019).

Our study observed a reduction of shoot length on the trees pruned less severely, which was also documented in other studies (Usenik *et al.*, 2008; Villasante *et al.*, 2012), and may be explained by better nitrogen use efficiency by extension shoots on pruned branches (Ayala *et al.*, 2018). 'Krupnoplidna' trees also had decreased values of this indicator when pruned in late summer compared to dormancy. A similar effect was observed for peach, where shifting the pruning term further (June - July - August - September) progressively decreased both the diameter and mean length of new shoots in the subsequent season (Ikinci *et al.*, 2014).

It should be noted that the effect of pruning treatments on most of the parameters of tree vigour was more pronounced during the first and second cycles of pruning. During the third year of the research, those indices were either statistically non-significant among treatments, or, at least, less pronounced due to the trees adapting to the treatments and exhibiting their inherent growth habit. So, from an agronomic point of view, if the vigour in the orchard is excessive, shifting the pruning to late summer or removing less wood during pruning may be a valuable short-term technique in reducing tree vigour and increasing precociousness. In the long term, however, vigour is more dependent on specific rootstock and cultivar combination than on pruning measures.

Both late summer pruning and lower pruning severity positively contributed to flower formation on sweet cherry trees. In the case of the pruning term, it is reported that pruning in August led to an increase in carbohydrate content in sweet cherry flower buds in the outer and upper part of the tree canopy, compared to dormant pruning (Vosnjak *et al.*, 2021). Late summer pruning also shifts source-sink relations in the tree, promoting flower bud initi-

ation, as there is a strong adverse correlation between bud initiation and vigour (Flore and Layne, 1999). Lower pruning severity with fewer heading cuts and no thinning cuts increased the number of flowers, which can be attributed to an overall increase in the points of growth on the trees, as well the fact that weaker, less vigorous shoots were formed in these variants. These points are supported by the fact that in the conditions of our trial, a negative correlation was observed between the mean shoot length in the previous growing season and the number of flowers per for both 'Krupnoplidna' (r= -0.623 in 2018 and r= -0.526 in 2019) and 'Melitopolska Chorna' (r = -0.550 in 2018 and r = -0.683 in 2019) cultivars. For 'Krupnoplidna', a positive correlation was also found between the number of shoots in the previous growing season and number of flowers per tree (r= 0.717 in 2018 and r = -0.593 in 2019). No such relationships were observed for other studied vigour indices.

The results of this study are consistent with other trials on sweet cherry that report a yield increase when trees were unpruned or lightly pruned (Villasante *et al.*, 2012; Claverie and Lauri, 2005). However, it should be noted that when trees enter full bearing, low pruning severity has a negative effect on fruit size and thus marketability of the yield (Gonkiewicz, 2011; von Bennewitz *et al.*, 2011; Ayala *et al.*, 2018), so more aggressive pruning may be needed to manage crop load and maintain fruit quality.

Cultivar genotype had a bigger effect on the number of flowers on the tree than any pruning treatments, further proving that a cultivar-based approach is essential for the cherry cultivation technology to be successful.

The reaction of the trees to stub cutting was more uniform among the studied cultivars compared to other treatments in this study. In general, both short and long stubs consistently produced new shoots in the subsequent season and mean shoot length was not affected by stub length. As the trees got older, the share of the dead shoots increased, mostly for short stubs, but was still minor. Another study, in which trees were grafted on a dwarfing rootstock, indicates that removing a large portion of the current year's shoots can lead to much larger proportion of dead shoots - up to 50-70% (Usenik *et al.*, 2008). Our further research of stub cutting of 1-year-old shoots will focus on the effect of this technique in different parts of the canopy (stubs in lower and upper zones of the tree, on the central leader and on lateral branches), as stubs of different length often produced mixed results depending on the shoot position in the tree.

In summary, we can conclude that pruning young sweet cherry trees in late summer and reducing pruning severity leads to a decrease in vigour, manifesting itself mostly in the reduction of 1-year-old shoot length, and has a positive effect on flower bud initiation and precociousness. The effect of pruning treatments on tree vigour was more pronounced during the first and second year of their application. Stub cutting of 1-year-old wood is a valuable alternative to thinning cuts: one can preserve more points of growth on the tree, and new shoots form more consistently in the desired locations of the canopy. The specific cultivar's growth habit should always be considered when choosing pruning strategies in the orchard.

Acknowledgements

This research did not receive any specific funding. The authors thank I. Hryntsiv for providing the orchard for the trial, O. Nosachenko, M. Shevchenko and V. Topov for their help with pruning and field data collection, and R. Williams for improving the quality of English in the manuscript.

P.B. is personally grateful to Laimburg Research Centre staff for providing him shelter during the Russian aggression against Ukraine and making the preparation of this article possible.

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Adv. Hort. Sci., 2023 37(3): 281-287 DOI: 10.36253/ahsc-14018

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Container volume and doses of maximum technical efficiency of controlled-release fertilizer on *Cordia alliodora* seedlings

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Key words: Dickson index, Freijó, forest nursery, Nitrogen.

Abstract The objective of this study was to determine the correlation between the morphological characteristics of *Cordia alliodora* seedlings produced as a function of container volume and controlled-release fertilizer (CRF) doses under nursery conditions in Northern Amazon. The experimental design was a 2 x 6 factorial scheme, corresponding to two container volumes (1.8 and 2.2 L) and six doses of Forth Cote® (0, 1, 2, 4, 8, and 12 g L^{-1} in medium sand), with five replicates. The evaluations were: height (H), stem diameter (SD), shoot dry mass (SDM), root dry mass (RDM), total dry mass, increment in stem diameter (ΔSD) and increment in height (ΔH) obtained from the data collected every fifteen days, from transplanting, encompassing the period of plant growth until the end of the experiment (three months), in addition to Dickson quality index (DQI). Container volume of 2.2 L is suitable for the formation of good-quality *Cordia alliodora* seedlings at 90 days after transplanting. Controlled-release fertilizer doses from 8.0 g L^{-1} are not indicated to obtain seedlings of this species in the northern region of Brazil, with quality, regardless of the container volume.

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Citation:
SOUZA A.G., SMIDERLE O.J., 2023 - *Container volume and doses of maximum technical efficiency of controlled-release fertilizer on Cordia alliodora seedlings.* - Adv. Hort. Sci., 37(3): 281-287.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 30 November 2022
Accepted for publication 30 August 2023

1. Introduction

The forest-based sector can be described as an important component of the Brazilian economy, because it contributes significantly to the generation of products, taxes, jobs and income (Smiderle *et al*., 2021 a). The growing expansion of this sector has driven forest investors to opt for the cultivation of native species in the state of Roraima (Smiderle *et al*., 2022). In this scenario, there arises a challenge to meet the demand for seedlings of high-quality standard and adequate nutritional status for the implementation of reforestation for economic purposes.

Among the potential species for the implementation of reforestation in the Northern region of Brazil, *Cordia alliodora* Ruiz & Pavon (Boraginaceae), popularly known as 'freijó' and 'louro-freijó', component

of the local flora of the state of Roraima, stands out (Smiderle and Souza, 2022). The species lacks information in the national and international literature that can provide important methods and techniques for producing seedlings in quantity and quality (Massad *et al*., 2017).

Regarding the growth of *Cordia alliodora*, Smiderle and Souza (2022) reported that under favorable conditions plants in the seedling stage can reach growth in height of approximately two meters in the first year in the field. In turn, height increments occur between the second and tenth year, and maturity is reached between 5 and 10 years (Smiderle and Souza, 2022). Smiderle *et al*. (2021 b), in studies with native forest species of Roraima, determined that their initial growth is slow and they require more time in the nursery to reach desirable minimum size, which in turn induces the use of larger containers, as well as the addition of adequate fertilization.

Controlled-release fertilizers (CRF) have been described as an alternative to conventional fertilizers for fertilization of seedlings in the nursery phase, because they induce rapid initial growth (Wang *et al*., 2016; AO *et al*., 2018; Shi *et al*., 2019) and favor survival and vigor after the field planting phase (Fu *et al*., 2017; Shi *et al*., 2019). Currently, research has shown a positive effect of controlled-release fertilizers on the production of native seedlings in northern Brazil. For instance, Smiderle *et al*. (2020) worked with CRF and container size and concluded that the CRF dose of maximum technical efficiency of 4.71 g L^{-1} in medium sand substrate under container volume of 2.2 L promotes greater increments of shoots, stem diameter and biomass of *Agonandra brasiliensis* Miers ex Benth. & Hook. f. seedlings.

For some species native to northern Brazil, controlled-release fertilizer has shown negative influence, as observed by Smiderle *et al*. (2022) when evaluating *Hymenaea courbaril* L. seedlings in a screened nursery. These authors reported that controlled-release fertilizer doses greater than 6.0 g L^{-1} are not indicated to obtain seedlings suitable for planting in the field and with quality. Likewise, Mota *et al*. (2021) also revealed that CRF doses above 8.0 g L^{-1} induce reduction in the variables related to the root system of pau-marfim plants.

Studies of this nature indicate the need for research related to the appropriate doses of CRF, in NPK 18-05-09 formulation, as well as the appropriate container volume for producing seedlings of native forest species of Roraima, which need to be determined. In view of the above, the objective was to correlate the morphological characteristics of *Cordia alliodora* seedlings produced as a function of container volume and controlled-release fertilizer doses under nursery conditions in Northern Amazon, aiming to obtain quality seedlings.

2. Materials and Methods

The seeds of *Cordia alliodora* used to obtain the seedlings were collected from trees located at Embrapa Roraima (2°45'22" North latitude, 60°43'55" West longitude and altitude of 80 m), located beside the BR-174 highway, km 8, in the municipality of Boa Vista, state of Roraima, Brazil.

After obtaining the seeds, they were manually processed and then sown in a bed containing washed sand of medium particle size as substrate for seedling emergence. The moisture of the sand substrate was maintained through automated irrigation, with four daily waterings. To irrigate the plants, the field capacity for the amount of substrate was determined before the experiment (1.8 and 2.2 litres); this was then taken as the reference for maintaining the supply of water to the plants throughout the experimental period.

Approximately 12 days after sowing, the seedlings began to emerge and, as soon as they homogeneously reached an approximate height of 5.0 cm, they were transplanted to polyethylene bags containing medium sand as substrate (inert material) and controlled-release fertilizer (Forth Cote®), in NPK 18-05-09 formulation, was incorporated into the surface, according to treatment. Then, the plants were arranged in a screened nursery with 50% shading and maintained under sprinkler irrigation three times a day for periods of 5 min.

The experimental design adopted was completely randomized in a 2x6 factorial scheme, corresponding to two container volumes (1.8 and 2.2 L of substrate) and six doses of Forth Cote® (0; 1; 2; 4; 8 and 12 g L^{-1} of fertilizer), with five replicates, each consisting of five seedlings (one in each container).

The morphological attributes evaluated at 90 days after transplantation were: stem diameter (SD, in mm) (5 cm from the plant collar, determined with a digital caliper), shoot height (H) (from the sand level to the seedling apex, measured with a graduated ruler, in cm), and survival rate (%).

Subsequently, the plants were collected and divided into roots and shoots (stem and leaves), and then dried in an oven with forced air circulation, at 70± 5°C, until reaching constant weight. After drying, the dry mass of the different plant parts was individually determined: shoot dry mass (SDM, g plant^{-1}) and root dry mass (RDM, g plant^{-1}), which were summed to obtain the total dry mass (TDM, g plant^{-1}). The results allowed the calculation of the Dickson quality index (DQI), following the formula proposed by Dickson *et al*. (1960).

$$DQI = \frac{TDM\ (g)}{[(H/SD)+ (SDM/RDM)]}$$

The increment in stem diameter (ΔSD) and the increment in height (ΔH) were obtained from the data collected every fifteen days, during the growing period of the plants, from transplanting to the end of the experiment.

Possible differences between treatments were checked by analysis of variance (ANOVA) in factorial scheme. Variables that showed significant differences were subjected to regression analysis in order to assess the growth response of the plants as a function of the increasing doses of CRF for the two container volumes. The dose of maximum technical efficiency (DMTE) was calculated using the equation proposed by Tiesdale *et al*. (1993) ($y=ax^2+bx+c$) and by the mathematical model x = -b/2a. Data analysis was performed with Sisvar statistical software (Ferreira, 2014).

3. Results and Discussion

At the end of the experiment (90 days after transplanting), the survival rate of *Cordia alliodora seedlings* was 100% for all treatments.

All morphological variables evaluated showed quadratic behavior in the fit of the regression equations. Figure 1A shows the height (H) of seedlings cultivated with the CRF dose of maximum technical efficiency of 4.54 g L^{-1}, corresponding to 24.30 cm, representing an increase of 17.7% when compared to H of plants cultivated without the addition of CRF. Smiderle *et al*. (2020), in a study conducted with paumarfim (*Agonandra brasiliensis*) seedlings in substrate, also recorded positive quadratic response for all morphological variables evaluated according to the increase in CRF doses up to dose of maximum

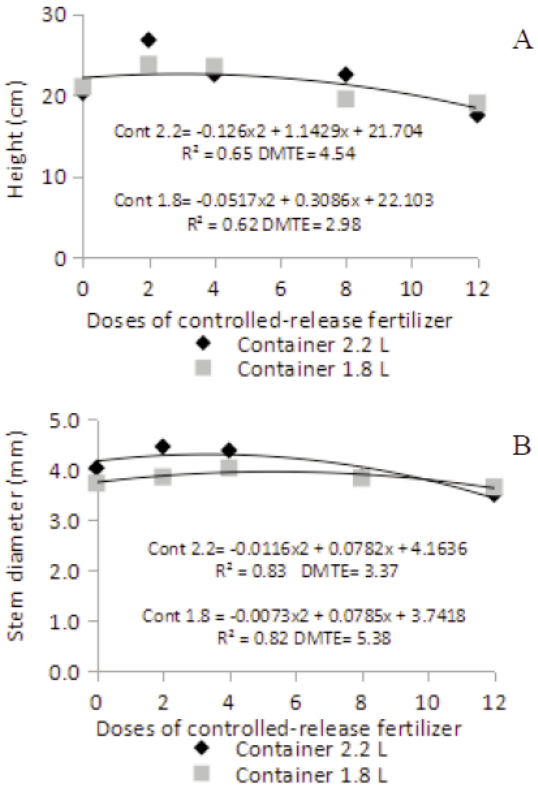

Fig. 1 - Combinations of CRF doses and container volume on the height (A) and stem diameter (B) of *Cordia alliodora* seedlings produced under nursery conditions, Boa Vista, RR.

technical efficiency (DMTE), suggesting that the application of doses higher than DMTE does not guarantee the absorption and mainly utilization of nutrients by the plant. In addition, DMTE for the 1.8 L container was 2.98 g L^{-1} of CRF, resulting in a height of 22.46 cm (Fig. 1B). According to Smiderle *et al*. (2022), shoot height combined with stem diameter is one of the most important morphological parameters to estimate the growth of native forest seedlings in northern Brazil, after definitive planting in the field.

For stem diameter (SD), the maximum estimated value was 4.35 mm at the DMTE of 3.37 g L^{-1} of CRF incorporated into the medium sand substrate in the container with volume of 2.2 L (Fig. 1B). In turn, the 1.8 L container led to the largest stem diameter (3.95 mm) of *Cordia alliodora* seedlings at the DMTE of 5.38 g L^{-1} of CRF (Fig. 1B). Conversely, without addition of CRF (control) the stem diameter was equal to 3.73 mm in the 1.8 L container. Thus, 3.37 g L^{-1} of CRF in the 2.2 L container (Fig. 1B) resulted in stem diameter 0.4 mm (10%) higher than the value found in the

1.8 L container, which even with 2.01 g L^{-1} more was not able to promote the same diameter, with a value 0.62 mm higher than that obtained without application of CRF (3.73 mm), an increase of 16%.

Determining the volume of container and the DMTE of fertilizers allows obtaining the ideal SD for planting the field in a shorter time, so it becomes of paramount importance for the production of *Cordia alliodora* seedlings, both to reduce the period of obtaining commercial seedlings, which is long in the traditional method, and to achieve efficiency in the use and utilization of fertilizer by the plant, thus ensuring maximum increment of plant organs in a short time when performing this management.

The highest increment in height - ΔH (Fig. 2A) occurred at the estimated DMTE of 4.70 g L^{-1} in the 2.2 L container, corresponding to a height of 21.92 cm, which represents an increase of 22.45% compared to the control treatment (substrate without addition of CRF), at 90 DAT (Fig. 1A). Certainly the increment in height was due to the higher dose of the controlled-release fertilizer, as well as the volume of the container, with a combination between the continuous supply of nitrogen (N) to the plant and factors such as solar radiation and temperature, thus resulting in greater photosynthetic efficiency and the production of new tissues in the plant organs. In addition, the DMTE for ΔSD was 4.64 g L^{-1}, corresponding to a value of 3.45 mm (Fig. 2B) in the 2.2 L container, values similar to those reported by Mota *et al*. (2020), who worked with *Agonandra brasiliensis* seedlings under different doses of controlled-release fertilizer and containers of different sizes in substrate and obtained seedlings similar to those of the present study.

Conversely, *Cordia alliodora* plants produced in the 1.8 L container showed lower ΔSD (2.94 mm) with the DMTE of 5.76 g L^{-1} compared to those grown in the 2.2 L container at the DMTE of 4.64 g L^{-1} (Fig. 2B). High N doses in 1.8 L containers affected the physiological quality of plants, causing negative effects on their development, especially those related to seedling diameter (Menegatti *et al*., 2022). Mota *et al*. (2020) commented that the N supply in containers with a volume of less than 1.8 L can easily have a negative effect on native forest species, which is not common with other nutrients.

However, a positive response was found for the 2.2 L container, for instance in shoot dry mass (SDM), which had DMTE of 5.82 g L^{-1} of CRF, with 24.6% gain in the SDM of *Cordia alliodora* when compared with

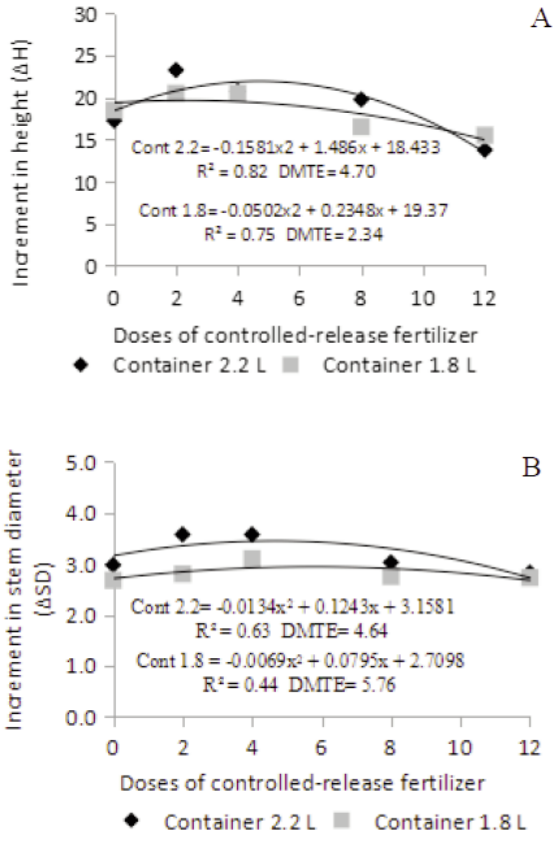

Fig. 2 - Increments in height (ΔH) (A, cm) and stem diameter (ΔSD) (B, mm) of *Cordia alliodora* seedlings as a function of the dose of controlled-release fertilizer, in two volumes of container, produced under nursery conditions, Boa Vista, RR.

the container volume of 1.8 L, with DMTE of 5.80 g L^{-1} of CRF (Fig. 3A). This result is probably related proportionally to the volume of the container, as well as the availability of greater space for root growth, thus ensuring greater expansion of the root system and utilization of nutrients.

According to Damasceno *et al*. (2019), shoot dry mass indicates the rusticity of a seedling, and the highest values, obtained in plants grown in the 2.2 L container, with the DMTE of 5.82 g L^{-1} of CRF (Fig. 3A), represented more lignified and rustic seedlings, with greater guarantee for establishment and survival in the field.

According to figure 3B, the maximum value (3.17 g $plant^{-1}$) of root dry mass was obtained at the DMTE of 5.54 g L^{-1} of CRF in the 2.2 L container, which represents a gain in root dry mass of 13.0%, compared with the 1.8 L container at the DMTE of 3.50 g L^{-1} of CRF. Chu *et al*. (2019) related the decrease in growth characteristics related to the root system to exces-

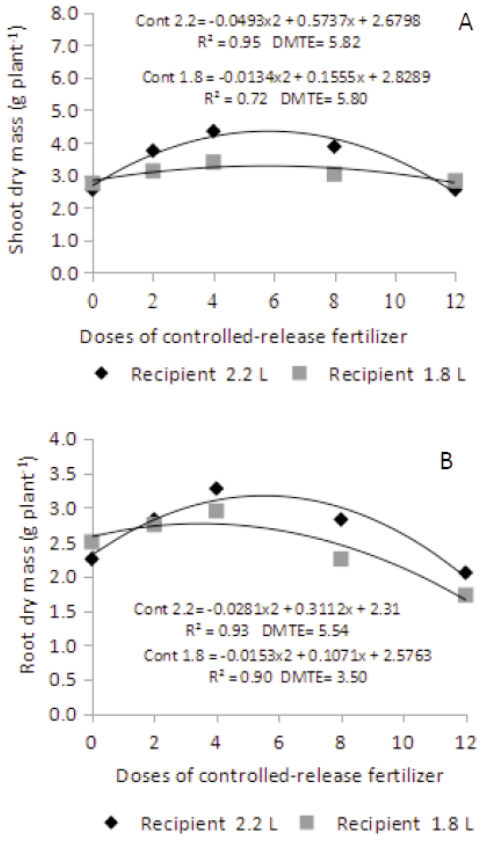

Fig. 3 - Shoot dry mass (A, g plant^{-1}) and root dry mass (B, g plant^{-1}) of *Cordia alliodora* seedlings as a function of the dose of controlled-release fertilizer and the container volume, produced under nursery conditions, Boa Vista, RR.

sive and toxic absorption of nutrients. For Fu *et al*. (2017), higher fertilization rates lead to a reduction in variables related to the root system of plants, due to the reduction in pH, which reduces the availability of some nutrients indispensable to the photosynthetic apparatus, such as phosphorus and magnesium, and favors excessive solubilization of elements, such as aluminum, making the substrate highly saline and toxic, especially to the organ in direct contact, the roots.

Menegatti *et al*. (2020) tested different CRF doses and also found higher production of root dry mass in *Prunus persica* seedlings up to DMTE of 4.82 g L^{-1} of CRF; above the dose of maximum technical efficiency, the other treatments became inferior to the control, that is, there was an inhibitory effect on the initial growth of the seedlings.

Regarding the total dry mass (TDM), a gradual increase was observed as a function of the doses up to DMTE of 5.75 g L^{-1} of CRF, followed by a reduction with the dose of 8 g L^{-1} of CRF, assuming a logistic form (Fig. 4A), regardless of the volume of the container in which *Cordia alliodora* seedlings were grown.

According to the results obtained for TDM, the increase in the CRF doses used caused reduction in its values, indicating a DMTE equal to 5.75 g L^{-1}, since positive responses in the gain of TDM were obtained at the dose of 4.0 to 5.75 g L^{-1} of substrate until 90 days of growth of *Cordia alliodora* seedlings.

The quality of *Cordia alliodora* seedlings was estimated using Dickson quality index, and the highest estimate was obtained for plants grown in the 2.2 L container, with DMTE of 6.24 g L^{-1} of CRF (Fig. 4B) incorporated into the medium sand substrate. Therefore, the seedlings of this treatment were again considered superior, with greater growth balance. According to Smiderle *et al*. (2021 b), this quality index is a good indicator of initial survival of seedlings in the field, because it considers important characteristics for evaluating the quality of the seedlings to be

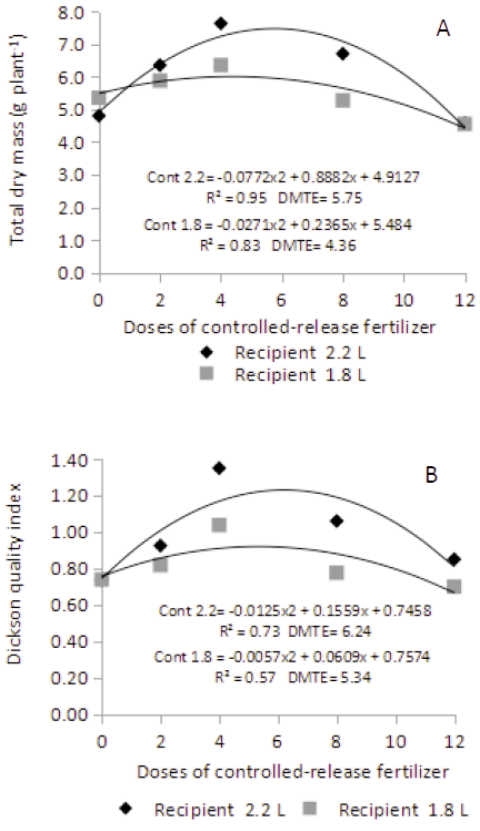

Fig. 4 - Total dry mass (A, g plant^{-1}) and Dickson quality index (B) of *Cordia alliodora* seedlings as a function of doses of controlled-release fertilizer and container volume, produced under nursery conditions, Boa Vista, RR.

transplanted, considering their robustness and balance of biomass distribution.

According to Souza *et al*. (2018), results obtained for native species of Northern Amazon are of great interest to producers of seedlings of forest species in the region, since there is an increase in the quality of seedlings produced, which is an advantage at the time of planting, since seedlings with better quality tend to have faster establishment and their growth is favored also in the field, in addition to contributing to minimizing the time of establishment.

According to Table 1, there was a positive and strong correlation (0.92) between the variables H and SD of *Cordia alliodora* seedlings, which can be attributed to their cultivation in containers, since the volume and depth of soil to be explored are limited, making the expenditure of energy and nutrients for root growth in length, as occurs in field growth, unnecessary. For Smiderle *et al*. (2021 a), the positive and strong correlation between H and SD demonstrates the balance of growth between the height and stem diameter of seedlings.

There was also a positive and weak correlation between SD and ΔH; the correlation is considered weak when it has a coefficient of variation of $0.1 \leq p < 0.5$ (Santos, 2010). The estimate of correlation between TDM and DQI at 90 days after transplantation was 0.95, a correlation considered positive and strong according to the criterion of Santos (2010), with coefficient of variation of $0.8 \leq p < 1$. Considering the results obtained in this study, it is possible to obtain *Cordia alliodora* seedlings with high quality standard with CRF incorporated into the substrate and the container volumes used.

In general, the correlation between the dose of maximum technical efficiency and container volume found in this study can be described as dependent on the container volume and CRF dose. All this information, if considered jointly, allows suggesting the improvement of the traditional system for the production of *Cordia alliodora* seedlings in suitable containers, through the use of fertilization of plants in nursery phase, considering the nutritional efficiency as a function of the container volume, aiming at better use of the input and reduction in the time for production.

4. Conclusions

Container volume of 2.2 L with controlled-release fertilizer in NPK 18-05-09 formulation is suitable for the formation of good-quality *Cordia alliodora* seedlings at 90 days after transplanting.

Controlled-release fertilizer at the maximum technical efficiency dose of 4.64 g L^{-1} in 2.2 L container is indicated to obtain *Cordia alliodora* seedlings with greater increment in stem diameter.

Container volume of 2.2 L at the maximum technical efficiency dose of 5.75 g L^{-1} of controlled-release fertilizer led to higher biomass in *Cordia alliodora* seedlings at 90 days after transplanting.

Controlled-release fertilizer doses from 8.0 g L^{-1} are not indicated to obtain *Cordia alliodora* seedlings in the northern region of Brazil, with quality, regardless of the container volume.

Acknowledgements

We thank the National Council for Scientific and Technological Development (CNPq) for granting the Scientific Initiation scholarship (CNPq/Embrapa - process: 122545/2021-4) to the first author and the research productivity grant to the second author.

Table 1 - Correlation matrix between phytotechnical variables, plant height (H), stem diameter (SD), increments in height (ΔH) and stem diameter (ΔSD), shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM) and Dickson quality index (DQI) of *Cordia alliodora* seedlings as a function of the doses of controlled-release fertilizer and container volume under nursery conditions, Boa Vista, RR

Variables	SD	ΔH	ΔSD	SDM	RDM	TDM	DQI
H	0.92 *	0.87 *	0.58 *	0.83 *	0.79 *	0.83 *	0.77 *
SD		0.54 *	0.72 *	0.84 *	0.73 *	0.74 *	0.73 *
ΔH			0.68 *	0.80 *	0.74 *	0.72 *	0.54 *
ΔSD				0.79 *	0.81 *	0.87 *	0.56 *
SDM					0.72 *	0.87 *	0.71 *
RDM						0.90 *	0.82 *
TDM							0.95 *

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Adv. Hort. Sci., 2023 37(3): 289-293 DOI: 10.36253/ahsc-14608

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Advances in Horticultural Science

New mutations of flower shape in *Nigella damascena* L., its pleiotropic effects and patterns of inheritance

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Key words: flower shape, inheritance, mutant, *Nigella damascena*, pleiotropic effect, shortened sepal.

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Citation:
LYAKH V., SOROKA A., 2023 - *New mutations of flower shape in Nigella damascena L., its pleiotropic effects and patterns of inheritance.* - Adv. Hort. Sci., 37(3): 289-293.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 13 April 2023
Accepted for publication 1 August 2023

Abstract: Two mutants with short sepals were identified after ethyl methanesulfonate treatment of *Nigella damascena* seeds. In one of them ("*shs1*" gene = short sepal 1), isolated from the line with double flowers, the sepals, in addition to reduced size, were divided into several rounded lobes, which granted the flower an original rose-like appearance of ornamental value. Another mutant with reduced sepals ("*shs2*" gene = short sepal 2) was isolated from the line with simple flowers. The allelism test showed that these two genes were non-allelic. Both mutants as pollen parents were crossed with the same line with single flowers. In a dihybrid cross, simple flower, non-reduced sepals (wild type) × double flower, reduced sepals ("*shs1*" gene) F_1 hybrids demonstrated a wild phenotype. F_2 progeny, in addition to two parental classes, showed two recombinant classes in a 9:3:3:1 ratio, indicating that flower shape and sepal size were inherited monogenously and independently, and the plant with rose-like flowers was a double recessive homozygote. Reduced sepals ("*shs2*" gene) in crosses with the single flower line of wild type were inherited as a monogenic recessive trait, showing a 3:1 segregation ratio in F_2. Both mutant genes had a number of similar pleiotropic effects, which, however, were different in strength. Thus, both mutant genes shortened leaf segments, divided the cotyledon leaves into several lobes, and caused disturbances in the female generative sphere, leading to a lack of seed setting. At the same time, the identification of mutants as early as at the cotyledon stage, due to the pleiotropic effect, makes it possible to select and maintain them, especially with regard to the mutant with rose-like flowers, which is highly decorative.

1. Introduction

Nigella damascena L. is an annual herbaceous plant of the Buttercup family (*Ranunculaceae*). This is a crop of wide application, the products of which are used in medicine, food industry, and perfumery. Its seeds contain about 50% fats, which consist mainly of unsaturated fatty acids, up to

20% protein, 2-3% essential oil, enzymes, more than 20 macro- and microelements, including essential ones (Riaz *et al.*, 1996).

Nigella is best known for the fact that nigedase is obtained from its seeds, a lipolytic enzyme preparation that is widely used in medical practice. The absence of animal protein and bile components in its composition permits to prescribe this enzyme preparation for allergies, as well as in cases where the presence of bile acids is highly undesirable. The literature contains information on the pharmacological activity of other biologically active compounds isolated from this plant, in particular, fatty oils and a number of essential oil components (Helvacıoğlu *et al.*, 2021; Salehi *et al.*, 2021; Benazzouz-Smail, 2023). The variety of applications of *Nigella* has initiated research to develop various *in vitro* biotechnologies for this crop from callus culture to protoplast culture (Klimek-Chodacka *et al.*, 2020).

In addition to the above, *Nigella damascena* has long been among the highly ornamental annual plants. Its high decorativeness is granted by rather large petal-like sepals of white, different shades of blue, purple, pink and even red colors. In floriculture, the shape of the flower is no less valuable than the color of the flower for giving the appearance to an ornamental plant. The presence of floral dimorphism in *Nigella damascena*, which ensures the shape of single or double flowers, and a variety of sepal colors allowed breeders to create a series of wonderful varieties.

It has long been shown that the floral dimorphism is monogenically controlled, with the 'single' morph being dominant and the 'double' morph being recessive (Toxopeus, 1927). In recent years, flower dimorphism and different types of petal modifications in *Nigella damascena* have served as the basis for using this plant as a model for elucidating the molecular control of floral dimorphism and identifying genes expressed during petal development (Jabbour *et al.*, 2015; Zhang *et al.*, 2020, Galipot *et al.*, 2021).

As a result of studies on induced mutagenesis in *Nigella*, we have identified two mutations affecting the size of the sepals, which ultimately alters the shape of the flower. These mutations, as well as their pleiotropic effects, are described in this article, which also presents the inheritance patterns for the mutant traits.

2. Materials and Methods

In our studies on chemical mutagenesis, two mutations with a similar phenotypic appearance, expressed in the deformation of the sepals, were identified in *Nigella damascena*. In one case the malformation was manifested in shortening the sepal and rounding its edge, so that the sepal instead of a pointed shape had an oval shape. It was by the presence of a shorter sepal and its rounded edge that this mutation was originally isolated. Another mutation was only designated by shortened sepals. Both mutations were identified in M_3 generation. The first mutant was isolated from a variety with double flowers after seed treatment with ethyl methanesulfonate at the concentration of 0.01% and exposure for 16 hours, the other was found from a variety with single flowers as a result of seed treatment with the same mutagen at the same concentration for 6 hours.

In order to check whether these two mutations are allelic, an allelism test was performed.

To study the inheritance of the mutant traits, both mutants, using them as pollen parents, were crossed with the same line with single flowers. F_1 hybrids were self-pollinated and in F_2 families the segregation ratios were analyzed. In the cross combination "single flower, non-reduced sepals × double flower, reduced sepals", four classes were considered, and in the combination "single flower, non-reduced sepals × single flower, reduced sepals", the F_2 population was divided into two classes.

To test if the observed frequencies of plants in F_2 populations correspond the expected ones a Chi-square test was used (Griffiths *et al.,* 2004).

3. Results and Discussion

Flowers of two mutants with deformed sepals are shown in figure 1. The flower of the mutant isolated as a result of mutagenic treatment of seeds of the double-flowered *Nigella* plant is shown in Figures 1a and 2b. As can be seen from the figures, the mutant, in contrast to the usual double flower (2d), was characterized by shortened and more rounded sepals ("*shs1*" = shortened sepals with rounded edges). The incompletely opened flower of the mutant plant resembled the shape of a rose flower.

In a cross combination of "single flower, non-reduced sepals (wild type) × double flower, reduced sepals (mutant type)", F_1 hybrids had a single flower and non-reduced elongated sepals like the wild type parent. That is, a single flower completely dominated

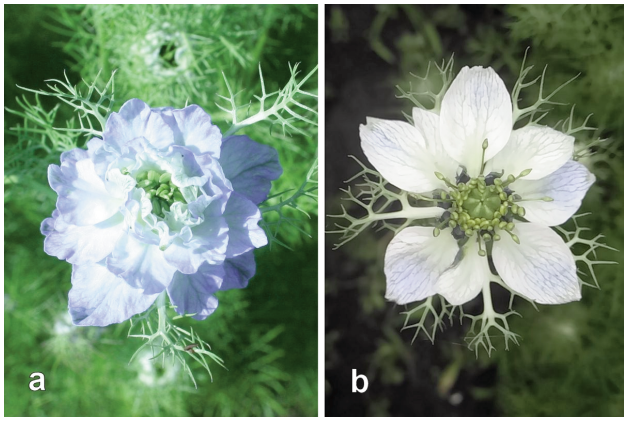

Fig. 1 - Flowers of two *Nigella damascena* mutants with reduced sepals: a) rose-like double flower at the beginning of opening (shs1 gene); b) single flower with reduced sepals (shs2 gene).

the double one, and non-reduced sepals over reduced ones ("*shs1*"). In this cross combination the parents differed by two genes and, if these genes are inherited independently, we have to obtain a typical dihybrid pattern with the four unique phenotypes in a 9:3:3:1 ratio in F_2. Two F_2 families showed a segregation ratio where, in addition to the parental classes of single flower, non-reduced sepals (2a) and double flower, reduced sepals (2b), two recombinant classes appeared - single flower, reduced sepals (2c) and double flower, non-reduced sepals (2d) in approximately equal proportions (Table 1, Fig. 2). In both F_2 families, there was a complete correspondence of the observed segregation ratios to the theoretically expected frequencies. The identified segregation model indicated an independent combination of flower morph and sepal shape traits and, consequently, the absence of linkage between the genes that determine those traits.

The flower of another mutant with deformed sepals, isolated after mutagenic treatment of seeds of a plant with simple flowers, in contrast to the first mutant, was characterized by a stronger shortening of the sepals and the absence of roundness at their ends ("*shs2*" = shortened sepals) (Fig. 1b). The reduction in the sepal length was accompanied by a significant deformation of the flower pistil, which was visually revealed in the strong shortening of the styloides. Some flowers of this mutant lacked them altogether.

Sepals reduced in length ("*shs2*") in crosses with the single flower line of wild type (with non-reduced sepals) were inherited in a monogenic recessive pat-

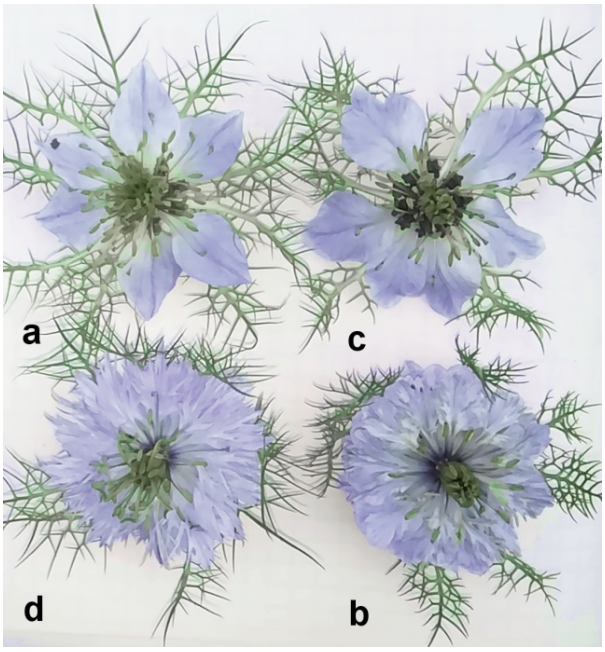

Fig. 2 - Phenotypic classes in F_2 *Nigella damascena* cross combinations single flower, non-reduced sepals (wild type) × double flower, reduced sepals (*shs1* mutant): a) single flower, non-reduced sepals; b) double flower, reduced sepals; c) single flower, reduced sepals; d) double flower, non-reduced sepals.

Table 1 - F_2 segregation for sepal shape and floral morph in cross of single flower, elongated sepals (wild type) and double flower, oval sepals (mutant type) plants in *N. damascena*

F_1 phenotype	Total F_2 plants	F_2 phenotypes				Segregation ratio tested	χ2 (P value)
		single flower, non-reduced sepals	single flower, reduced sepals	double flower, non-reduced sepals	double flower, reduced sepal		
Single flower, non-reduced sepals	100	61	17	16	6	(3:1) × (3:1) = 9:3:3:1	0.29 (0.59)
Single flower, non-reduced sepals	68	40	9	13	6	(3:1) × (3:1) = 9:3:3:1	1.39 (0.24)

$\chi 2_{05}$ (d.f. 3) = 7.82.

tern, showing complete dominance of the wild type over the mutant in F_1, and a 3:1 segregation ratio in F_2 (Table 2).

The allelism test performed showed that these two genes, which determine the shortening of the sepals, are non-allelic. However, they have a number of similar pleiotropic effects. Both genes, without affecting plant height, cause shortening of true leaf segments. The bracts of both mutants are also shortened and more densely attached to the ripening boll than in the wild type. It should be noted that the *shs2* gene as compared with the *shs1* gene causes stronger changes (Fig. 3). Shortening the leaves and bracts changes the habit of the plant, making it more compact.

Both mutant genes affect not only true leaves, but also cotyledons, causing them to be dissected into lobes. The division of one or two cotyledons into two lobes is characteristic of the mutant with *shs2* gene (Fig. 4 b), while the multi-lobed state of both cotyledons is inherent for the mutant carrying *shs1* gene (Fig. 4 a).

The negative effect of both mutant genes on the main function of the flower, which is reproduction, was also noted. The mutants were successfully used in various crosses as a source of pollen, but their involvement in hybridization as female parents was problematic. Sometimes such crossings were successful with the *shs2* mutant when using late flowers, but it was not possible to obtain seeds from the *shs1* mutant even after free pollination. This indicates serious disturbances in the female generative sphere of the flowers of both mutants.

Previously, in our studies with *Linum grandiflorum* Desf., a mutant with short petals, resembling a wild carnation flower, was identified (Lyakh, 2018). After mutagenic treatment of immature sunflower embryos, a mutant with shortened petals (ray flowers) was also obtained (Soroka and Lyakh, 2009). In both cases, as for Nigella, ethyl methanesulfonate was used. The mutation identified in sunflower had a strong pleiotropic effect, affecting the stem, leaf, and even cotyledons. At the same time, true and cotyledon leaves had, in contrast to the elongated, rounded end of the leaf blade.

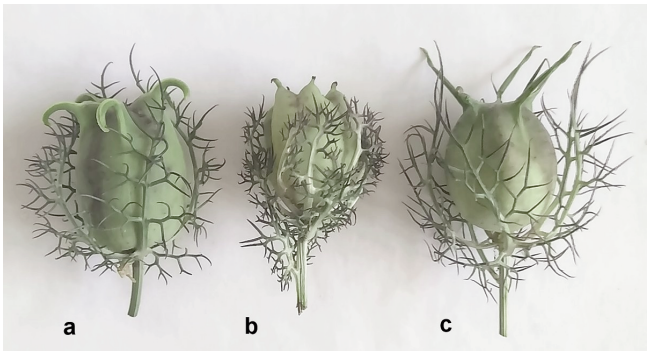

Fig. 3 - Bracts and capsules of two *Nigella damascena* mutants with reduced sepals compared to the wild type: a) *shs1* mutant, b) *shs2* mutant, c) wild type (single flower).

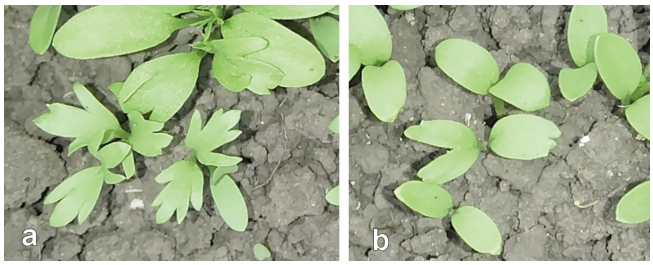

Fig. 4 - Cotyledons of two *Nigella damascena* mutants: a) *shs1* mutant, b) *shs2* mutant.

Two-locus genetic control of petal shape was revealed in *Linum grandiflorum* and sunflower, where a shortened petaled plant is a double recessive homozygote (Soroka and Lyakh, 2017; Lyakh, 2018). In turn, a simpler genetic system is known that controls the shape of plant organs, in particular leaves. Thus, it was found that the shape of the leaflet in cowpea is monogenously controlled, with the lanceolate leaflet shape dominant over the ovoid one (Nwofia, 2014). The same monogenic control of leaf shape, but with a co-dominant interaction of

Table 2 - F_2 segregation for sepal shape in cross of single flowered plants with non-reduced (wild type) and reduced sepals (mutant type) in *N. damascena*

F_1 phenotype	Total F_2 plants	F_2 phenotypes		Segregation ratio tested	$\chi 2$ (P value)
		non-reduced sepals	reduced sepals		
Non-reduced sepals	62	51	11	3:1	1.74 (0.19)
Non-reduced sepals	59	50	9	3:1	2.94 (0.09)

$\chi 2_{05}$ (d.f. 1) = 3.84

alleles, was found in caladium (Deng and Harbaugh, 2006).

As noted above, both mutations caused a partial reduction in the size of sepals of *Nigella* flowers. There is an opinion that the size of the flower organs is controlled by one genetic program, while the number of flower organs is determined by another genetic system, independent of the first one. At the same time, they both regulate the size of the generative organ itself, the flower (Weiss *et al.*, 2005). Our data on the independent combination of genes that determine the number of sepals and their size support the above judgment.

Of the two mutations of reduced sepals identified in *Nigella*, only one (*shs1* gene) affected the shape of the flower, turning an ordinary double flower into a rose-like flower with a decorative value.

4. Conclusions

The preservation and reproduction of plants with such a flower shape for ornamental use in the usual way is problematic due to the inferiority of the female generative sphere.

A partial way out of this problem could be the use of the pleiotropic effect detected at the cotyledon leaf stage. Then the screening of the offspring of heterozygous plants that are the part of a self-pollinated family, carrying the rose-like flower gene, and a subsequent elimination of seedlings with wild-type cotyledons, will not only preserve but also allow to use this unique genotype in ornamental floriculture.

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Adv. Hort. Sci., 2023 37(3): 295-305 DOI: 10.36253/ahsc-13849

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Advances in Horticultural Science

Response of hydroponic baby lettuce to UV-B radiation exposure during the growing period

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Key words: Carotenoids, chlorophyll, dry matter, leaf area, polyphenols.

Abstract: Lettuce (*Lactuca sativa* L.) has a nutritional contribution comparable to other vegetables. It is produced in soil and hydroponics systems, outdoors or indoors, and in some cases, with the management of radiation. UV-B radiation exposure can influence the functional quality of vegetables and is becoming more frequent. Cultivars Kristine RZ and Versaï RZ were exposed to four radiation doses: UV-B0 (0 µW·cm^{-2}), UV-B16 (16 µW·cm^{-2}), UV-B33 (33 µW·cm^{-2}) and UV-B58 (58 µW·cm^{-2}), during 30 min for 10 days. Lettuce leaves were harvested twice. The leaf area of 'Versaï RZ' was not affected by radiation in the first harvest, while the high doses (33 and 58 µW·cm^{-2}) reduced the leaf area of 'Kristine RZ' between 15-30%, respectively. The radiation did not significantly impact the percentage of dry matter and the color parameters. However, functional compounds were affected. In general, the cv. Kristine RZ responded positively to the dose of 16 µW·cm^{-2} while 'Versaï RZ' to 58 µW·cm^{-2}. An increase in the content of functional compounds was also observed in 'Versaï RZ' in the second harvest, and a reduction in the levels measured in 'Kristine RZ' indicated a different adaptation to UV-B radiation that must be studied individually.

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Citation:
SILVEIRA A.C., RIVERA MARCHANT L., ESCALONA V.H., 2023 - *Response of hydroponic baby lettuce to UV-B radiation exposure during the growing period.* - Adv. Hort. Sci., 37(3): 295-305.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 31 October 2022
Accepted for publication 25 July 2023

1. Introduction

Lettuce (*Lactuca sativa* L.) is one of the most consumed leafy vegetables worldwide. Within the species, there are four botanical varieties with different characteristics: A) *L. sativa* var. *capitata*; B) *L. sativa* var. *longifolia*; C) *L. sativa* var. *crispa*; and D) *L. sativa* var. *acephala* (Kim *et al*., 2016). It has a high water content (~95%), and despite its vast consumption, it is not considered an essential source of nutrient supply. However, its nutritional contribution is comparable to that of other vegetables because it is consumed raw, implying that the cooking processes do not affect its composition (Xiao *et al*., 2012). Lettuce is low in calories, fat, and sodium and provides minerals, fibers, provitamin A or β-carotene, vitamins C, K, and folate (vitamin B9), and phenolic compounds to the diet, among others

(Kim *et al*., 2016).

The plasticity of the crop determines that it can be produced in soil and hydroponics systems. In addition, it can be harvested with different degrees of development ranging from the first leaves (cotyledons), seedlings (baby leaf), or fully developed plants (Xiao *et al*., 2012).

Hydroponics is a production system in which nutrients are supplied to plants artificially through water (Sharma *et al*., 2018). Three large hydroponic cultivation systems are differentiated into substrate, water, and air (aeroponics). The most used are water crops, which include two types: floating root system, where the crop is in continuous contact with the nutrient solution, and which have the advantages of being easy to perform, low cost, and do not require extra energy use; and the Nutrient Film Technique (NFT), which is a closed system where plants grown in a constant recirculation of a thin layer solution through the roots, with no loss or leakage of nutrient solution (Magwaza *et al*., 2020).

In addition to being the essential energy source for photosynthesis, light is one of the environmental factors that determine plants' growth, development, morphology, and synthesis of secondary metabolites. The relationship between light and plants has different conceptions and levels of complexity that involve aspects such as quality and quantity of light, which directly influence photosynthesis, but also the responses of plants to environmental stimuli. In this sense, many works have been developed on the ability of plants to detect and respond to the moment, duration, wavelength, dose, and direction of light, which involves the processes of photoperiodicity, phototropisms, and the photomorphogenesis (Robson *et al*., 2015).

The visible spectrum region, which goes from 400-700 nm, corresponds to the range of emissions they use and is known as photosynthetically active radiation (PAR). However, plants require a broader range for their development, which goes from 300 to 800 nm, which includes, in addition to PAR radiation, UV, and far red (Li and Kubota, 2009; Chory, 2010). One of the components of light is UV radiation, which according to its wavelength, is divided into UV-C (100-280 nm), UV-B (280-315 nm), and UV-A (315-400 nm). UV-C radiation and much of UV-B radiation (wavelengths less than 290 nm) do not reach the earth. UV-A radiation contributes approximately 5% of the photons in the photosynthetically active radiation (400-700 nm, PAR); it is highly variable, constituting no more than 0.33% of the photons in PAR. Although it represents a tiny fraction of the radiation that reaches the earth, it plays a fundamental role in regulating metabolic pathways in the development of the associated specific photomorphogenic responses (Robson *et al*., 2015; Robson *et al*., 2019). The study of the relationship of plants with UV-B radiation allowed the identification of a specific photoreceptor, the UV RESISTANCE LOCUS 8 (UVR8), which allowed a substantial advance in the understanding of signaling and response processes (Rai *et al*., 2021).

Exposure to UV-B radiation has a negative effect on photosynthesis due to damage at the level of DNA, proteins, and especially in the photosystems (PSI and PSII) and the light-harvesting complexes, which result from the increase in the levels of ROS. However, it is an effective elicitor to increase the content of bioactive compounds since one of the responses to exposure to UV-B radiation involves the induction and biosynthesis of phenolic compounds, including flavonoids, which act as UV protection components, and have antioxidant potential (Neugart and Schreiner, 2018). Different works mentioned increases in the concentrations of individual phenylpropanoids, such as hydroxycinnamates and flavonoids, in plants exposed to UV-B radiation. These changes are generally believed to positively impact the antioxidant capacity and UV protection (Moreira-Rodríguez *et al*., 2017 a, b; Dou *et al*., 2019; Rodríguez-Calzada *et al*., 2019; Castillejo *et al*., 2021; Loconsole and Santamaria, 2021). In the last 20 years, the consumption of vegetables has focused on the contribution of compounds of high nutritional value. Vitamins (E, C); hundreds of chemical compounds, such as sulfur and selenium; polyphenols such as flavonoids, stilbenes, and ellagic acid; and carotenoid compounds, such as lycopene, lutein, and ß-carotene among others are included in this group (Kyriacou *et al*., 2016). Consumers are looking for new products that promote health and longevity combined with gastronomic delight. Consequently, the way is opened to develop exceptional products that may be new, as in the case of microgreens or traditional products whose production systems have been modified (light management, for example) to influence their functional quality positively. Advances in the knowledge of physiological processes mediated by light have allowed the safe, healthy, and sustainable production of different plant species within controlled environments known as plant factories (SharathKumar *et al*., 2020; Yoon *et al*., 2022). Based

on the above, the objective of this work was to evaluate the effect of the application of ultraviolet-B (UV-B) radiation under greenhouse conditions on the antioxidant characteristics of red and green "baby" lettuce leaves grown in a hydroponic system.

2. Materials and Methods

Plant material production

The study was carried out in a greenhouse at the Centro de Estudios de Postcosecha (CEPOC), at the Facultad de Ciencias Agronómicas de la Universidad de Chile (32°40' south latitude and 70°32' west longitude and 625 m a.s.l. altitudes, Santiago, Chile). Lettuce (*Lactuca sativa* L.), cultivars 'Kristine RZ' and 'Versaï RZ', both of oak leaf, green and red respectively, were used for the experiment. The sowing was carried out at the end of autumn in alveolate trays of 200 units, with a substrate of perlite with rock wool in a 1:1 ratio. At the first stages of the seedling, irrigation was made by tap water, depending on the requirements of the crop. Upon reaching the phenological stage of the first true leaf, the seedling was watered with a Hoagland II-modified nutrient solution, diluted to 50% with a pH between 5.5 and 5.8 measured with a potentiometer (Hi99301, Hanna Instruments, USA). Before sowing, a germination test was carried out according to ISTA Standards, obtained 96.7% for 'Kristine RZ' and 100% for 'Versaï RZ'. When the lettuces reached the stage of the third to fourth true leaf, they were transplanted to a 1.5x7 m NFT table with 8 profiles, a slope of 2.5%, and the height of the nutrient solution sheet of 0.005 m. Plant density was 53 plants m^{-2}. Once the transplant was carried out, the crop was irrigated continuously with tap water for 5 days to reduce the stress of the transplant. When plants were in the phenological stage of the four to fifth true leaf, they were irrigated with a Hoagland II-modified nutrient solution diluted to 50%. During cultivation, pH conditions were between 5.5 and 5.8, measured with a potentiometer (Hi99301, Hanna Instruments, USA).

UV-B irradiation treatments

Before treatments applying, photosynthetically active radiation (PAR, µmol $m^{-2}s^{-1}$) was measured with a PAR radiation meter (Fieldscout, Model 3415, Spectrum Technologies, Inc., Illinois, USA) to determine the best time for UV-B irradiation. The chosen time was at 7:00 p.m., which corresponded to the moment closest to the point of light compensation of sun plants (20-30 µmol m^{-2} s^{-1}); where the photon flux in the net exchange of the leaf is zero, equalizing the rates of production and consumption of CO_2 (Yin *et al*., 2011).

UV-B radiation was applied with 13 UV radiation lamps (Q-Panel 313, Cleveland, USA), arranged in a steel structure of 1.5x1.8 m. The lamps were covered with a 0.11 mm mica (Socomish, Santiago, Chile) to isolate any other type of radiation other than UV-B radiation. Four radiation doses corresponding to: UV-B0 (0 µW·cm^{-2}), UV-B16 (16 µW·cm^{-2}), UV-B33 (33 µW·cm^{-2}) and UV-B58 (58 µW·cm^{-2}) were evaluated. Radiation was applied for 30 min daily for 10 days when the lettuces reached the fifth to sixth true leaf. Greenhouse growth conditions were 22.4±4.5°C mean daily temperature and 48.8±6.5% mean daily relative humidity.

After 10 days, when the lettuces were in the phenological stage of 8^{th} to 9^{th} true leaf, 7 to 8 outer leaves were harvested, leaving 2 to 3 leaves per plant. These plants remained in growing conditions until they reached 5 to 6 fully extended leaves the moment they were irradiated. When plants reached the same condition as in the first harvest, they were harvested again (second harvest). The time difference between the first and second harvests was 19 days.

At each moment of analysis, the following determinations were made:

Leaf area (cm^2). Measured in a total of 30 leaves per variety, with a leaf area determiner (Area Meter, LI-COR 3000, USA).

Fresh and dry matter of the aerial part (g). Weight of whole leaves was determined with a precision balance (Radwag, AS 100/C/2, Poland) and corresponded to the fresh matter. After that, leaves were dried in an oven (Labtech, LDO-150F, Korea) with forced air ventilation at 70°C until constant mass to obtain the dry mass. Values were expressed in percentages.

Colour. Measured in the adaxial part of the distal sector of the lamina in 30 leaves per variety, using a compact tristimulus colorimeter (Minolta Chroma meter, CR-300, Ramsey, NJ, USA) with a D65 light source, an angle observed from 0° and calibrated with a white standard, using the CIELab system. Parameter values were expressed as hue (h_{ab}), chroma (C*), and lightness (L*).

Chlorophyll a, b, and carotenoids determination

(mg g^{-1}). It was carried out according to the methodology proposed by Lichtenthaler and Wellburn (1983). For the extraction, 0.4 g of the distal part of the leaf blades were weighed (Radwag, AS 100/C/2, Poland), and 15 mL of 80% (v/v) acetone were added. Subsequently, the mixture was homogenized at 3,500 rpm for 30 s (IKA T18 basic, Ultra Turrax, Wilmington, USA), filtered with gauze, and centrifuged (HERMLE Labortechnik, Z326K, Wehingen, Germany) for 15 min at 3,630 gx. The determinations were made in the supernatant that was measured in a plate spectrophotometer (Asys, UVM340, Eugendorf, Austria) at 470, 646, and 663 nm. For the quantification, the following expressions were used:

$$Ca = 12.25\ A_{663} - 2.79\ A_{646}$$

$$Cb = 21.5\ A_{646} - 5.1\ A_{663}$$

$$C_{x+c} = \frac{1000\ A_{470} - 1.82\ Ca - 85.02\ Cb}{198}$$

Where C*a* is the chlorophyll *a* content, C*b* the chlorophyll b content and C_{x+c}, the carotenoid content.

Extraction of bioactive compounds. It was made following the methodology proposed by Swain and Hillis (1959) with some modifications. For this, 5 g of sample were weighed (Radwag, AS 100/C/2, Poland), mixed with 20 mL of methanol, and homogenized at 3,500 rpm for 45 s (IKA T18 basic, Ultra Turrax, Wilmington, USA). The homogenate was stored at 5°C for 24 h. Subsequently, it was filtered with gauze and centrifuged (HERMLE Labortechnik, Z326K, Wehingen, Germany) for 20 min at 3,630 x *g*. Measurements were made on the supernatant.

Total phenolic compounds (µg GAE g^{-1}). It was determined according to the colorimetric method of Folin Ciocalteu (Singleton and Rossi, 1965), placing 19.2 µL of extract/blank, together with 29 µL of Folin-Cioclateu reagent (1:8 v/v with distilled water) in each cell of the Elisa plate. After 3 min, 192 µL of 1N Na_2CO_3 were added, and 10 min after, the time at which the reaction was complete as previously determined; absorbance was measured at 750 nm. For the calculation, a calibration line was made with gallic acid (R^2= 0.9958). The values were expressed in µg of gallic acid equivalent (GAE) g^{-1} of fresh weight.

Antioxidant capacity by DPPH (µg ET·g^{-1}). It was determined according to the methodology of Brand-Williams *et al*. (1995), placing 21 µL of sample and 194 µL of DPPH solution in each cell (previously adjusted to 1.1 absorbance at 515 nm). After 2 h, at which time the reaction was complete, the absorbance was measured. For the calculation, a calibration curve was made with Trolox (R^2= 0.9992). The results were expressed as µg Trolox equivalent (TE) g^{-1} fresh weight.

Antioxidant capacity by FRAP (µg ET·g^{-1}). For the analysis, the methodology proposed by Benzie and Strain (1996) was followed. To 6 µL of sample, 198 µL of FRAP reagent was added (buffer acetate 300 mM pH 3.5 + ferric chloride 20 mM aqueous solution + 2,4,6-Tripyridyl-s-Triazine 10 mM in HCl 40 mM). After 30 min, time in which the reaction stabilized, the absorbance at 593 nm was measured. A calibration curve was made with Trolox (R^2= 0.9951) to express the results as µg Trolox (ET)·g^{-1} fresh weight.

Statistical analysis

The experimental design was a 4x2 factorial, completely randomized with 3 repetitions. The factors corresponded to the level of UV-B radiation and cultivar, being distributed randomly within each repetition. The experimental unit used was 8 plants per replicate and cultivar.

An analysis of variance (ANDEVA) was performed, and when statistically significant differences were found, Tukey's multiple range comparison test was used, with a significance level of 5%.

The percentage values were corrected prior to statistical analysis using the following formula:

$$Corrected\ value = arcsen\sqrt{y/100}$$

where *y* is the percentage values (0 to 100).

3. Results

The foliar leaf area showed differences between genetic materials and treatments in the first harvest (Fig. 1A). The foliar leaf of cv. 'Kristine RZ' was more significant than cv. 'Versaï RZ on control and lower UV-B radiation (16 µW.cm^{-2}). When supplemented with UV-B radiation, no response was found in the cv. Versaï RZ. However, the higher doses of radiation (33 and 58 µW·cm^{-2}) reduced the leaf area of 'Kristine RZ'. Nevertheless, UV-B radiation doses applied to each cv. in the second harvest did not differ. Differences were only observed between 'Kristine RZ' and 'Versaï RZ', being the leaf area higher in the first one (Fig. 1B).

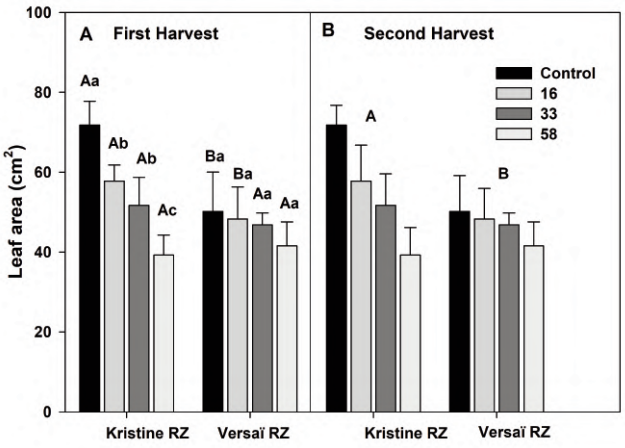

Fig. 1 - Effect of UV-B radiation on leaf area (cm^2) of baby lettuce 'Kristine RZ' and 'Versaï RZ' in a hydroponic system, on first (A) and second harvest (B). Values are means (n= 10). Different letters, uppercase for cultivars and lowercase for treatments, indicate significant differences according to Tukey's test (p≤0.05).

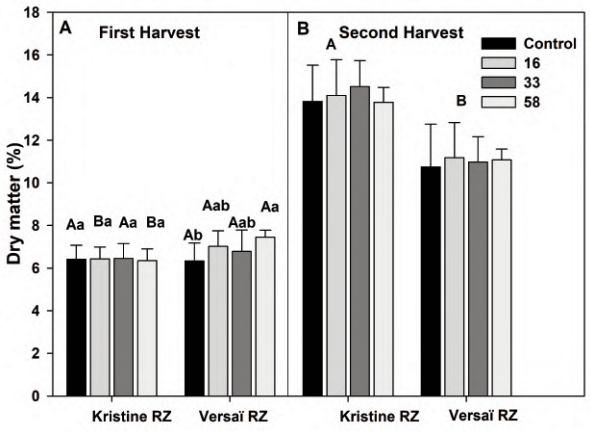

Fig. 2 - Effect of UV-B radiation on dry matter (%) of baby lettuce 'Kristine RZ' and 'Versaï RZ' in a hydroponic system, on first (A) and second harvest (B). Values are means (n= 10). Different letters, uppercase for cultivars and lowercase for treatments, indicate significant differences according to Tukey's test (p≤0.05).

The Kristine RZ cultivar dry matter did not show significant differences between the intensities of UV-B radiation in the first harvest, with an average of 6.4% (Fig 2A). In 'Versaï RZ,' differences were only observed between the control and 58 $\mu W \cdot cm^{-2}$. The cultivar effect was expressed in a higher dry matter/fresh matter ratio by the cultivar 'Versaï RZ' compared to 'Kristine RZ' in the UV-B radiation intensities of 16 and 58 $\mu W \cdot cm^{-2}$; while at 0 and 33 $\mu W \cdot cm^{-2}$, there were no significant differences between cultivars. In the second harvest, the cv. Kristine RZ presented a higher percentage of dry material (~13%) compared to 'Versaï RZ' (~11%), without differences related to the intensity of the radiation being observed in any of them (Fig. 2B).

UV-B radiation did not significantly affect the different color parameters of the evaluated lettuce (Table 1). The 'Kristine RZ' presented h_{ab} values of 120° corresponding to green coloration, while 'Versaï RZ' presented values of 90° indicating red-yellowish coloration. The cv. Kristine RZ did not show variations in this parameter either in the first or the second harvest. In the case of 'Versaï RZ,' the behavior was

Table 1 - Lettuce cultivars used in this experiment

Treatment	First harvest		Second harvest	
	'Kristine RZ'	'Versaï RZ,'	'Kristine RZ'	'Versaï RZ,'
*L**				
Control	121.81 ± 1.17 Aa	72.14 ± 0.88 Ba	117.85 ± 1.57 Aa	30.92 ± 1.78 Bc
16	121.89 ± 1.38 Aa	67.03 ± 1.13 Bb	117.41 ± 1.14 Aa	25.96 ± 0.67 Bd
33	122.53 ± 1.06 Aa	72.41 ± 0.88 Ba	117.81 ± 1.27 Aa	33.42 ± 0.23 Bb
58	121.47 ± 1.04 Aa	67.52 ± 0.96 Bb	117.69 ± 1.65 Aa	37.66 ± 0.18 Ba
hab				
Control	41.25 ± 0.57 Aa	8.45 ± 1.11 Ba	45.94 ± 1.63 Aa	7.33 ± 0.95 Bb
16	40.12 ± 0.72 Aab	8.33 ± 0.88 Ba	46.73 ± 0.16 Aa	7.99 ± 1.16 Bab
33	38.41 ± 2.69 Abc	7.95 ± 0.66 Ba	45.44 ± 2.38 Aab	8.58 ± 1.21 Bab
58	37.72 ± 1.23 Ac	8.09 ± 0.85 Ba	44.14 ±0.28 Ab	9.06 ± 0.28 Ba
*C**				
Control	60.79 ± 0.53 Aa	34.32 ± 0.47 Ba	64.78 ± 2.16 A ns	32.01 ± 3.74 B ns
16	59.77 ± 0.81 Aab	33.74 ± 0.72 Bab	64.02 ± 2.51 A	32.42 ± 2.27 B
33	57.31 ± 1.55 Ab	33.66 ± 1.63 Bb	62.81 ± 1.27 A	32.31 ± 3.27 B
58	57.16 ± 1.38 Ab	33.11 ± 0.16 Bb	64.14 ± 1.72 A	32.72 ± 2.69 B

Each value was indicated by mean±standard error (n=8). Different letters indicate significant differences by Tukey's multiple test with a significance level of 0.05.

quite erratic since, in the first harvest, the leaves of the control and the 33 µW·cm^{-2} treatment were the least red. Compared to the first harvest, in the second, the leaves of 'Versaï RZ' were redder, with values between 25-35, especially those of the control and the 16 µW·cm^{-2} treatment.

Regarding saturation expressed by C*, 'Kristine RZ' presented higher values than 'Versaï RZ' in both harvests, indicating more vivid colors. It also presented greater luminosity (L*). Higher UV-B radiation intensities in the first harvest in 'Kristine RZ' generally reduced saturation and lightness (C* and L* respectively). While in 'Versaï RZ,' only a decrease in luminosity was observed. In the second harvest, the behavior of 'Kristine RZ' was like that of the first. However, in 'Versaï RZ,' the effect was the opposite since the greater intensity of radiation determined a greater saturation. However, the parameter L* was not affected by radiation in either of the two cultivars.

Chlorophyll *a* content showed differences between genetic materials and radiation levels. In the first harvest, 'Kristine RZ' presented about 10 times more than 'Versaï RZ' in all treatments. In 'Kristine RZ,' the highest values were measured in control and 16 µW.cm^{-2} , while the lowest was in those with higher radiation intensities, with no differences between them. On the contrary, the treatments did not affect chlorophyll's *a* level of 'Versaï RZ.' (Fig. 3A).

In the second harvest, the behavior was practically the opposite. In most treatments, 'Kristine RZ' presented lower levels of chlorophyll than cv. Versaï RZ. No response to treatments was found in 'Kristine RZ,' while in 'Versaï RZ,' there was an increase in treatments of 33 and 58 µW·cm^{-2}, respectively (Fig. 3B). Comparing the values measured in the first and second harvest, in the cv. Kristine RZ, the values were practically halved. At the same time, in 'Versaï RZ,' it increased between 3 and 9 times, indicating a very different response to radiation linked not only to the genotype but also to the age of the plant.

Chlorophyll *b* values measured at the first harvest in cv. Kristine RZ were around 10 higher than those of 'Versaï RZ.' In both, an effect of UV-B radiation levels was observed. In 'Kristine RZ,' the 16 µW·cm^{-2} treatment determined an increase, while in 'Versaï RZ,' the increase was observed in the 58 µW·cm^{-2} treatments (Fig. 4A). In the second harvest, only differences between varieties were observed (Fig. 4B). However, contrary to the first, the values measured

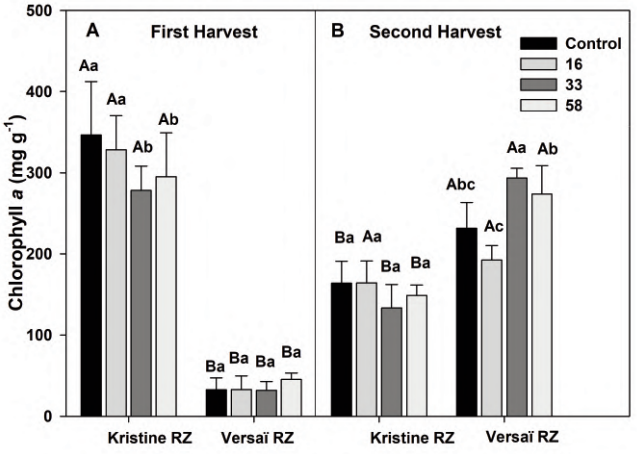

Fig. 3 - Effect of UV-B radiation on chlorophyll a content (mg·g^{-1}) of baby lettuce 'Kristine RZ' and 'Versaï RZ' in a hydroponic system, on first (A) and second harvest (B). Values are means (n= 3). Different letters, uppercase for cultivars and lowercase for treatments, indicate significant differences according to Tukey's test (p≤0.05).

in 'Kristine RZ' were around half of those measured in 'Versaï RZ.'

Also, in the case of carotenoids, both in the first and in the second harvest, differences were found between genetic materials. While, in the first harvest, 'Kristine RZ' presented around 10 times more, in the second, it presented between 37-62% less (Fig. 5A and B). Regarding the effect of the intensity of the radiation, in the first harvest the two highest doses of UV-B radiation had a negative effect on the carotenoids of 'Kristine RZ', while the intensity did not affect the carotenoid levels of 'Versaï RZ'. On the

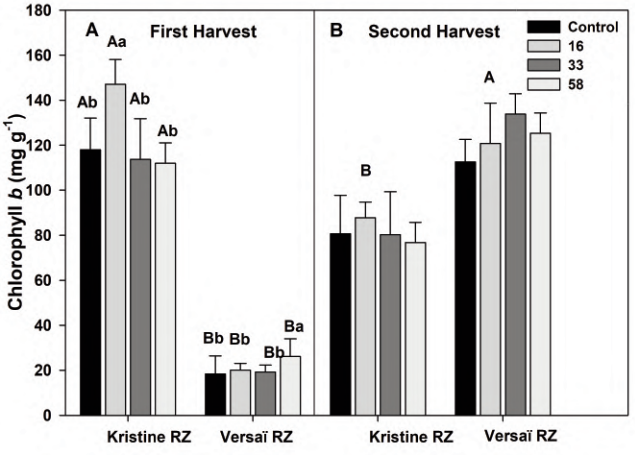

Fig. 4 - Effect of UV-B radiation on chlorophyll b content (mg·g^{-1}) of baby lettuce 'Kristine RZ' and 'Versaï RZ' in a hydroponic system, on first (A) and second harvest (B). Values are means (n= 3). Different letters, uppercase for cultivars and lowercase for treatments, indicate significant differences according to Tukey's test (p≤0.05).

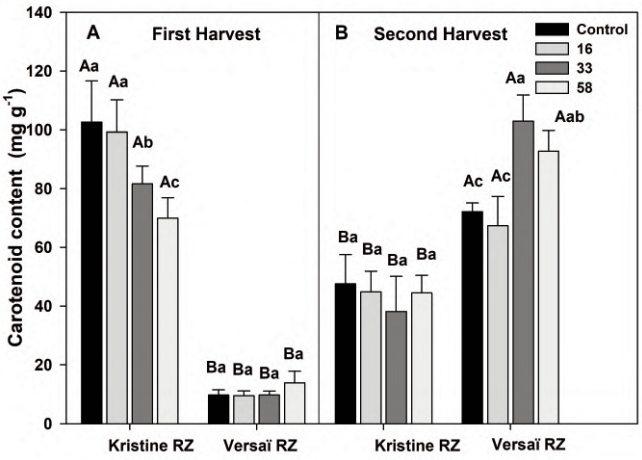

Fig. 5 - Effect of UV-B radiation on carotenoid contend (mg·g^{-1}) of baby lettuce 'Kristine RZ' and 'Versaï RZ' in a hydroponic system, on first (A) and second harvest (B). Values are means (n= 3). Different letters, uppercase for cultivars and lowercase for treatments, indicate significant differences according to Tukey's test (p≤0.05).

contrary, in the second harvest the radiation did not cause differences in the content measured in 'Kristine RZ' but determined in 'Versaï RZ' lettuces exposed to 33 and 58 µW·cm^{-2} an increased.

At both harvest times, 'Versaï RZ' presented about 70-80% more total phenolic compounds than 'Kristine RZ.' In 'Versaï RZ' from the first harvest, the radiation intensity did not affect the values (Fig. 6A). In the second harvest, an increase in phenolic compounds was observed in both cultivars, independent of the radiation dose (Fig. 6B).

Regarding CAT measured by the DPPH method, both in the first and second harvest, 'Versaï RZ' surpassed 'Kristine RZ,' which presented between 3 and 5 times fewer antioxidant compounds (Fig. 7). In the first harvest, there was no response to radiation in the case of 'Kristine RZ,' while in 'Versaï RZ' the intensities of 16 and 33 µW.cm^{-2} reduced the levels of these compounds (Fig. 7A). However, in the treatment of 58 µW.cm ^{2}, there were no differences with the control. In the second harvest, the response to the dose was somewhat erratic in 'Kristine RZ'. At the same time, in 'Versaï RZ', the control treatment presented the lowest levels while the radiation determined an increase, reaching the highest values at 16 µW·cm^{-2} (Fig. 7B).

On the other hand, when CAT was measured by the FRAP method, both in the first and in the second harvest, differences were observed between genetic materials, with 'Versaï RZ' being superior to 'Kristine RZ' (Fig. 8).

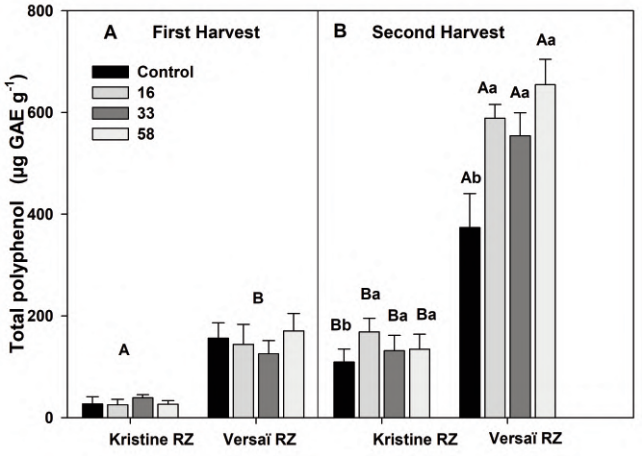

Fig. 6 - Effect of UV-B radiation on total polyphenol content (µg GAE. g^{-1}) of baby lettuce 'Kristine RZ' and 'Versaï RZ' in a hydroponic system, on first (A) and second harvest (B). Values are means (n= 3). Different letters, uppercase for cultivars and lowercase for treatment, indicate significant differences according to Tukey's test (p≤0.05).

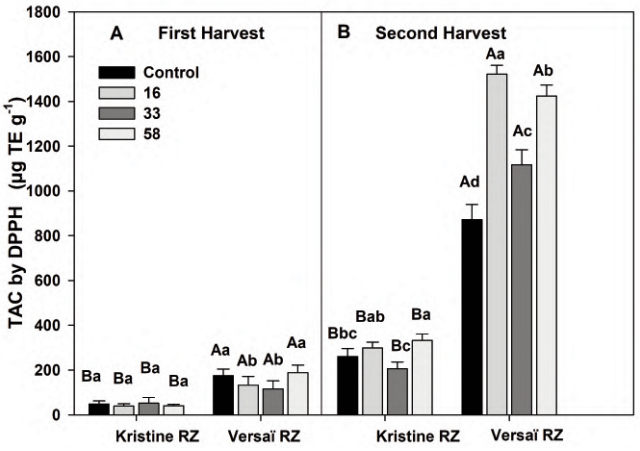

Fig. 7 - Effect of UV-B radiation on total antioxidant capacity by DPPH method (µg TE·g^{-1}) of baby lettuce 'Kristine RZ' and 'Versaï RZ' in a hydroponic system, on first (A) and second harvest (B). Values are means (n= 3). Different letters, uppercase for cultivars and lowercase for treatments, indicate significant differences according to Tukey's test (p≤0.05).

4. Discussion and Conclusions

There are reports that the application of supplemental UV-B radiation has a negative effect on vegetative growth in general and consequently on the yield of different plant products. The most frequently reported alterations include a decrease in the leaf area and/or an increase in the thickness of the leaves. Plant exposure to UV-B radiation, both in the field and in controlled environments, reduced leaf development in lettuce, peas, corn, and sweet pepper, among others (Choudhary and Agrowal, 2014;

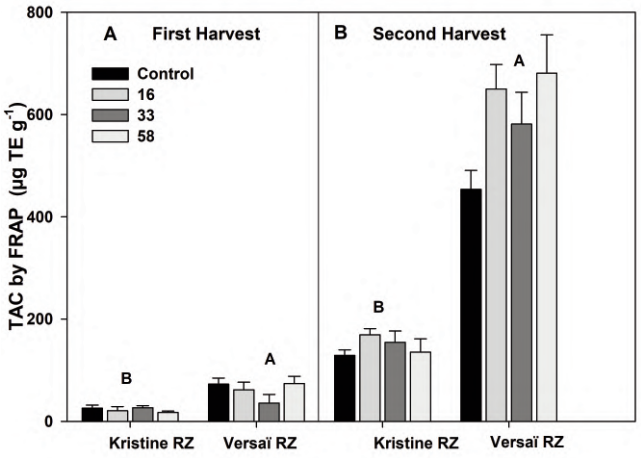

Fig. 8 - Effect of UV-B radiation on total antioxidant capacity by FRAP method (µg TE·g^{-1}) of baby lettuce 'Kristine RZ' and 'Versaï RZ' in a hydroponic system, on first (A) and second harvest (B). Values are means (n= 3). Different letters, uppercase for cultivars and lowercase for treatments, indicate significant differences according to Tukey's test (p≤0.05).

Fina *et al*., 2017; Rodríguez-Cazalda *et al*., 2019). Although alterations occur because of exposure to UV-B radiation, it is mentioned that they could be transitory since, once the acclimatization stage has been overcome, which involves processes such as the positive regulation of ROS elimination, the detection UV and DNA repair capabilities; any interruption in leaf development is overcome. This may allow leaf development to resume its original pattern, or even produce a compensatory response whereby greater expansion is matched by reduced division (Héctors *et al*., 2010; Robson *et al*., 2015). This could be explaining the behavior observed in the second harvest.

On the other hand, and contrary to what was found in this work, it is indicated that exposure to UV-B radiation reduces plants' biomass due to the lower capacity for photosynthesis, mainly due to the effect that UV-B radiation could have. B on photosystem II (PSII) (Bornman, 1989; Mittal *et al*., 2021). Another factor involved is the reduction in the amount of chlorophylls that negatively affects biomass (Kataria and Guruprasad, 2012) as well as the lower turgor pressure that prevents cells from increasing their water content (Choudhary and Agrawal, 2014; Fina *et al*., 2017). More recently, Rizi *et al*. (2021) pointed out that exposure to UV-B rays negatively affects many compounds and biochemical processes in plants, including chlorophyll content and photosynthesis, which reduces carbohydrate production, with the consequent adverse effect on growth and biomass.

The changes observed in color are related to those observed in the different compounds, both pigments, chlorophyll a, b, and carotenoids, as well as phenolic compounds, which include others also linked to color, such as anthocyanins (Goto *et al*., 2016; Sytar *et al*., 2018; Gurdon *et al*., 2019). The differential response found in the varieties studied was also observed by other authors. UV-B radiation induces physiological, biochemical, and morphological stress responses in plants, which are species-specific and even differ between cultivars. In a study where two blueberry cultivars (Legacy and Bluegold) were analyzed, a different response was found where in Legacy (resistant to UV-B radiation) there was an increase in photoprotective pigments during the first week of exposure (19 µW·cm^{-2}) and from the second there was a reprogramming of its metabolism that determined an increase in phenolic compounds and its antioxidant capacity (Luego Escobar *et al*., 2017).

The differences found between the first and second harvests may be linked to the lettuce varieties presenting differential acclimatization mechanisms. In this sense, 'Kristine RZ' has an immediate response, but tolerates low levels of radiation. On the other hand, 'Versaï RZ' takes longer to adapt to UV-B radiation. However, it is capable of responding to higher radiation doses. The differential response is because after exposure to radiation, plants need to reprogram their metabolism to alleviate stress (Barnes *et al*., 2015; Wargent *et al*., 2015). On the other hand, the answer will depend on the type of pigment being considered, which in the case of carotenoids comprises different molecules with different sensitivity to UV-B radiation (Badmus *et al*., 2022).

In a study carried out on broccoli sprouts, the application of 0.042 W·m^{-2} for 4h + 24h of adaptation did not determine variations in carotenoids or chlorophylls (Mewis *et al*., 2012). However, when broccoli sprouts were treated with 7.16 W·m^{-2} for 120 min, photoreceptor pigments were differentially affected, determining increases in carotenoids, lutein and mainly neoxanthin, and in chlorophyll *a*, in relation to control (Moreira-Rodríguez *et al*., 2017 a). According to León-Chan *et al*. (2017), the daily exposure of pepper, during growth (days), to 72 kJ·m^{-2} for 6 h, did not alter the levels of chlorophyll *a* and *b* in relation to the control. However, it determined a notable increase in carotenoids (from 0.02 to 2.18 mg·100 g^{-1} FW).

To protect themselves from the damage generated by UV-B radiation, plants activate their defense mechanisms to avoid excess ROS and maintain the stability of their cellular structures. The biosynthesis of antioxidant compounds, among which are those of a phenolic nature (phenolic acids, flavonoids, among others) as well as vitamins, is one of the defense mechanisms (León-Chan *et al*., 2017; Moreira-Rodríguez *et al*., 2017b; Neugart and Schreiner, 2018). In a study carried out with basil exposed to different doses of UV-B radiation (8.5, 34, 68, 102 kJ m^{-2} day^{-1}), it was found that discontinuous applications for long periods (about 6 days) determined an increase in phenolic compounds without altering the photosynthetic process, directly proportional to the dose of radiation used (Mosadegh *et al*., 2018). In a similar work with purple and green basil exposed to 18.7 kJ m^{-2} h^{-1} for different exposure times, increases in the concentrations of anthocyanin, phenols, and flavonoids were found that even reached 169% (Dou *et al*., 2019). Therefore, it is expected that exposure to UV-B radiation will increase, as observed in the lettuce varieties studied. Castillejo *et al*. (2021) applied doses of 5, 10, and 15 kJ·m^{-2} to kale sprouts during germination at 3.5, 7, and 10 days (25% of the dose at each moment) and found variations in the levels of antioxidant compounds. Doses of 10 and 15 kJ .m^{-2} increased phenol levels by 30%. In addition, TAC experienced increases of 10% (measured by DPPH) and 20% (measured by FRAP) because of the protection mechanism of plants against the stress factor constituted by UV-B radiation.

In work carried out by Hao *et al*. (2022) in Pak Choi, an increase in the amount of phenolic compounds measured by DPPH and FRAP was found, depending on the applied radiation dose. Doses of 0.7 W.m^{-2} for 4 and 8h determined increases. However, no response was observed when the radiation increased to 1.4 W·m^{-2} or the exposure time was greater than 8 h. The authors attribute this to the fact that different signaling pathways are activated depending on the dose.

A response linked to genetic characteristics and plant age was also observed, but it did not follow the same pattern as for chlorophylls and carotenoids. In this case, both varieties took longer to acclimatize, so the most critical response corresponded to the second harvest. In this sense, Rizi *et al*. (2021) reported an increase in both phenolic compounds (1.34 times) and flavonoids (2 times) concerning the control and after 5 days of exposure to radiation of 10.97 kJ m^{-2} day^{-1}, especially in the young leaves of *salvia verticillata*.

Therefore, controlled doses of UV-B radiation can be used to develop products with added value as they are rich in functional compounds, as shown in this and other works. These applications must be evaluated in each genetic material to adjust the dose, as well as the behavior of the plants since it has been demonstrated once again that the response is specific.

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Adv. Hort. Sci., 2023 37(3): 307-315 DOI: 10.36253/ahsc-14158

AHS
Advances in Horticultural Science

Growth and yield performance of carrot (*Daucus carota* L.) as influenced by plant population density under irrigation condition

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Key words: cv. Nantes, marketable yield, plant spacing, row distance, taproot, unmarketable yield.

Abstract: Poor agronomic practices, such as inadequate plant density, can result in suboptimal carrot (*Daucus carota* L.) yield and quality in some regions. In 2020, a field experiment was conducted under irrigation conditions in Gerado, South Wollo administrative zone, Ethiopia, using the Nantes variety as a test crop, to investigate the impact of inter-row and intra-row spacing on carrot yield. The treatments involved three inter-row (row) spacing levels (10, 15, and 20 cm) and three intra-row (plant) spacing levels (5, 10, and 15 cm) in a randomized complete block design with three replications. Row and plant spacing significantly affected (P<0.05) total yield, plant height, leaf fresh weight, root length, root diameter, and root fresh weight. The highest marketable yield (490.4 q ha^{-1}) was achieved with a plant density of 20 x 5 cm. In contrast, the highest unmarketable yield (36.3 q ha^{-1}) was obtained with a spacing of 20 x 15 cm. Hence, a plant density of 20 x 5 cm is recommended for optimal marketable carrot yield in the study region and similar agroecologies, although further research across multiple locations and seasons is necessary to validate the results.

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Citation:
MUHIE S.H., YIMER H.S., 2023 - *Growth and yield performance of carrot (Daucus carota L.) as influenced by plant population density under irrigation condition.* - Adv. Hort. Sci., 37(3): 307-315.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Author Contribution:
The corresponding author, Seid Hussen Muhie developed the research idea, drafted the proposal, and wrote the manuscript
The co-author, Hussen Seid Yimer, developed the proposal, conducted the research, did data collection and analysis, and wrote the draft manuscript.

Competing Interests:
The authors declare no competing interests.

Received for publication 1 January 2023
Accepted for publication 30 August 2023

1. Introduction

Carrot (*Daucus carota* L.) is a short duration vegetable crop. In terms of production areas and market value, it is among the top ten most economically significant vegetable crops in the world. Carrots are widely cultivated because they offer a low-cost source of vitamins (particularly Vitamin A), minerals, and fibre in the human diet (Nuez and Prohens, 2008). The taproot contains high amount of carotene (10 mg per 100 g), thiamine (0.04 mg per 100 g) and riboflavin (0.05 mg per 100 g). Additionally, it contains protein, fat, minerals and vitamin C. Due to these several uses, carrot consumption has increased from time to time (Tegen and Jembere, 2021).

In the world's main carrot-growing nations, yields of carrots can range from 30 to 100 t ha^{-1}. Carrot yields per unit area in the majority of developing nations like Ethiopia (whose average fresh carrot yield per ha is 5.6 t) continue to be below the global average (Kassa *et al.*, 2018). Numerous factors, such as poor production techniques, a lack of technical inputs, pests, and postharvest losses, are linked to low productivity (Tegen and Jembere, 2021; Tschirley *et al.*, 2004). Abiotic stress can also contribute to the decline in quantity and quality of horticultural products, such as carrot (Muhie *et al.*, 2021).

One of the key elements affecting marketable carrot root yield and root size is plant population density (Lana, 2012). In previous research investigations, it was reported that plant population of 450,000 and 300,000 is ideal for fresh market and processing carrots, respectively (Tegen and Jembere, 2021). In addition, it was also revealed that narrow spacing resulted in a higher marketable carrot root yield (Da Silva *et al.*, 2008; Shiberu and Tamiru, 2016). On the other hand, another group of researchers reported that crops planted with wider spacing produced the highest total yield. This discrepancy between the result findings of researchers came from the purpose of production of carrots (for fresh market or for industrial use), as each purpose has its own specific root size range (Kabir *et al.*, 2013; Lana and Carvalho, 2013). Researchers also looked into the possibility of producing baby carrots that are more suited for commercialization through the use of high population density cropping and early harvesting. Farmers typically sow carrots by broadcast at a rate of 4-5 kg/ha, although some of them prefer inter-row spacing ranging from 20 to 30 cm and intra-row spacing of 10 to 20 cm. Crops such as carrot (*Daucus carota*) and Chinese jute (*Abutilon theophrasti*) exhibit plasticity in their morphology and modular growth, making it challenging to determine a suitable unit for population density (Wang *et al.*, 2017; Ford and Sorrensen, 2018), a crucial variable that connects individuals to crops. Some authors have provided details on agronomic practices used in carrot production (Bender *et al.*, 2020; Reginaldo *et al.*, 2021). However, there is limited information available on the plant population density of irrigated carrots that can ensure an optimal marketable yield. Farmers in the study area use broadcasting method of sowing under rainfed production system. Recent research finding also recommended the need to investigate the effects of plant density on carrot (Biratu *et al.*, 2022). The objective of the current study was to identify the optimum population density by adjusting inter- and intra-row spacing for marketable root yield and root size of carrot under irrigation.

2. Materials and Methods

Descriptions of the study area

The experiment was conducted at Gerado, South Wollo administrative zone, Northeastern Amhara region, Ethiopia in 2020 cropping season under irrigation. The area is located at distance of 401km away from the capital to the Northeastern part of the country. Geographically, the study area is found at the intersection of 11°8' N and 39°38' E (Fig. 1). It falls within semi-arid climatic zone with an average monthly minimum and maximum temperature of 12.37°C and 26.27°C, respectively. The area receives an annual rainfall amount of 1291.3 mm/year with erratic nature. Due to this the area is characterized as moisture deficit unless there is supplementary irrigation. The soil type of the area is sandy-loam. It has three permanent rivers which have the potential to irrigate throughout the year.

Experimental design and treatments

The experiment included nine treatments involving three different inter-row distances (10, 15, and 20 cm), also referred to as row distance (Rd), and three different intra-row distances (5, 10, and 15 cm), also referred to as plant distance in the row (Pd). The experiment was arranged in a randomized complete block design with three replications following the procedures of Gomez and Gomez (2010). The treat-

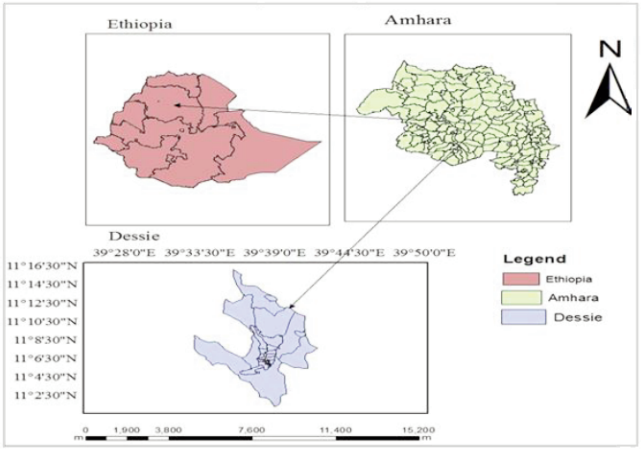

Fig. 1 - Map of the study area.

ments were assigned randomly to the experimental plots within a block.

Experimental materials and procedures

Carrot, cv. Nantes was used as a test crop for this experiment. This cultivar of carrot is well adapted in the study areas. The land was well prepared to a fine tillage to a depth of 30 cm following the conventional tillage practice, using oxen to plough. Thereafter, a field layout was prepared, and each treatment was assigned randomly to the experimental plots. Seeds were sown in raised beds with 20 cm height at a spacing based on treatment assigned to the plot. Carrot seeds were sown by drilling in 1.2 m x 1 m long rows in each plot. The complete amount of phosphorus (175 kg P_2O_5 ha^{-1}) was applied at once, while the nitrogen in the form of urea (150 kg ha^{-1}) was applied in two parts: half of the amount was applied during sowing, and the remaining half was manually top-dressed in the inter-row spaces during the mid-tillering crop stage, which occurred 35 days after emergence (DAE). Irrigation and other required cultural practices were applied equally to all plots. During the experimental periods, a successful crop was produced by applying furrow irrigation at seven days interval and consistently performing all recommended cultural practices. Weeds were manually removed and collected from the crop fields, while harvesting was carried out at crop maturity using a hand hoe.

Data collection

Phenological data. Data such as days to 50% emergence and days to 90% physiological maturity were recorded by counting the number of dates to the respective phenological parameters.

Growth parameters. Plant height, leaf number, leaf fresh weight, and canopy cover were recorded appropriately using five randomly selected plants. Canopy cover was determined as the perimeter of the plant at its widest horizontal plane. It typically assumes that there are a few minor gaps in the leaves and that an average crown perimeter will smooth out any uneven edges.

Yield parameters. Root length and root diameter were measured from five randomly selected plants using a calliper. The fresh weight of roots per plant was determined by measuring the weight of five randomly selected plants using a sensitive balance and the average value was calculated and used for analysis.

The yield of marketable roots was calculated per unit plot excluding border effects. The yield per unit area was converted to marketable yield per hectare. The unmarketable roots were identified based on cracked, branched, small size with diameter of approximately 1-1.5 cm and rotten. Then, the unmarketable roots were calculated per unit plot excluding border rows. The yield per unit area was converted to unmarketable yield per hectare.

Statistical analysis

The collected data underwent Analysis of Variance (ANOVA) using SAS 9.1, which was appropriate for the design of the experiment. The means of significant treatment effects were separated using the Least Significant Difference (LSD) test at a 5% level of significance.

3. Results and Discussion

Phenology

Plant density is considered as one of the most important factors affecting crop phenology (Shafi *et al.*, 2012; Khan *et al.*, 2017). In the present experiment, plant spacing did not significantly ($P \leq 0.05$) affect days to 50% emergence. This could be attributed to the fact that the viability of the seed, moisture availability, and air conditions are the essential elements required for germination, rather than spacing. Similarly, Tesfu and Charles (2010) found that neither sowing date nor planting density significantly affected the number of days required for 50% crop emergence.

In general, carrot plants grown with a narrower row distance tended to mature faster than those grown with wider spacing. The fastest time to reach maturity for carrot plants (73.3 days) was observed with a spacing of 10 x 5 cm, while the slowest time (134.3 days) was observed with a spacing of 20 x 15 cm, followed by a spacing of 20 x 10 cm (121.3 days) (Table 1). This suggests that row spacing has a more significant effect than plant spacing on maturity in this specific case study. Indeed, when the distance between rows was increased from 10 cm to 20 cm, the number of days to reach physiological maturity increased by 34 days. Other investigations have also found that plant density has a significant impact on the time it takes to reach 90% maturity (Da Silva *et al.*, 2008; Tegen *et al.*, 2021). Tesfu and Charles (2010) proposed that lower plant density may allow for more space and resources per plant, which could lead to extended vegetative growth and a longer

time to reach maturity.

Growth and yield

The result of analysis of variance (ANOVA) showed that all growth and yield parameters of Nantes carrot were significantly affected (P <0.05) by plant and row distances and their interaction (plant spacing).

Plant height

It is well known that growth parameters, such as plant height, can be influenced by plant population density (Abuzar *et al.*, 2011; Rahman *et al.*, 2011). Based on our results, the highest plant height of carrot (73.0 cm) was recorded from a spacing of 20 x 15 cm, followed by 15 x 15 cm spacing (60.0 cm). Conversely, the shortest plant height (17.3 cm) was recorded when carrots were sown with a spacing of 10 x 5 cm (Table 2).

Increasing the distance between rows from 10 cm to 20 cm resulted in a 26.7 cm increase in plant height. This may be due to the availability of essential resources necessary for growth and development, as well as the presence of adequate free space between plants to reduce competition in the higher spacing. These findings are consistent with previous research, such as Dawuda *et al.* (2011), who reported that taller plants were observed at higher spacing, and Kabir *et al.* (2013), who reported that taller plants were observed at a spacing of 30 x 20 cm compared to a spacing of 20 x 10 cm. According to Kabir *et al.* (2013), plants sown in higher spacing had enough space for vegetative growth and experienced less competition for nutrients compared to those sown in lower spacing treatments, such as 20 x 10 cm and 25 x 15 cm. When crops have to compete with their neighbouring plants for soil nutrients and sunlight, their health and growth can be negatively impacted. Poorly functioning plants will not attain their desired height or canopy and their roots will have to compete not only for nutrients and water but also for space. Furthermore, high planting density can inhibit photosynthesis.

Leaf number, leaf weight and canopy cover

Plant density have been reported to affect leaf number, canopy development, plant architecture, early ground cover and competitive ability of crops with weed (Bonaparte and Brawn, 1976; Deressegn and Telele, 2017; Hou *et al.*, 2019; Bernhard and Below, 2020). Moreover, leaf weight can be affected by the accumulation and the partitioning of synthesized food to non-photosynthetic parts (Halford, 2010; Osorio *et al.*, 2014).

The highest values of leaf count (21.0), leaf weight

Table 1 - Mean days to maturity of carrot as influenced by plant spacing

Row distance (cm)	Plant distance in the row (cm)			Mean
	5	10	15	
10	73.33 e	81.67 de	87.67 d	80.89 c
15	82.00 de	89.67 d	108.33 c	93.33 b
20	89.67 d	121.33 b	134.33 a	115.11 a
Mean	81.67 c	97.56 b	110.11 a	
CV (%) = 7.44%				

Mean values within rows and columns followed by different letter(s) are significantly different at 5% probability level.
CV= coefficient of variation.

Table 2 - Mean plant height (cm) of carrot as influenced by inter and intra row spacing

Row distance (cm)	Plant distance in the row (cm)			Mean
	5	10	15	
10	17.33 f	23.67 ef	31.33 de	24.11 c
15	21.17 ef	41.60 cd	60.00 b	40.92 b
20	27.33 ef	52.00 bc	73.00 a	50.78 a
Mean	21.94 c	39.09 b	54.78 a	
CV (%) = 17.81%				

Mean values within rows and columns followed by different letter(s) are significantly different at 5% probability level.
CV= coefficient of variation.

(15.0 g) and canopy spread (52.7 cm) were observed at the widest plant spacing (20 x 15 cm), while the lowest values (6.3, 9.1 g, and 14.3 cm, for leaf number, leaf weight and canopy cover, respectively) were found with the narrowest spacing of 10 x 5 cm (Table 3).

Table 3 - Mean leaf number, leaf weight and canopy cover of carrot (*Daucus carota* L.) plants as influenced by inter and intra row spacing

Distances (cm)	Leaf number (n)	Leaf weight (g)	Canopy cover (cm)
Row distance (Rd)			
10	8.22 c	11.23 c	27.89 c
15	12.44 b	12.51 b	33.89 b
20	15.33 a	14.15 a	39.67 a
Plant distance in the row (Pd)			
5	8.89 c	11.18 c	24.56 c
10	11.33 b	12.94 b	31.44 b
15	15.78 a	13.81 a	45.44 a
Plant spacing (Rd × Pd)			
10 × 5	6.33 g	9.06 e	14.33 f
10 × 10	8.00 gf	12.19 cd	31.33 de
10 × 15	10.33 def	12.57 cd	38.00 c
15 × 5	9.33 ef	12.57 cd	27.33 e
15 ×10	12.00 cd	12.36 cd	28.67 de
15 × 15	16.00 b	13.89 ab	45.67 b
20 × 5	11.00 de	12.19 bc	32.00 cde
20 × 10	14.00 bc	14.27 ab	34.33 cd
20 × 15	21.00 a	14.98 a	52.67 a
CV (%)	11.74%	5.74%	10.37%

Mean values within rows and columns followed by different letter(s) are significantly different at 5% probability level.
CV= coefficient of variation.

When the row distance was increased from 10 cm to 20 cm, it was observed that approximately seven additional leaves could develop, indicating that wider spacing can result in higher leaf area for maximum assimilate synthesis. This could be attributed to the greater free space available for plant growth between rows, which reduces competition for nutrients. This finding is consistent with previous studies by Dawuda *et al.* (2011) and Kabir *et al.* (2013), which reported that wider spacing can lead to more leaves and larger canopies, potentially intercepting more light for better growth and yield. Lower crop population density, as reported by Demisie and Tolessa (2018) and Van Delden *et al.* (2021), may allow foliage to receive maximum photosynthetically active radiation (PAR) and synthesize assimilates, ultimately contributing to greater leaf growth. An increase in canopy size is likely to enhance photosynthesis, leading to the production of more leaves, which has been supported by the findings of Appiah *et al.* (2017) and Tesfu and Charles (2010) in other plants. Similar findings have also been reported in carrot (Alam *et al.*, 2020) and radish (Sandipan and Rawat, 2020).

Root length, root diameter and root weight

The number of plants per unit area can influence the yield and quality of horticultural crops (Rodriguez *et al.*, 2007; Lencha and Buke, 2017; Demisie and Tolessa, 2018; Sinta and Garo, 2021; Tegen *et al.*, 2021). In the current investigation, the maximum root length (22.2 cm), root diameter (5.9 cm) and root weight (136.3 g) were recorded at spacing of 20 x 15 cm followed by 15 x 15 cm spacing (19.1 cm for root length, 5.2 cm for root diameter and 115.4 g for root weight). On the contrary, the lowest values (13.7 cm, 3.4 cm, and 64.2 g for root length, diameter and weight, respectively) were recorded when plants were cultured in rows spaced 10 cm apart with 5 cm between plants within rows (Table 4). Increasing plant spacing resulted in longer roots having larger diameter, thus in higher plant yield, potentially due

Table 4 - Mean root length, root diameter and root weight of carrot (*Daucus carota* L.) plants as influenced by inter and intra row spacing

Distances (cm)	Root length (cm)	Root diameter (cm)	Root weight (g)
Row distance (Rd)			
10	15.51 c	3.97 c	79.14 c
15	17.03 b	4.37 b	104.60 b
20	19.31 a	4.87 a	114.46 a
Plant distance in the row (Pd)			
5	15.34 c	3.68 c	85.26 c
10	17.36 b	4.43 b	98.03 b
15	19.06 a	5.09 a	114.92 a
Plant spacing (Rd × Pd)			
10 × 5	13.77 h	3.40 f	64.23 f
10 × 10	16.57 ef	4.33 cd	80.23 ef
10 × 15	16.20 f	4.18 d	92.98 de
15 × 5	14.73 g	3.63 ef	96.09 cde
15 ×10	17.23 de	4.30 cd	102.26 bcd
15 × 15	19.10 b	5.17 b	115.45 b
20 × 5	17.53 cd	4.02 de	95.45 de
20 × 10	18.23 c	4.65 c	111.62 bc
20 × 15	22.17 a	5.93 a	136.32 a
CV (%)	2.83%	5.87%	28.6%

Mean values within rows and columns followed by different letter(s) are significantly different at 5% probability level.
CV= coefficient of variation.

to the availability of sufficient resources for root growth and development, and reduced competition for available soil resources.

The results of our study are in line with previous research, which suggests that wider spacing of plants can lead to increased nutrient uptake and photosynthesis rates, resulting in improved areal and root growth as well as fresh root weight in carrot production (Kabir *et al.*, 2013; D'hooghe *et al.*, 2018; Appiah *et al.*, 2021). Kharsan *et al.* (2019) reported a gradual increase in root diameter with increasing spacing, observing a 1.4 cm increase in root diameter when plant spacing was increased from 5 cm to 15 cm. Similar results and arguments were reported more recently also by Tegen and Jembere (2021), Kwiatkowski *et al.* (2022) and Searight *et al.* (2022). It was suggested that plants sown with wider spacing had more room to develop their roots in the soil, leading to an increase in root diameter compared to those planted with lower spacing.

Marketable and unmaketable root yield (q ha^{-1})

According to Pant and Sah (2020) and Sandhu *et al.* (2021), the success of crop establishment, yield, and profitability are all affected by plant density. Poor plant stand is a major factor in reducing yield, and increasing planting density can further exacerbate this problem by decreasing the plant's net photosynthetic rate (Pn), stomatal conductance (Gc), and leaf chlorophyll content, ultimately leading to decreased yield (Zhang *et al.*, 2021).

The highest marketable yield (490.4 q ha^{-1}) was recorded at spacing of 20 x 5 cm followed by 15 x 5 cm spacing (359.5 q ha^{-1}), as the smallest root marketable yield (83.4 q ha^{-1}) was recorded at 20 x 15 cm spacing (Table 5). Thus, the highest marketable root yield of carrot per hectare was obtained with the smallest plant distance within the row and the largest distance between rows, while the carrot yield was the lowest when plants were grown using the largest intra- and inter-row spacings. This result is supported by Appiah *et al.* (2021), who observed that narrow spacing resulted in small and uneven root sizes which are rejected from the market. The reason for the lower marketable yield resulting from wider spacing can be indirectly attributed to the number of plants per unit area. Each plant has the chance to produce marketable root. Hence, the density of plants per unit area has a direct impact on the available number of roots, which ultimately affects the yield. Moreover, an increase in row spacing causes excessive branching and cracking of the roots, making them less desirable to consumers or in the market, as stated by Connors (2022) and Searight *et al.* (2022).

Table 5 - Marketable and unmarketable root yield of carrot (*Daucus carota* L.) as influenced by inter and intra row spacing

Distances (cm)	Marketable yield (qha^{-1})	Unmarketable yield (qha^{-1})
Row distance (Rd)		
10	166.98 c	5.85 c
15	225.67 b	9.66 b
20	286.10 a	16.97 a
Plant distance in the row (Pd)		
5	338.11 a	5.61 c
10	227.74 b	9.29 b
15	112.90 c	17.58 a
Plant spacing (Rd × Pd)		
10 × 5	164.42 def	2.70 d
10 × 10	185.98 cde	6.38 cd
10 × 15	150.55 def	8.47 cd
15 × 5	359.48 b	9.72 c
15 ×10	112.82 cd	11.29 bc
15 × 15	104.70 ef	17.99 b
20 × 5	490.43 a	4.40 cd
20 × 10	284.43 bc	10.21 bc
20 × 15	83.43 f	36.29 a
CV (%)	25.74%	32.89%

Mean values within rows and columns followed by different letter(s) are significantly different at 5% probability level.
CV= coefficient of variation.

According to Haque and Sakimin (2022), exceeding a certain planting density threshold can lead to decreased yield and quality due to inadequate resource supply, resulting in produce that is unsuitable for the market. In our experiment, larger planting distances of 20 x 15 cm and 15 x 15 cm resulted in the highest unmarketable yields of 36.3 q ha^{-1} and 17.9 q ha^{-1}, respectively. Conversely, reducing the distances to 10 x 5 cm minimized the unmarketable yield to just 2.7 q ha^{-1}, as shown in Table 5.

Dawuda *et al.* (2011) also reported maximum unmarketable yield from plants that were grown adopting wide spacing up to 30 × 5 cm. In agreement with the present finding, Adem Seid *et al.* (2019) reported a decline in unmarketable yield as plant spacing increased. It was suggested that percentage of root cracking might increase in the wider spacing due to more fluctuation of available soil moisture as

absorbed by the plants. According to some authors (Merfield, 2006; Adem Seid *et al.*, 2019; Tegen and Jembere, 2021; Mahaffee *et al.*, 2023), the only disadvantages of high-density plantings include producing fewer jumbo carrots and lack of airflow through the field that can increase the incidence of foliar diseases, but this can be managed via integrated pest management. In a previous investigation on carrot cultivation, it has been reported that the yield of boxed sized root increases with plant density to a maximum and then decreases, being maximum yield achieved with higher plant density (Tegen and Jembere, 2021).

4. Conclusions

The growth and productivity of carrots are significantly affected by agronomic practices, with plant population being a vital management factor. Results from our experiment showed that spacing had a significant influence on all parameters except for days to emergence. The lowest sowing density with a spacing of 20 x 15 cm resulted in the highest values of plant height (73.0 cm), number of leaves (21.0 cm), diameter of root (3.4 cm), and length of root (22.2 cm). However, the highest root yield per hectare (490.4 q ha^{-1}) was recorded from a spacing of 20 x 5 cm, while the lowest yield (83.4 kg ha^{-1}) was from the widest spacing of 20 x 15 cm. Although wider spacing resulted in greater root length, leaf and root fresh weights, and plant height, it also led to maximum unmarketable yield, demonstrating the significance of plant distance on carrot productivity. Thus, our research suggests that the optimal plant density for maximum marketable yield of carrots in similar agroclimatic conditions and irrigated production systems is 5 cm intra-row spacing and 20 cm inter-row spacing. Going beyond this optimum level may cause branching, cracking, and unsuitability of the carrot roots for the market. However, it's important to note that these findings are from a single season and location, and further research across various locations and seasons is necessary for more reliable recommendations.

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Adv. Hort. Sci., 2023 37(3): 317-327 DOI: 10.36253/ahsc-13944

AHS
Advances in Horticultural Science

Improving onion productivity and producer income through nitrogen management

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Key words: *Allium cepa*, application frequency, bulb, fertilizer, yield.

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Citation:
DINEGA T.M., HAILE A., BESHIR H.M., 2023 - *Improving onion productivity and producer income through nitrogen management.* - Adv. Hort. Sci., 37(3): 317-327.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Ethical Approvals:
This study does not involve experiments on animals or human subjects.

Funding:
The research was conducted as a work product of the employee, Alage Teachers Vocational Education Technical College.

Competing Interests:
The authors declare no competing interests.

Received for publication 14 January 2023
Accepted for publication 21 March 2023

Abstract: Intensifying nitrogen (N) management can improve yield and bulb quality in onions. A field experiment was conducted to determine the optimum N rate and application frequency for high onion productivity. Treatments comprised levels of nitrogen (N): 50, 100, 150, 200, or 250 kg ha^{-1}, and nitrogen application frequency: all at once, twice, three times, or four times. The N rate and application frequency affected growth performance, bulb characters, yield, and bulb quality of onions. The supplement of 150 kg ha^{-1} N at three times the application frequencies generated the highest marketable yield (46.5 t ha^{-1}) with the highest net benefit (626317 ETB ha^{-1}). Application of 150 kg ha^{-1} of inorganic nitrogen with three times the application frequency improves the marketable yield of onions with the highest and most acceptable net benefit. The intensive and economical use of inorganic nitrogen and its frequency of application increased the growth and economic yield of onions in field conditions.

1. Introduction

Intensive nutrient management for onions (*Allium cepa* L.) involves using fertilizer as efficiently as possible. The principle behind nutrient management is balancing soil nutrient inputs with crop requirements (Fekadu and Dandena, 2006). Nitrogen (N) is an essential nutrient whose deficiency limits crop productivity. Compared to other vegetables, onions require high amounts of nitrogen and are applied at different times during the growth (Geisseler *et al.*, 2022). The recovery of fertilizer can be low, 30 to 40% (Halvorson *et al.*, 2002; Sharma *et al.*, 2012), due to the shallow onion root system. Low N fertilizer recovery in onions is, in part, due to the variable amounts of mineral N present in the soil before sowing (Brewster, 2008). Appropriate N fertilizer management requires knowledge of crop demand and the time it is needed (IPNI, 2012).

A low rate of N causes a low yield of onions due to a shortage of N required for the chlorophyll pigment that is responsible for photosynthe-

sis (Jilani *et al.*, 2004; Khan *et al.*, 2021). Excessive N is hazardous to the environment, weaken the foliage and predispose the plants to pathogenic diseases (Geary *et al.*, 2015). These factors decreased the potential of processing photosynthesis that resulted in yield reduction due to a decrease in assimilates in plant leaves (Reay *et al.*, 2012).

The frequency of N application affects the productivity of onions (Grant *et al.*, 2012). When applying N fertilizer all at once, more N is susceptible to denitrification, leaching, or volatilization. When N fertilizer is applied faster than plants can use it, soil bacteria convert it to nitrate and decrease N use efficiency (Zhang *et al.*, 2013).

The majority of small-scale farmers use less nitrogen and apply it all at once, which is sub-optimal (Shura *et al.*, 2022). There is often excessive application of N fertilizer by large-scale commercial farmers near harvest. It is assumed that synchronization of crop demand for N fertilizer and time of application with sufficient amounts improves yield of onions and reduces waste of N, reducing production costs and environmental pollution due to unused excess N leaching in different forms (Geisseler *et al.*, 2022). However, the optimum amount of N that matches the crop demand during the growth period is little explored. We hypothesized that the low N use efficiency of the onion due to its shallow and sparse root system can be improved by controlled application of N fertilizer during the growth period. Therefore, this work was conducted to determine the optimum N rate and proper application frequency during the growth period for economically feasible onion production.

2. Materials and Methods

Site description

The study was conducted at Alage Agricultural Technical Vocational Educational and Training College, near Bulbula Town, Ethiopia, under supplemental irrigation (when precipitation is lacking) from June to October 2021. The area is situated between 7°65" N latitude and 38°56" E longitude at 1600 m above sea level in the dry plateau of the southern part of the Ethiopian rift valley system. The area is characterized by a bimodal rainfall pattern where a short rainy season occurs during March and April and the main rain starts in June and extends to September, with high rainfall in July and August. The mean annual rainfall is 800 mm, and the annual mean minimum and maximum temperatures are 11 and 29°C, respectively. The soil of the area ranges from sandy loam to sandy clay loam, with some clay loam and a few clay soils, and is slightly alkaline, pH 7.8 (Alemayehu and Bewket, 2016).

Physico-chemical properties of the experimental field soil

Before planting, five soil samples were randomly taken from the field at a depth of 0 to 20 cm in a zigzag pattern using an auger. Samples were mixed to produce a representative composite sample of 1 kg. The soil sample was air-dried and ground to pass 2 and 0.5 mm (for total N) sieves and analyzed for total P, total N, pH, organic carbon (OC), exchangeable cations, and physical properties at the Batu Agricultural Research Centre Soil Laboratory.

The soil was a silty clay loam with 18.0% sand, 50.5% silt, and 31.5% clay. The soil was slightly alkaline in reaction, with a pH (H_2O 1:2.5) of 7.82, which is within the range of ideal soil pH for onion bulb production (Graham *et al.*, 2004). Total N, available P, organic carbon (OC), and CEC of the soil before planting were 0.12%, 11.48 mg kg^{-1}, 1.39%, and 31.74 cmol (+) kg^{-1}, respectively. The total N content of the soil was within the range of low, according to Havlin *et al.* (1999). The cation exchange capacity (CEC, 31.74 meq/100 g) of the soil was high according to the rating of Jackson (1975). The carbon-to-nitrogen ratio (C:N) was 11.5%.

Planting materials

The onion, cv. Bombay Red, was used. It is adapted to areas of 700 to 2000 m above sea level. The size of the bulb of this variety ranges from 85 to 90 g, with a yield potential of 25 to 30 t ha^{-1} under research conditions (Lemma and Shimeles, 2003). Sources of fertilizers were urea (46% N) and Triple Super Phosphate (TSP) (46% P_2O_5) for N and phosphorus, respectively. The nitrogen fertilizer was applied immediately after weeding. Onion seeds were sown 56 days before transplanting on a 1 m wide seed bed 10 m in length (area 10 m^2). Seedlings were grown under suitable conditions of fertilization, weeding, and pest control. Seven days before transplanting, seedlings were gradually exposed to field conditions and withheld from the water supply (hardened off) in the nursery, and then manually transplanted at 56 days old. The TSP was applied during transplantation. All cultural practices and crop protection measures (diseases and insect control) were

carried out uniformly for all plots (EARO, 2004).

Treatments, design and experimental procedure

Treatments were arranged in factorial combinations in a randomized complete block design with three replications. While transplants were being developed, the soil was manually pulverized twice with an oxen-driven plough. After the soil was pulverized, levelling and ridge preparation were done with hoes and spades. Rates of N were: 50, 100, 150, 200, or 250 kg ha^{-1} in band application according to application frequencies, which were: NAF1 (all at once 1 week after transplanting), NAF2 (half of the N at 1 week after transplanting, another half of the N at 21 days after transplanting), NAF3 (one-third of the N at 1 week after transplanting, one-third of the N at 21 days after transplanting, and one-third of the N at 42 days after transplanting), or NAF4 (one-fourth at 1 week after transplanting, one-fourth of the N at 21 days, one-fourth of the N at 42 days after transplanting, and one-fourth of the N at 63 days after transplanting). Treatment combinations were assigned randomly to experimental units within each block. The national blanket recommendation of N fertilizer for onion production is 100 kg ha^{-1}, which could be considered a control treatment. Double row planting was done by hand on ridges about 20 cm high at a spacing of 40 cm for water furrows, 20 cm between rows on raised beds, and 5 cm between plants within rows. There were 60 plots corresponding to the 20 treatment combinations with three replications. The unit plot size of the experiment was 2 x 2.5 m (5 m^2). Blocks were separated by 1.5 m, and the space between each plot within a block was 1 m. In each plot, 10 rows were prepared, and in each row, 50 seedlings were manually planted. Generally, 500 onion seedlings were planted per plot. The outer 2 rows on both sides of the plot and the 2 plants at both ends of the rows were border plants. The plants in the six central rows were used for measurements.

Data collection

Ten plants were randomly selected from each plot's central six rows, and data on growth performance, quality indicators, and yield components were recorded for each of them. For the data on bulb yield, all of the plants in each plot were harvested. Thus, the following information was collected:

Maturity and growth parameters

Days to maturity were recorded as the number of days from seedling transplanting to a day at which more than 80% of the plants in each plot showed yellowing of leaves or attained physiological maturity. Plant height was measured from the ground to the tip of the leaves on 10 randomly selected plants from the central rows in each plot at maturity. Leaf length was recorded at physiological maturity from the sheath to the tip of the leaf from the third youngest leaves of ten representative plants, which was used to count the number of leaves per plant using a ruler. Leaf diameter was measured from the third-youngest leaves at the bottom, middle, and tip parts of the leaves from ten randomly selected plants using a veneer caliper. Leaf number per plant was counted as the total number of leaves from 10 randomly selected plants at maturity, and the average of the ten plants was taken. The aboveground biomass was harvested by cutting the plant at the crown part, drying it in an oven at 650°C until a constant weight was attained, and the shoot dry matter was determined and expressed in grammes at harvest. Additionally, the total dry biomass was determined by summing the shoot and bulb dry weights of the sample.

Yield and yield related parameters

The mean bulb diameters of ten sample bulbs were measured at the maximum wider portion of matured bulbs using calipers. The bulb length of ten sample bulbs was measured along the length of the bulb from the basal end to the top end, at which the bulb neck was removed from matured bulbs using calipers after harvest. The average fresh weight of ten randomly taken mature bulbs was measured using a sensitive balance and finally expressed in grammes. Fir bulb dry weight Ten bulbs were randomly taken from each plot and chopped, mixed thoroughly, placed in an aluminum paper bag, and put in the oven to dry at 650°C until a constant dry weight was attained. Then each sample was immediately recorded as a bulb's dry weight. The bulb dry matter concentration (%) was determined by randomly selecting ten bulbs from each plot and chopping them, mixing them thoroughly, then weighting them and recording the fresh weight. Then each subsample was placed in an aluminum paper bag and put in an oven at 650°C until constant dry matter was attained. Each sub-sample was then immediately weighed and recorded as a dry matter yield. The dry matter concentration was determined using the loss weight, and the fresh sample was weighed to the nearest gramme using the formula set by Ruck (1969) and Dantata (2014):

$$\text{Bulb dry matter concentration} = \frac{\text{Bulb dry weight}}{\text{Bulb fresh weight}} \text{ x } 100$$

Marketable bulb yield (t ha^{-1}) was determined from the weight of healthy and marketable bulbs that range from 20 g to 160 g in weight (Lemma and Shimeles, 2003). The marketable yield was determined from the net plot at the final harvest. Unmarketable bulb yield (t ha^{-1}) was measured as the total weight of unmarketable bulbs that are undersized (<20 g), diseased, decayed, and bulbs from plants with physiological disorders such as thick neck and split were measured from a net plot at final harvest.

Undersized bulb yield (t ha^{-1}) was determined by taking under sized bulbs (<20 g) as unmarketable bulbs per net plot and converted to t ha^{-1} as determined. Total bulb yield (t ha^{-1}): The total bulb yield was measured from the total harvest of net plot as a sum weight of marketable and unmarketable yields that was measured in kg per plot and finally converted into t ha^{-1}. Harvest index (%) was expressed as the ratio of total bulb dry weight to the total biomass dry weight and expressed in percentage.

$$\text{Harvest index (HI)} = \frac{\text{Bulb dry weight}}{\text{Total dry biomass}} \text{ x } 100$$

The TSS was determined at harvesting time from ten randomly selected bulbs per plot using the procedures described by Waskar *et al.* (1999). Aliquot juice was extracted using a juice extractor, and 50 ml of the slurry was centrifuged for 15 minutes. The TSS was determined by a hand refractometer (ATAGO TC-1E) with a range of 0 to 32 Brix and resolutions of 0.20 Brix by placing 1 to 2 drops of clear juice on the prism, washing it with distilled water, and drying it with tissue paper.

Data analysis

Data were subjected to analysis of variance using Stat-8 software (ver. 8.1.1, Analytical Software, Tallahassee, FL). Assumptions of ANOVA were tested, and no violation was observed. The data analysis was done with a generalized linear model. The N rate and application frequency were fixed effects, and the block was random. If the interaction was significant, it was used to explain the results. If the interaction was not significant, the main effects were separated using LSD (Walter and Duncan, 1969).

Economic analysis

A partial budget analysis was made to determine the exact rate of return that producers gain on their investment by changing existing cultural practices to alternative ones. The potential response of added fertilizer corresponding to labor costs and the price of fertilizers (variable costs for urea fertilizer at 16.2 birr) throughout the crop growing season was evaluated. The birr is the local currency. 1 birr is equal to 0.031 US during the growth period. The economic outcome was analyzed using agronomic indices for N and its frequencies. The economic analysis was computed using accepted procedures (CIMMYT, 1988), where: Avy (gross average bulb yield, average yield of each treatment); Ajy (adjusted yield, the average yield adjusted downward by 10% to reflect the difference between experimental yield and yield of the farmers); GFB (gross field benefit determined by multiplying field price that farmers receive for the crop by adjusted yield); NFB (net field benefit calculated by subtracting total costs from gross field benefit; GFB for each treatment); and MRR (marginal rate of return %) calculated by dividing change in net benefit by the marginal cost reflecting change in cost (CIMMYT, 1988).

3. Results and Discussion

The main effects of N rate and application frequency influenced plant height, leaf length, leaf width, and number of leaves. The interaction effect of two factors was not significant for these parameters. Plant height increased with increasing N rate from 50 to 250 kg ha^{-1} N. The tallest and shortest onion plants were at 250 kg ha^{-1} and 50 kg ha^{-1}, respectively. Onions treated with N at 250 kg ha^{-1} exceeded their mean height by 34.23% compared with the 50 kg ha^{-1} N-treated onions (Fig. 1A). In response to An N application frequency, the tallest plants were for 3 time's N application (Table 1). Application of N at all frequencies (once, twice, and four times) was not different for plant height. The increase in plant height with the addition of higher N fertilizer could be attributed to the increased availability of N for growth as a result of protein synthesis and the accumulation of carbohydrates (Rizk, 2012). The tallest plants recorded at three applications of N may be because of increased N use efficiency, decreasing N loss by leaching and volatilization (Rizk, 2012). Application of N three times better matched the availability of N with crop N demand compared to application all at once, or 2 or 4 applications of N (Brewster, 2008). This result is consistent with the findings of Morsy *et al.* (2012) and Nasreen *et al.* (2007), who reported onion plant height increased as

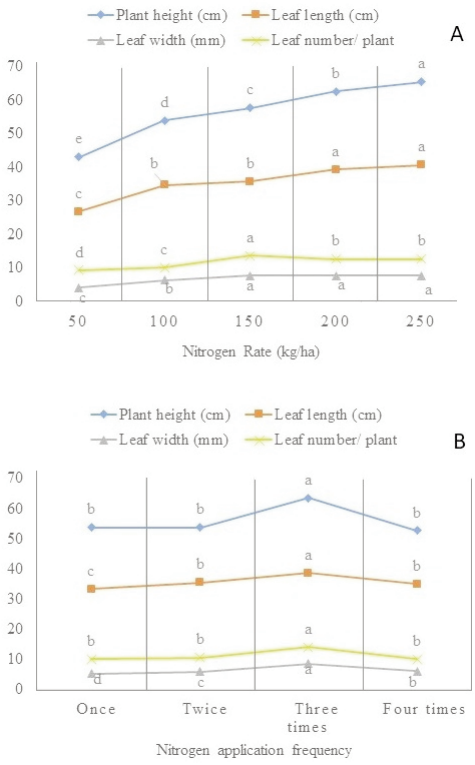

Fig. 1 - The effect of nitrogen rate (A) and application frequency (B) on plant height, leaf length, leaf diameter and leaf number of onion.

N fertilizer rate increased.

Shorter leaves were from the 50 kg ha^{-1} N that decreased by about 31.56% and 33.95% compared to values obtained from 200 and 250 kg ha^{-1} N, respectively (Fig. 1). The effects of 100 and 150 kg ha^{-1} N were similar. The influence of N rate at 200 and 250 kg ha^{-1} was not significant for leaf length. The longest and shortest leaves were when N fertilizer was applied three times and all at once, respectively (Fig. 1B). These values agree with Rao *et al.* (2013), who reported that higher N fertilization increased onion leaf length, and Khan *et al.* (2002), where the longest leaves were when N was applied three times. Increased leaf length at three times N applications could be due to increased recovery of N by onions and decreased N loss (Ali and Ceyhan, 2001).

The narrowest leaves were from plants treated with 50 kg ha^{-1} N. Leaf diameter increased with increased N, from 50 to 150 kg ha^{-1}. The N rates of 150, 200, and 250 kg ha^{-1} had similar leaf diameters (Fig. 1A). Leaf diameter was influenced by the frequency of N applications. Three times N application increased leaf diameter by 38.99% compared to applying N all at once (Fig. 1B). The increase in leaf diameter with an increase in N rate from 50-150 kg

Table 1 - Interaction effects of N rate and application frequency on bulb size, marketable bulb yield, total bulb yield, bulb dry weight, total dry biomass, and total soluble solids of onions

Treatments		Average bulb size (g plant^{-1})	Marketable bulb yield (t ha^{-1})	Total bulb yield (t ha^{-1})	Bulb dry weight (g plant^{-1})	Total dry biomass (g plant^{-1})	Total soluble solids (°Brix)
Nitrogen rate (kg ha^{-1})	Nitrogen application frequency						
50	Once	31.55 k	25.75 j	28.00 k	5.01 i	3.65 i	9.27 i
	Twice	40.23 j	31.17 hi	33.17 ij	6.12 h	3.83 h	10.17 h
	Three times	44.25 ij	33.58 gh	35.00 hij	7.08 g	4.58 gh	10.47 fg
	Four times	43.44 ij	30.43 i	32.38 j	7.45 g	4.94 g	10.03 gh
100	Once	53.00 hi	29.52 i	32.33 j	8.72 f	6.00 f	10.73 efg
	Twice	56.27 gh	34.42 fg	36.00 ghi	8.95 f	6.15 ef	11.05 def
	Three times	57.88 fgh	39.48 de	41.12 de	10.15 e	6.92 de	11.59 cd
	Four times	63.52 defg	36.27 fg	38.00 fg	9.19 f	6.13 ef	11.0 2def
150	Once	62.92 efgh	30.78 hi	33.32 ij	10.42 e	7.37 cd	10.62 fg
	Twice	82.33 b	45.17 bc	46.97 b	12.01 cd	8.83 b	11.06 def
	Three times	94.00 a	46.95 a	48.84 a	13.95 a	9.79 a	12.30 b
	Four times	66.39 cdefg	43.5 bc	45.16 bc	11.39 d	7.85 c	11.14 def
200	Once	69.67 cde	33.75 fgh	36.18 gh	11.67 d	7.87 c	10.42 fg
	Twice	75.35 bc	42.5 bcd	44.37 bc	12.15 cd	8.19 bc	11.36 de
	Three times	70.29 cde	42.50 bc	45.08 bc	12.95 b	8.81 b	12.36 b
	Four times	70.79 cde	43.83 bcd	45.57 bc	12.79 bc	8.25 bc	11.37 de
250	Once	66.00 defg	36.73 ef	39.33 ef	11.93 cd	7.87 c	10.73 efg
	Twice	67.92 cdef	42.92 bc	39.32 ef	12.74 bc	8.19 bc	11.15 def
	Three times	73.51 bcd	47.88 bcd	45.04 bc	12.77 bc	8.81 b	13.13 a
	Four times	69.59 cde	43.92 ef	43.60 cd	12.72 bc	8.25 bc	12.24 bc
LSD		10.28	3.124	2.84	0.9	0.9	0.74
α		**	**	**	*	**	**
CV (%)		9.88	5.02	4.33	5.17	7.64	4.02

Means followed by the same letters within a column are not significantly different at P<0.05.

ha^{-1} and 3 applications of N could be associated with a better supply of N and better N use efficiency. Application of N with three or more applications and a higher supply of N could increase leaf thickness, capture resources for photosynthesis, and promote better growth and development. The lowest leaf diameter was due to the low N rate applied to onions (Abdissa *et al.*, 2011; Woldeyohannes *et al.*, 2013; Seid *et al.*, 2014).

Increasing N from 50 to 150 kg ha^{-1} increased leaf number. Beyond 150 kg ha^{-1}, the number of leaves decreased. Onion leaf number increased as the N fertilizer rate increased from 50 to 150 kg N ha^{-1} (Fig. 1A). Increasing N frequency from one to three applications increased leaf number. At 4 N applications, leaf number decreased (Fig. 1B). This may be due to a lack of enough nitrogen at the early growth stage at which leaf formation was initiated. When N is applied four times during the growth period, a quarter of it will not be used by the onion plants for the initiation of leaf formation due to late application (Geisseler *et al.*, 2022). Increases in the number of leaves with a further increase in the rate of N could be attributed to enhanced photo-assimilate production, cell division, and vegetative growth (Suthar, 2009). The N plays a role in leaf production and vegetative growth (Nasreen *et al.*, 2007). Increases in the number of leaves per plant with up to 3 applications of N may be attributed to increasing N use efficiency (Geisseler *et al.*, 2022). This indicates that one-third of the N fertilizer in the first application was enough for leaf number production and vigorous vegetative growth, which agrees with Mengel *et al.* (2006).

Bulb diameter and bulb length were influenced by N rate and N application frequency, but the interaction of N rate and frequency was not significant. Increasing the N rate from 50 to 150 kg ha^{-1} increased bulb diameter by 43.66%. Increasing the N rate beyond 150 kg ha^{-1} did not increase bulb diameter (Fig. 2B). The widest bulbs were for the 3-times N application (Fig. 2B), which agrees with Nasreen *et al.* (2007). Development of wider bulbs with increasing frequency and rate of N fertilizer could be associated with the availability of more growth resources due to efficient N use as bulbs develop. Increasing N rate and split application are associated with promoting cell elongation, above-ground vegetative growth, and the synthesis of chlorophyll, resulting in dark green leaves (Geisseler *et al.*, 2022). These results agree with Soleymani and Shahrajabian (2012) and Ghaffoor *et al.* (2003).

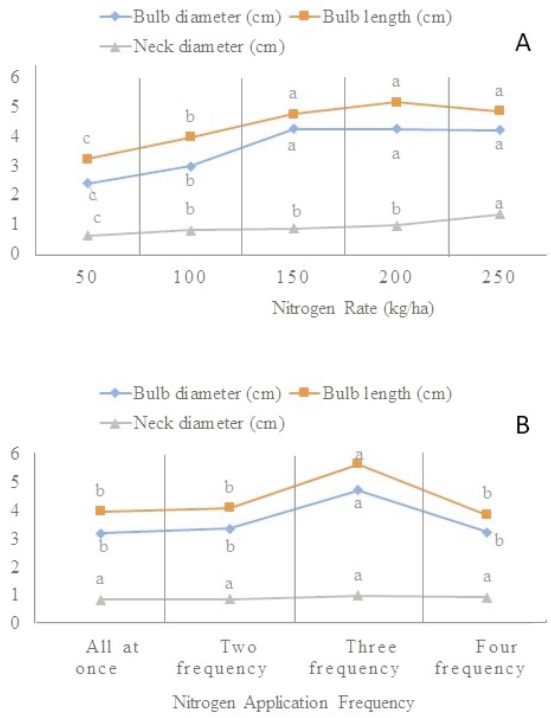

Fig. 2 - The effect of nitrogen rate (A) and application frequency (B) on bulb diameter, bulb length and bulb neck of onion.

Increasing the rate of N application from 50 to 150 kg ha^{-1} increased bulb length. At N rates of 150, 200, or 250 kg ha^{-1} bulb lengths were similar (Fig. 2A). Increasing application frequency up to three times increases bulb length. When all N fertilizer was applied at once, bulb length was reduced (Fig. 2B). The reason for the longest bulbs at 150 kg ha^{-1} N could be the supply of optimum N (Fageria and Baligar, 2005). The increase in bulb length at 3 N applications might be due to the recovery of N by onions and its subsequent use for growth (Singh and Chaure, 1999; Bahadur and Singh, 2005; Mengel *et al.*, 2006).

Bulb neck thickness was only affected by the N rate. The widest bulb necks were due to the application of 250 kg ha^{-1} N, and the narrowest bulb neck diameter was 50 kg ha^{-1} N (Fig. 2A). The reason for the widest neck diameter at 250 kg ha^{-1} N rate might be due to high N that resulted in excessive vegetative growth and delayed maturity, resulting in a large neck size (Grant *et al.*, 2012; Morsy *et al.*, 2012). The increase in neck diameter might show its involvement in the synthesis of amino acids, as they link together to form proteins and make up metabolic processes required for plant growth, including neck thickening (Jilani, 2004).

The main effects of N rate and N application frequency influenced the shoot dry matter weight and harvest index of onion plants. The interaction effect of an N application and its application frequency was not significant. The unmarketable yield was only affected by the N rate. Bulb dry matter, dry total biomass weight, bulb fresh weight, marketable bulb yield, total soluble solids, and total bulb yield were affected by the interaction effect of N rate and application frequency in addition to the main effects.

The highest shoot dry matter weight was 250 kg ha^{-1} N. As the N rate increased from 50 to 250 kg ha^{-1}, the shoot dry matter of onions increased by 50.51% (Fig. 3A). This might be due to excessive vegetative growth that resulted from the application of excess N (Kandil *et al.*, 2013). The lowest shoot dry weight yield on 50 kg ha^{-1} N might also be because of N deficiency, which limits cell division and expansion, chloroplast development, chlorophyll concentration, and enzyme activity (Soleymani and Shahrajabian, 2012). The highest shoot dry matter weight per plant was at 3 N applications. The lowest shoot dry matter weight was from treatment at N applied once (A). Shoot dry matter yield from 3 applications of N increased by 17.42% compared to shoot dry matter produced when N was applied at one time (Fig. 3B). Three applications of N fertilizer increased the N use efficiency of onions (Sharma, 1992). The present finding agrees with Nasreen *et al.* (2007), who indicated the shoot dry matter weight increased with an increment of N fertilizer rate applied three times.

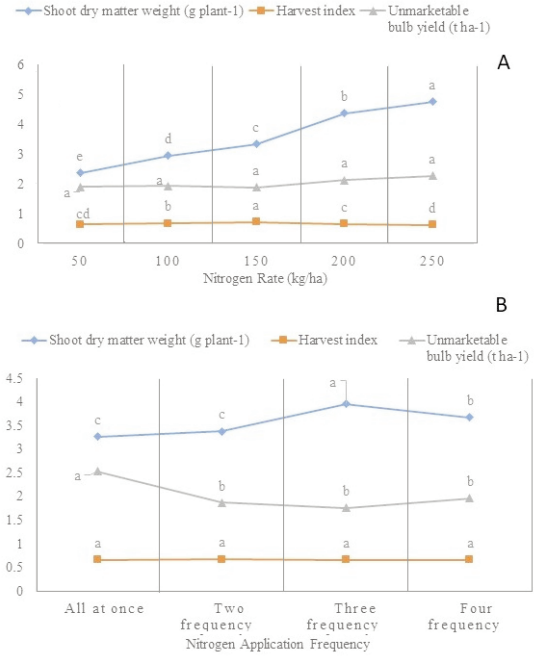

Fig. 3 - The effect of nitrogen rate (A) and application frequency (B) on shoot dry matter, harvest index and unmarketable yield of onion.

The harvest index was increased from 63.74 to 71.51% as N was increased from 50 to 150 kg ha^{-1}. Beyond 150 kg ha^{-1} N, the harvest index decreased (62.2% at 250 kg ha^{-1} N) due to increase above-ground dry biomass compared to the bulb weight of onions. The harvest index recorded at 50 kg ha^{-1} N was similar to that recorded at 200 and 250 kg ha^{-1} N (Fig. 3A). The average harvest index across all N rates was 66.2%. The highest harvest index at 150 kg ha^{-1} N might be due to increased bulb weight due to enough N sufficient for photosynthesis and assimilate production that increased bulb dry weight with optimum above ground biomass resulting in a higher harvest index (Geisseler *et al.*, 2022). The low harvest index from treatment with 50 kg ha^{-1} was due to low bulb dry matter weight due to N deficiency (Nasreen *et al.*, 2007). The low results obtained from 200 and 250 kg ha^{-1} might be because of etiolated growth of above-ground biomass and low bulb growth performance resulting from the application of excess N (Negash *et al.*, 2009; Abdissa *et al.*, 2011).

With increasing frequency of N up to 3 applications, the unmarketable bulb yield of onions decreased. The highest value of unmarketable bulb yield was all N applied once. This was followed by the 4N applications. The lowest unmarketable bulb yield was 3 N applications (Fig. 3B). The unmarketable yield from the application of all N at once was exceeded by the unmarketable yield from three applications of N. This may be due to the loss of N because of volatilization and leaching of N, which decrease N use efficiency if N supply is sufficient. Sustainable application of N during the active growth period can decrease unmarketable bulb yield (Balemi *et al.*, 2007; Biesiada and Kołota, 2009; Soleymani and Shahrajabian, 2012).

Increasing the rate of nitrogen application up to 150 kg ha^{-1} increased the average bulb weight by increasing the frequency of nitrogen application up to three times. The highest average fresh bulb weight was recorded at an N rate of 150 kg ha^{-1} applied three times (Table 1). This treatment approximated the expected bulb weight for the cultivar. The lowest bulb size was for the 50 kg ha^{-1} N all applied at once (Table 1). Application of 150 kg ha^{-1} N three times exceeded the lowest fresh bulb weight recorded at 50 kg ha^{-1} N all applied at once by 66.4%.

The increase in fresh bulb weight with an increase

in nitrogen fertilizer rate might be due to a sufficient supply of nitrogen that enhances cell division and expansion, chloroplast development, chlorophyll concentration, and enzyme activity. The increase in fresh bulb weight with increasing nitrogen application frequency up to 3 times might be due to the increasing matching of the crop demand for N with the plant requirement for N (Khan *et al.*, 2002; Yadav *et al*., 2003). When all of the nitrogen is supplied ahead of crop growth, more of that nitrogen is susceptible to denitrification, leaching, or volatilization (Brady, 1985).

Increasing the N from 50 to 150 kg ha^{-1} increased the production of marketable bulbs across the board by increasing the N application frequency up to three times. Beyond that, marketable bulb yield was not increased. The highest marketable bulb yield was from onions, which provided 150 kg ha^{-1} N in 3 applications. The lowest marketable bulb yield was in response to 50 kg ha^{-1} N applied at once (Table 1). This might be attributed to an optimum rate of N fertilizer and sustained application of N in relation to the demand of the crop, with reduced loss of N. Split applications can be timed to match the N available with crop demand. This reduces the residence time of fertilizer N in the soil and the risk of N being lost. The marketable bulb yield of onions per unit area is a function of the N dose supplied and the application frequency of N fertilizer (Naik and Hosamani, 2003; Latif *et al.*, 2010). A higher marketable bulb yield was achieved at a 150 kg ha^{-1} rate of N fertilization applied three times (Balemi *et al.*, 2007; Soleymani and Shahrajabian, 2012).

Total bulb yield increased in response to increasing N rates up to 150 kg ha^{-1} across increasing frequency of N application up to 3 times. The highest total bulb yield was obtained from onion plants at 150 kg ha^{-1} N and 3 applications (Table 1). The lowest total bulb yield was in response to the application of N at 50 kg ha^{-1} all at once. The increased total bulb yield in response to 150 kg ha^{-1} N and 3 applications might be due to plants receiving enough N. High N use efficiency and crop recovery occurred when N was applied 3 times during active growth (Tsai *et al.*, 2012). The decrease in total bulb yield with application of N beyond 150 kg ha^{-1} might be due to luxury consumption of N that affects onion plant metabolism by decreasing the ability of the root surface to absorb phosphorus and decreasing the assimilate preparation (Mahdieh *et al.*, 2012). The highest bulb yield due to the application of 150 kg ha^{-1} N applied three times agrees with Gebremedhin *et al.* (2018). Enhanced leaf number and length may lead to increased assimilate production and increased bulb yield (Geisseler *et al.*, 2022). The dose of N up to 120 kg ha^{-1} increased total bulb yield, but below this rate, total bulb yield decreased (Jilani *et al.*, 2004). A low rate of N applied all at once produced lower total yields compared to higher N doses applied in 3 splits (Balemi *et al.*, 2007; Soleymani and Shahrajabian, 2012).

Plants treated with 150 kg ha^{-1} N applied three times produced the highest total dry biomass. Plants treated with 50 kg ha^{-1} N all at once produced the lowest dry total biomass weight (Table 1). The total dry biomass from plants treated with 150 kg ha^{-1} N applied three times was about 64.09% higher than the lowest total dry biomass weight produced by onion plants treated with 50 kg ha^{-1} N applied all at once (Table 1). An increase in total dry biomass in response to an increasing rate of N may be associated with sufficient supply and efficient use, which enhance vegetative growth and contribute to an improved rate of photosynthesis and assimilate production (Nasreen *et al.*, 2007; Sikder *et al.*, 2010; Daniel *et al.*, 2021).

Total soluble solids increased with increasing N across the frequency of application. The highest total soluble solids were for plants grown at 250 kg ha^{-1} N with 3 applications. The lowest total soluble solids were for plants grown at 50 kg ha^{-1} N applied all at once (Table 1).

Total soluble solids from treatment with 250 kg ha^{-1} N applied three times exceeded total soluble solids obtained at 50 kg ha^{-1} N applied all at once by about 29.4% (Table 1). The possible reason for increasing total soluble solids with a higher application rate of N along with increasing N application frequency might be increased chlorophyll content and dry weight per plant (Naik and Hosamani, 2003; Mengel *et al.*, 2006; Moursy *et al.*, 2007; Morsy *et al.*, 2012).

The minimum acceptable MRR is 100% (CIMMYT, 1988). The labor cost for applying N fertilizer was increased depending on the labor required for each N fertilizer application frequency. The cost of labor for N fertilizer application was 100, 200, 300, and 400 birr ha^{-1} for 1, 2, 3, or 4 applications of N fertilizer, respectively. The field price of onions during harvesting was 15 birr per kg. All total variable costs were subtracted from the gross benefit to obtain the net benefit (Table 2).

Table 2 - Cost benefit analysis[z] of nitrogen and its application frequencies

Nitrogen rate	Nitrogen application frequency	Average marketable yield	Adjusted marketable yield [y]	Gross field benenit	Nitrogen cost	Labour cost for nitrogen application	Total variable cost	Net benefit	Dominance	Marginal rate of return (%)
50	Once	25.75	23.18	347700	1761	100	1861	345839	-	-
50	Twice	31.17	28.05	420750	1761	200	1961	418789	N	729.50
50	Three times	33.58	30.22	453300	1761	300	2061	451239	N	324.50
50	Four times	30.43	27.39	410850	1761	400	2161	408689	D	
100	Once	29.52	26.57	398550	3522	100	3622	394928	D	
100	Twice	34.42	30.98	464700	3522	200	3722	460978	N	5.90
100	Three times	39.48	35.53	532950	3522	300	3822	529128	N	681.50
100	Four times	36.27	32.64	489600	3522	400	3922	485678	D	
150	Once	30.78	27.43	411450	5283	100	5383	406067	D	
150	Twice	45.17	40.65	609750	5283	200	5483	604267	N	45.24
150	Three times	46.95	42.26	633900	5283	300	5583	628317	N	240.50
150	Four times	43.5	39.15	587250	5283	400	5683	581567	D	
200	Once	33.75	30.38	455700	7044	100	7144	448556	D	
200	Twice	42.5	38.25	573750	7044	200	7244	566506	D	
200	Three times	42.97	38.673	580095	7044	300	7344	572751	D	
200	Four times	43.83	39.45	592750	7044	400	7444	585306	D	
250	Once	36.73	33.06	495900	8805	100	8905	486995	D	
250	Twice	42.92	38.63	579450	8805	200	9005	570445	D	
250	Three times	42.5	38.25	573750	8805	300	9105	564645	D	
250	Four times	41.25	37.125	556875	8805	400	9205	547670	D	

[z] The analysis was performed according to accepted procedures (CIMMYT, 1988).
[y] Adj. yield = adjusted marketable yield downward by 10%.
[w] D = dominated (any treatment that has net benefits that are less than or equal to those of a treatment with lower costs that vary is dominated; CIMMYT, 1988).
N = non-dominated, *= the exchange rate of the US dollar to birr was 38 birr in 2021.

Partial budget analysis indicated the highest MRR of 7.295% was from an application of 50 kg ha^{-1} N rate in 2 applications. For every 1 birr invested in 100 kg ha^{-1} N applied three times, growers can expect to recover the 1 birr and obtain an additional 7.295 birr. The higher net benefit with an acceptable MRR of 240.50% was from 150 kg ha^{-1} N applied three times (Table 2). The most attractive combinations for farmers were in response to an application of 150 kg ha^{-1} N, which provided three times the highest marketable yield and net benefit. Onion producers may maximize their net benefit by using 150 kg ha^{-1} of N applied three times.

4. Conclusions

The interaction of N rate and application frequency resulted in the highest total bulb yield (48.84 g plant^{-1}), marketable bulb yield (46.95 t ha^{-1}), total dry biomass weight (13.5 g plant^{-1}), and bulb dry biomass yield (9.7 g plant^{-1}), when the combination of N rate at 150 kg ha^{-1} and three times application frequencies of N was realized. The highest value of total soluble solids (13.13°Brix) was recorded at the treatment combination of 250 N kg ha^{-1} with three times the application frequency. The partial budget analysis revealed that the highest net benefit of Birr 628317 with an acceptable MRR of 240.5% was obtained from the application of N at 150 kg ha^{-1} and three frequencies of N application. Generally, the treatment combination of an N rate of 150 kg ha^{-1} with three times the N application frequency can be recommended to achieve a high bulb yield of onions with the highest net benefit.

Acknowledgements

The authors acknowledge the management in

charge of the Alage Teachers Vocational Education Technical College, which partly supported this research by providing field space.

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Adv. Hort. Sci., 2023 37(3): 329-341 DOI: 10.36253/ahsc-15064

AHS
Advances in Horticultural Science

Characterization of Italian honeys: integrating volatile and physico-chemical data

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Key words: Honey, honey characterization, honey origin, honey properties, honey volatiles, monofloral honey, Proton Transfer Reaction Time-of-Flight Mass Spectrometer.

Abstract: This article focuses on the comprehensive characterization of Italian honeys using various physico-chemical analyses and their volatile organic compounds (VOCs) fingerprint obtained through the PTR-ToF-MS technology. Honey characteristics, including pH, electrical conductivity, moisture content, hydroxymethylfurfural (HMF), and sugar content, were analyzed to assess their quality and origin. Honey samples from different flowers, including acacia, chestnut, citrus, linden, and multifloral, were collected and investigated. Furthermore, a few aged honeys were collected and analyzed and compared with the fresh ones. Physico-chemical analysis revealed that chestnut honey is characterized by high pH and EC values. Acacia honey has a higher fructose content, while aging appears to influence HMF levels, a vital indicator of honey quality, with aged samples exhibiting significant increases in HMF content. The VOC profiles have been found to vary among different honey types, suggesting that VOCs could be used as indicators of honey origin. Multivariate statistical analyses, such as partial least squares discriminant analysis (PLS-DA), have been applied to the VOCs data to differentiate honey types based on their volatile profiles. Acacia honey exhibited different physicochemical parameters but on the contrary, in the VOCs analysis, it displayed similarities with the linden honey due to their shared low emissions of volatile compounds. Citrus honey had similar chemical parameters to linden and multifloral honeys, but its distinctive VOCs emission allowed for a more accurate identification. In conclusion, the analysis performed with the PTR-ToF-MS was successful in obtaining specific volatile fingerprints of those samples and was effective for improving the characterization of honeys.

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Citation:
TAITI C., GUARDIGLI G., BABBINI S., MARONE E., MASI E., COMPARINI D., MANCUSO S., 2023 - *Characterization of Italian honeys: integrating volatile and physico-chemical data.* - Adv. Hort. Sci., 37(3): 329-341.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 24 August 2023
Accepted for publication 20 October 2023

1. Introduction

Honey is a natural product known and used by humans since antiquity

(Nikhat and Fazil, 2022). The Italian legislation, transposing Directive 2001/110/EC, defines honey as "the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature". Honey characteristics, such as flavour and physico-chemical properties, can vary substantially depending on botanical and geographical origin (Zhou *et al*., 2002; Warui *et al.*, 2019). The Italian legislation (D. lgs. 21/05/2004, n. 179) has established thresholds and values for physico-chemical criteria, including moisture, electrical conductivity, hydroxymethylfurfural (HMF), sugar, and others, to evaluate the marketability and quality of honey, which were added to the aromatic profile of honey. Nonetheless, there are roughly 320 distinct types of honey available on the market, which can be grouped into monofloral and multifloral varieties (Vîjan *et al.*, 2023). In Italy, there is a rich assortment of honeys, and this diversity is the result of the unique combination of regional production, climate conditions, and a multitude of floral sources (Castiglioni *et al.*, 2017). Monofloral honey is obtained from bees that have mainly visited a unique botanical species, these honeys are particularly valuable on the market (Schuhfried *et al.*, 2016). As reported by ISMEA, the cost of multifloral honey differs from the cost of monofloral honey (ISMEA, 2023). However, European legislation does not specify the properties of monofloral honey, so countries like Italy imposed a national regulation with a minimum percentage of pollen required for the identification as monofloral, which varied from floral origin depending on the pollen production, position and flower structure of each botanical species (Tedesco *et al.*, 2022). On the other hand, multifloral honey is produced from several types of flowers, and its characteristics and properties can differ greatly depending on the visited flowers and the geographical origin. Melissopalynological analysis is the official method for identifying the botanical and geographical origin of honey (Aronne and De Micco, 2010). However, this analysis is time-consuming and cannot be applied to filtered honey. Moreover, the execution requires palynological competence, which is a limiting factor (Mureșan *et al.*, 2022). In previous studies, PTR-ToF-MS has been used for the categorization of honey types based on their aromatic profiles, such as the monofloral classification (Kuś and van Ruth, 2015; Schuhfried *et al*., 2016) and for the discrimination of their botanical origin (Ballabio *et al.*, 2018).

Thus, the primary objective of this study was to comprehensively characterize Italian honeys by employing a combination of volatile compound analysis, alongside conventional physico-chemical analyses. By integrating volatile profiling using PRT TOF-MS with established analytical techniques, the aim was to determine whether this analysis could serve as an additional, complementary or substitute method for discriminating different botanical origins of Italian honey.

2. Materials and Methods

Sample collection

Honey samples were gathered in 2022 from May to August directly from beekeepers from different natural geographical macro-areas (districts) of Italy to have a variety of sources that include region, province, altitude, and botanical origins. A total of 84 samples of honey were collected, 78 of these were obtained in 2022, and 6 were collected between 2020 and 2021 (aged samples). Each sample was stored in the dark in a cool and dry place. The collection focused mostly on Italian artisan-produced honey as reported in Table 1. In addition, to achieve even more powerful results, we collected 12 Italian commercial samples. The types of honey were 49 multifloral (of which 6 commercial), 16 acacia (of which 2 commercial), 11 chestnut, five citrus (of which 4 commercial) and three lindens.

Physico-chemical analysis

All the physico-chemical analysis were performed according to the guidelines of the Italian regulation DM 25/07/2003 GU number 185 (Gazzetta Ufficiale, 2003).

Determination of pH. To assess the pH, 10 g of sample was thoroughly mixed in 40 ml ultrapure distilled water (dilution 1:5) from a Millipore Milli-Q lab water system. The resulting solution was measured using a PHM 210 Standard pH Meter (MeterLab, Radiometer Copenhagen), which was previously calibrated with standard pH 4 and pH 7 solutions.

Electrical conductivity. The EC of honey was obtained from the same diluted solution used to assess the pH. The measurement was done using a conductometer (Conductimeter GLP 31 CRISON) cali-

Table 1 - Description of the traits of the samples analyzed, considering the different botanical origin, geographical area, and year of production

Botanical Origin	Region	Province	Production source	Harvest year	No. of samples
Acacia	Tuscany	Firenze	Beekeeper	2022	5
Acacia	Tuscany	Livorno	Beekeeper	2022	1
Acacia	Tuscany	Arezzo	Beekeeper	2022	1
Acacia	Abruzzo	Pescara	Beekeeper	2022	1
Acacia	Tuscany	-	Commercial	2022	1
Acacia	Italy	-	Commercial	2022	1
Acacia	Lombardy	Cremona	Beekeeper	2022	1
Acacia	Abruzzo	Teramo	Beekeeper	2022	1
Acacia	Tuscany	Prato	Beekeeper	2022	1
Acacia	Piedmont	Torino	Beekeeper	2022	1
Acacia	Tuscany	Pisa	Beekeeper	2022	1
Acacia	Emilia-Romagna	Forlì	Beekeeper	2022	1
Chestnut	Tuscany	Firenze	Beekeeper	2022	4
Chestnut	Tuscany	Livorno	Beekeeper	2022	1
Chestnut	Tuscany	Arezzo	Beekeeper	2022	1
Chestnut	Lombardy	Cremona	Beekeeper	2022	1
Chestnut	Piedmont	Torino	Beekeeper	2022	1
Chestnut	Tuscany	Pisa	Beekeeper	2022	1
Chestnut	Emilia-	Forlì	Beekeeper	2022	1
Chestnut	Campania	Salerno	Beekeeper	2022	1
Citrus	Calabria	-	Commercial	2021	1
Citrus	Italy	-	Commercial	2021	1
Citrus	Italy	-	Commercial	2022	2
Citrus	Sicily	Ragusa	Beekeeper	2022	1
Linden	Tuscany	Firenze	Beekeeper	2022	1
Linden	Lombardy	Cremona	Beekeeper	2022	1
Linden	Emilia-	Forlì	Beekeeper	2022	1
Multifloral	Tuscany	Firenze	Beekeeper	2020	1
Multifloral	Tuscany	Firenze	Beekeeper	2021	1
Multifloral	Tuscany	Firenze	Beekeeper	2022	21
Multifloral	Tuscany	Prato	Beekeeper	2021	1
Multifloral	Tuscany	Livorno	Beekeeper	2022	1
Multifloral	Tuscany	Arezzo	Beekeeper	2022	3
Multifloral	Abruzzo	Pescara	Beekeeper	2022	1
Multifloral	Italy	-	Commercial	2021	1
Multifloral	Italy	-	Commercial	2022	5
Multifloral	Lombardy	Cremona	Beekeeper	2022	4
Multifloral	Abruzzo	Teramo	Beekeeper	2022	1
Multifloral	Sicily	Ragusa	Beekeeper	2022	3
Multifloral	Piedmont	Torino	Beekeeper	2022	1
Multifloral	Umbria	Todi	Beekeeper	2022	1
Multifloral	Tuscany	Pisa	Beekeeper	2022	1
Multifloral	Lazio	Roma	Beekeeper	2022	2
Multifloral	Campania	Salerno	Beekeeper	2022	1
Total					84

brated with appropriate standard solutions. The results were expressed in mS/cm. The maximum EC for honey is 0.8 mS/cm according to Italian law (Directive 2001/110/EC), while for the honeydew, multifloral/mixed, and chestnut honey the EC values must be greater than 0.8 mS/cm.

Moisture content. The water content of the honey samples was determined with a handheld refractometer (HHTEC) with automatic temperature compensation. The samples were measured as-is, and the results are expressed as moisture content percent. The legal threshold for selling honey is 20%, but in competitions for premium/quality honeys, the limit is usually lowered to 18%.

Hydroxymethylfurfural (HMF) and furfural (F) quantification by HPLC. The HMF and F were quantified following the HPLC method, which had been previously described in other studies with a few adjustments in accordance with Italian legislation guidelines (Fallico *et al.*, 2004; Truzzi *et al.*, 2012). Briefly, 5 g of honey was diluted with ultrapure distilled water (1:5) and mixed. Then, within 12h, samples have been filtered on a 0.45 µm syringe filter and 20 µl were injected into the HPLC system (Azura, Knauer, Berlin, Germany) coupled to a UV detector (Analytical UV Flow Cell detector UVD 2.1S, Knauer). The chromatographic column was Eurospher II 100-5 C18 150 x 4 mm, and the analysis conditions were: isocratic mobile phase, water-methanol 90:10 v/v; flow rate 0.6 mL/min; column temperature 30°C. The detector wavelength was fixed at 285nm, the identification of HMF and F was done by comparing the retention time of standard solution, and the quantification was done using a calibration curve specific for each molecule (Fig. 1 A). The calibration curve for HMF was made with five solutions at different concentrations (0.0005, 0.005, 0.01, 0.05, 0.1 mg/ml), while the F calibration curve was 0.0006, 0.001, 0.002, 0.006, 0.01 mg/ml. According to the law, the results were expressed in mg/kg, and the legal limit for HMF in commercial honey is 40 mg/kg.

Sugars determination

Brix determination. Brix degrees of the honey samples was measured with the same refractometer of moisture content measurement. Brix degrees represent the percentage of sugar content in honey by weight, with 1 Brix degree equivalent to 1 g of sucrose in 100 g of solution (Geană *et al.*, 2020).

Sugar quantification by HPLC. HPLC coupled to a refractive index detector was used for the qualitative

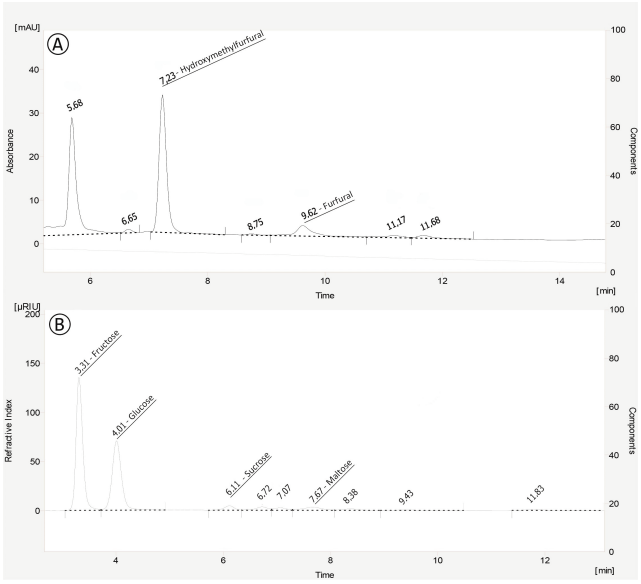

Fig. 1 - Chromatographic profiles of analyzed compounds (A) Chromatogram representing the analysis of hydroxymethylfurfural (HMF) and furfural (F) content in honey samples. The retention times and identified compound names are indicated. (B) Chromatogram illustrating the sugar analysis in honey samples. Retention times and identified sugar names are provided.

and quantitative analysis of sugars (AZURA RID 2.1L, Knauer, Berlin, Germany). The chromatographic column was Eurospher II 100-3 NH2 150 x 4 mm, employing a mobile phase 80:20 of acetonitrile-water and an isocratic flow rate of 1.5 ml/min at 35°C. Honey samples were prepared by placing 0.5 g in 10 mL of H_2O (1:20), mixing for 12-24h, then filtering and diluting 1:1 using the same solution as the mobile phase. Calibration curves were prepared using fructose, glucose, sucrose, and maltose standards for quantification, and retention times were used for identification (Fig. 1 B). Six distinct solutions, each with a different concentration, were employed to construct the calibration curve. The concentrations used were 0, 2.5, 3.75, 5, 7.5, 10, and 15 mg/ml.

Defect identification by sensory analysis

Before to test, each samples were homogenized by mixing with a glass rod, filtered and left until completely clear, after which they were subjected to organoleptic analysis (consistency, color, smell and taste) according to the national standard SR 784-3:2009 (Council European Union, 2001). Particular attention was directed towards identifying any potential defects in the honey samples, with a specific focus on the detection of fermentation. To confirm the conformance of honeys and eventually exclude samples with imperfections, the odour, colour, taste, and texture of honey were assessed. All the samples were found to be conforming and free from defects based on that assessment.

PTR-ToF-MS measurements and data analysis

Using a PTR-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) with $H3O^+$ as the reagent ion and over the mass range of m/z 20-250, volatile fingerprints of 84 samples were acquired. The benefits of the PTR-MS technology are fully and completely described in a previous study (Blake *et al.*, 2009).

Volatile headspace from each sample was analyzed follow the setup previously proposed by Schuhfried *et al*. (2016) with some modification. In short, 5 g of honey (±0.1 g) were placed into a 250 ml glass jar with two Teflon septa on the cap's opposing sides for the VOCs analysis. Then, each jar has been sealed and fluxed with clean air for 60 seconds before the incubation time, in order to remove all the VOCs accumulated during the sample preparation. Subsequently, the samples have been incubated at 37°C for 30 min in order to allow VOCs to fill the head-space. Finally, the volatile compounds were analyzed using the PTR-ToF-MS in its standard configuration. The zero air-generator (Peak Scientific Instruments) supplied clean air at a flow rate of 0.5 lpm (lpm = liter per minute) to the entry of the sampling device during all analyses, and the same flow rate was set for the PTR-MS inlet flow. To prevent the systematic memory effect, clean air was fluxed for five minutes in the tool apparatus between measurements. For each sample run, 120 s worth of mass spectra were captured. The instrument's settings of 2.20 mbar for the drift-tube pressure, 60°C for the drift temperature, and 550 V for the drift voltage produced an electric field strength to number density ratio (E/N) of 120 Td. Every sample was examined twice. Internal calibration of ToF spectra was performed off-line after dead time correction in order to achieve high mass resolution (Cappellin *et al.*, 2011).

The PTR-ToF-MS's better resolution offers a sum formula and a rough identification of each mass peak found. The TofDaq programme (Tofwerk AG, Thun, Switzerland) was used to collect, record, and analyze the data. Data were expressed in ppbv using a process outlined by Lindigner and Jordan (Lindinger and Jordan, 1998). Finally, all the VOC data were filtered using a threshold of 0.50 ppbv and by eliminating any signals that may be attributed to the chem-

istry of the water or to interfering ions, which are thought to be challenging to precisely quantify. Statistics were applied to the filtered data.

Statistical analysis

Multivariate partial least square-discriminant analysis (PLSDA) (supervised method) was applied to the spectra obtained from 84 honey samples produced by different genotypes, comprehensive of 38 protonated masses, for exploring the possibility of correctly classifying the botanical origin of the honeys (acacia, chestnut, citrus fruits, linden, and wildflower, this last coming from a mix of species). As a pre-processing step, data were submitted to logarithmic transformation and auto-scaling. The whole data set was split into training and validation subset, optimally chosen with the Euclidean distances based on the algorithm of Kennard and Stone (1969). The training set consisted of about 85% of the samples, used for selection of the optimal number of latent variables (LVs), model calibration and cross validation (internal validation). The test set, used to predict the class membership (external validation), included 15% of samples removed from the data set. The training set was used to build a model based on venetian blinds cross validation procedures, evaluated by the number of correct predictions and the root-mean-square error of cross-validation (RMSECV), subsequently validated with the removed samples (external validation set). External validation of the model was quantified by the root-mean-square error of prediction (RMSEP). The optimal number of LVs was selected as those associated to the minimum error and misclassification rate of the calibration dataset. Confusion matrices were used to study the reliability of the models. The threshold to assign a sample to a class was chosen minimizing the number of false positives and false negatives (Bayes theorem). Variable Importance in Projection (VIP) scores (p = 0.01) were also calculated.

PLS-DA analysis was performed using PLS-Toolbox v. 8.0.2 (Eigenvector Research Inc., West Eaglerock Drive, Wenatchee, WA) for MATLAB R2015b (Mathworks Inc., Natick, MA, USA).

In addition, to study the relationships between the different samples as a function of different physico-chemical variables, a Factor Analysis (FA) was applied, considering as factors the content of the different analyzed sugars (glucose, fructose, sucrose, maltose), pH, electrical conductivity (mS/cm), and HMF (5-hydroxymethylfurfuraldehyde) level. This last parameter is essential to evaluate the compliance of honey with current legislation. The level of HMF is used as an indicator of the heating or high temperature storage of the honey. In fact, it is generally not present in fresh honey, while its content increases during conditioning and storage (Zappalà *et al.*, 2005). Furthermore, it is inversely proportional to the fructose content and the fructose/glucose ratio (Kesić *et al.*, 2014).

Factor Analysis (FA) allows to visualize variables and samples simultaneously in a two or three-dimensional space and to study the relationships between the observations (honey samples) and the variables (Greenacre, 1984; Escofier and Pagès, 1992). Computations were performed by XLSTAT Version 2014.5.03.

3. Results and Discussion

pH

The pH values of the examined honey samples fell within the acidic range, varying between 3.5 and 6, reported in Table 2 as the mean and the standard deviation (mean ± SD). Acacia honey exhibited the lowest average pH of 3.77 ± 0.13, closely followed by citrus honey with an average pH of 3.90 ± 0.51.

Table 2 - Physico-chemical honey characteristics. The table reports the value of pH, electrical conductivity (EC), moisture content, hydroxymethylfurfural (HMF), and furfural (F)

	pH	EC (mS/cm)	Moisture content (%)	HMF (mg/kg)	F (mg/kg)
Acacia	3.77 ± 0.13	0.27 ± 0.10	16.44 ± 0.74	0.95 ± 0.45	0.95 ± 0.54
Chestnut	5.19 ± 0.49	1.58 ± 0.33	16.60 ± 0.73	1.78 ± 0.48	2.21 ± 1.15
Citrus	3.90 ± 0.51	0.57 ± 0.54	17.20 ± 0.86	3.50 ± 1.64	2.74 ± 1.38
Linden	4.16 ± 0.06	0.92 ± 0.12	15.33 ± 1.15	2.40 ± 0.20	3.70 ± 1.53
Multifloral	4.12 ± 0.36	0.80 ± 0.42	16.16 ± 1.12	1.67 ± 0.56	1.53 ± 1.30
Aged	4.00 ± 0.19	0.70 ± 0.51	16.48 ± 1.85	6.82 ± 3.04	2.96 ± 1.17

Data are reported as the mean ± standard deviation.

Linden and multifloral honeys demonstrated slightly higher average pH values of 4.16 ± 0.06 and 4.12 ± 0.36, respectively. As expected, multifloral honeys displayed a considerable range of pH values (3.44 to 5.05), reflective of their inherent compositional diversity. In contrast, chestnut honey exhibited the highest pH value of 5.19 ± 0.49 among the tested varieties. Notably, the pH of aged honey, at 4.00 ± 0.19, aligned closely with the pH values of other honeys of corresponding botanical origins, such as citrus and multifloral. The acidic nature of the honey samples has implications for their antimicrobial activity (Acquarone *et al.,* 2007). The observed pH variations align with previous findings, with acacia and citrus honeys consistently displaying lower pH levels while chestnut the highest (Bertoncelj *et al.*, 2011; Živkov-Baloš *et al.*, 2018). The near 4 pH level observed in other honey types is consistent with results reported in earlier studies (Truzzi *et al.*, 2014).

Electrical conductivity (EC)

The EC of honey samples ranged between 0.1 and 2 mS/cm, depending by the botanical origin (Table 2). Acacia honeys were characterized by a considerably low EC (with an average value of 0.27 ± 0.10 mS/cm); in contrast, chestnut honeys had high EC values, with an average of 1.58 ± 0.33 mS/cm and a maximum value of 1.96 mS/cm. Citrus showed a mean of 0.57 ± 0.54 mS/cm, and linden of 0.92 ± 0.12 mS/cm. On the other hand, multifloral honeys exhibited a wide range of values, from 0.21 mS/cm to 1.86 mS/cm, the overall mean was 0.80 ± 0.42 mS/cm. Additionally, aged honey samples did not show significant differences when compared to honey of the same botanical origin (0.69 ± 0.51 mS/cm). The EC values of most honey samples fell within the standard limit (Table 2), with the exception of two honeys. Notably, linden honey exhibited an average electrical conductivity (EC) that exceeded the established threshold. However, when considering a limit of 0.8 mS/cm, the EC values for linden honey (0.92 mS/cm) remained acceptable, thanks to an exemption stated in D.lgs. 179/04, which allows EC levels above 0.8 mS/cm. Nevertheless, EC is considered a reliable indicator of the botanical origin of honey. Chestnut honey is characterised by a high EC value, followed by linden honey, which also displayed a relatively high value, as reported in other studies (Truzzi *et al.*, 2014; Živkov-Baloš *et al.*, 2018). Excluding the outlier value of citrus honey, the average EC of the samples was similar to findings in other studies (0.24 mS/cm) (Di Marco *et al.*, 2017; Di Rosa *et al.*, 2019). Conversely, multifloral honeys showed a wide range of EC values, ranging from 0.21 mS/cm to 1.86 mS/cm, reflecting the variation in floral sources visited by the bees.

Moisture content

The moisture content of all the honey types ranged from 13.4% to 19.6%, and all the samples were under the maximum limit of 20% (Table 2). Moreover, 94% of the samples meet the criteria for quality competitions (value ≤ 18%). The highest average value was 17.20 ± 0.86% of citrus honey, while the lower was 15.33 ± 1.15% of linden honey. Acacia, chestnut, multifloral, and aged honeys had similar values of 16.44 ± 0.74%, 16.60 ± 0.73%, 16.16 ± 1.12%, and 16.48 ± 1.85%, respectively. Honey moisture content is an important factor and a parameter used to evaluate the product's quality. Values that are too low can cause processing problems, while values that are too high could lead to the onset of fermentation processes, altering its quality, shelf life, taste, and composition (El Sohaimy *et al.,* 2015). There was no discernible difference between the water content of various varieties of honey when the samples were compared, despite the significant variety and botanical origin of the samples. Indeed, there is a relationship between moisture content and honey maturation, production season, ventilation of the beehive, meteorological conditions and work processes (Kirs *et al.*, 2011; Escuredo *et al.*, 2014; De Sousa *et al.*, 2016; Lazarević *et al.*, 2017).

Hydroxymethylfurfural (HMF) and furfural (F)

The HPLC quantification of HMF (hydroxymethylfurfural) showed that all samples had HMF content within the standard thresholds. Acacia honey exhibited an average HMF content of 0.95±0.45 mg/kg, while chestnut honey showed a higher value of 1.78 ±0.48 mg/kg of HMF. Citrus honey recorded an even higher content, with 3.50±1.68 mg/kg of HMF with the higher value represented from the aged commercial sample. Samples of linden honey displayed an average HMF content of 2.40±0.20 mg/kg, while multifloral honey showed a mean value of 1.67±0.56 mg/kg of HMF. However, the main difference was highlighted between fresh and aged honey. Indeed, samples of aged honey showed a considerable rise in HMF content, with an average of 6.82 ± 3.04 mg/kg. All honey harvested in 2022 had values from 0.5 to 3 mg/kg, while aged honey had significantly higher values from 4.6 to 12.12 mg/kg (Table 2).

These results clearly indicate that ageing process can significantly influence HMF levels, which serve as

an important indicator of honey quality and freshness. Indeed, as reported in previous studies, these compounds are related to the heating practices and preservation conditions of honey and derive from the degradation of fructose (Aronne and De micco, 2010; Tedesco *et al.*, 2022).

In the same chromatographic run of HMF, furfural (F) data was also obtained. Acacia honey showed a mean of 0.95 ± 0.54 mg/kg, representing the honey with the lowest average F content, ranging from 0 to 1.89 mg/kg. Multifloral honey followed with a content of 1.53-1.30 mg/kg. Chestnut honey exhibited an average F content of 2.21 ± 1.15 mg/kg. Both citrus and aged honey displayed similar values of F: 2.74 ± 1.38 mg/kg and 2.96 ± 1.17 mg/kg, respectively. The highest mean value was found in linden honey (3.70 ± 1.53 mg/kg); however, the sample with the highest F content was multifloral honey with 6.74 mg/kg. Also, if neither restrictions nor indications are reported for furfural in the legislation, it is related to storage and of honey, since both F and HMF are usually produced by the Maillard reaction (Zhang *et al.*, 2009). However, the average furfural content in the different honey types was in line with other studies (Gaspar and Lopes, 2009; Apriceno *et al.*, 2018; Tedesco *et al.*, 2022).

Brix

The degree Brix analysis, representing the total sugar content in honey, revealed that all honey samples exhibited values ranging from 78.8% to 85.5%. Among the varieties, citrus honey displayed the lowest mean value (81.10±0.84%), while linden honey showcased the highest (82.93 ± 1.10%) (Table 3). Similarly, in line with previous studies, our Brix values were found to be comparable to those reported, reaffirming the absence of significant distinctions in sugar content among different honey botanical origins.

However, similar to what was reported in earlier studies that also found similar brix values, the study of sugars using the refractometer did not reveal any appreciable differences between different types of honey (Oroian and Ropciuc, 2017; Geană *et al.*, 2020).

Sugar quantification

Quantitative analysis of fructose, glucose, sucrose, and maltose was conducted using HPLC, with results expressed as percentages (g/g). Comprehensive data, including the sum of fructose and glucose, as well as individual sugar levels, are presented in Table 3. Acacia honey exhibited the highest fructose content at 49.08 ± 1.71%, while aged honey displayed the lowest fructose content (39.18 ± 3.50%). The fructose content across all samples ranged from 33.1% to 52.8%. Glucose content ranged from 22% to 40.2%, with chestnut honey demonstrating the lowest average value (27.24 ± 2.93%) and aged honey the highest (33.71 ± 4.76%). The range of maltose concentration was 0.9% to 4.8%, with chestnut honey having the highest level and linden honey the lowest (Table 3). Additionally, according to Council Directive 2001/110/CE of December 20, 2001, in unadulterated honeys, the sum of glucose and fructose should not fall below 60 g/100g for nectar honey, while sucrose must not exceed 5 g/100g. More specifically, 5g/100g for Acacia (*Robinia pseudoacacia*), Lucerne (*Medicago sativa*), Banksia (*Banksia menziesii*), Sulla (*Hedysarum coronarium*), Eucalyptus (*Eucalyptus camaldulensis*), not more than 10 g/100 g for Citrus (*Citrus* spp.) honey, and no more than 15 g/100 g for Lavender (*Lavandula* spp.) and Borage (*Borago officinalis*). In reference to these criteria, all 84 samples were unadulterated, in accordance with the legislation. The sum of glucose and fructose ranged from 63.80 to 84.90%, affirming the high quality of the honey samples, while sucrose was always lower than

Table 3 - The table reports the value (average and standard deviation) of degree Brix of honey (%) that represents the total sugar content and Fructose, Glucose, and Maltose expressed as percentages (g/g). In the table it is also reported the sum of Glucose and Fructose (G+F) and the Fructose Glucose ratio (F/G)

	Brix	Fructose	Glucose	Maltose	G + F	F/G
Acacia	81.90 ± 0.72	49.08 ± 1.71	30.05 ± 2.08	3.12 ± 0.45	79.12 ± 2.68	1.64 ± 0.13
Chestnut	81.71 ± 0.77	45.34 ± 2.43	27.24 ± 2.93	3.36 ± 0.82	72.58 ± 4.76	1.68 ± 0.15
Citrus	81.10 ± 0.84	42.65 ± 2.57	32.79 ± 5.24	2.90 ± 1.02	75.44 ± 6.74	1.32 ± 0.17
Linden	82.93 ± 1.10	43.28 ± 2.02	28.39 ± 8.25	2.48 ± 0.80	71.66 ± 8.22	1.61 ± 0.42
Multifloral	82.19 ± 1.16	43.85 ± 3.71	30.26 ± 3.79	2.98 ± 0.87	74.11 ± 5.25	1.47 ± 0.23
Aged	82.24 ± 1.89	39.18 ± 3.50	33.71 ± 4.76	2.74 ± 0.62	72.89 ± 7.81	1.17 ± 0.10

1.8%, detected in only 15 samples of the total, with a maximum value of 1.8% (7 acacia, 1 citrus and 7 multifloral). Moreover, the Fructose/Glucose (F/G) ratio was calculated. The F/G ratio determines whether honey may crystallise; therefore, a ratio higher than 1 suggests a fluid honey, whereas a ratio lower than 1 indicates honey crystallizing more quickly (Geană *et al.*, 2020). The highest values were found in acacia, chestnut, and linden honeys (1.64, 1.67, and 1.60, respectively), indicating honey's ability to remain liquid for a longer amount of time. Citrus, multifloral flowers, and aged honeys, on the other hand, showed lower ratios (1.32, 1.47, and 1.17, respectively). Additionally, no samples had a value lower than 1, however aged honey with an F/G ratio of 1.17 is most likely to have crystallized. In the current study, the fructose and glucose values found across different honey samples are, on average, higher compared to those reported in other studies. However, the F/G ratio for acacia, citrus, and multifloral honeys remains consistent with literature values (Oddo and Piro, 2004; Geană *et al.*, 2020).

Factor analysis (FA)

In figure 2, the FA biplot simultaneously represents the relationship between the different sugars analyzed (glucose, fructose, sucrose, maltose), the level of HMF, the pH and the electrical conductivity, highlighting the relative distances among the 84 honey samples. The first axis explains 37.18% of the total variability in the spectral data, the second axis 18.01%. From the FA graph, some groups of samples emerge which seem to be related to a compound or

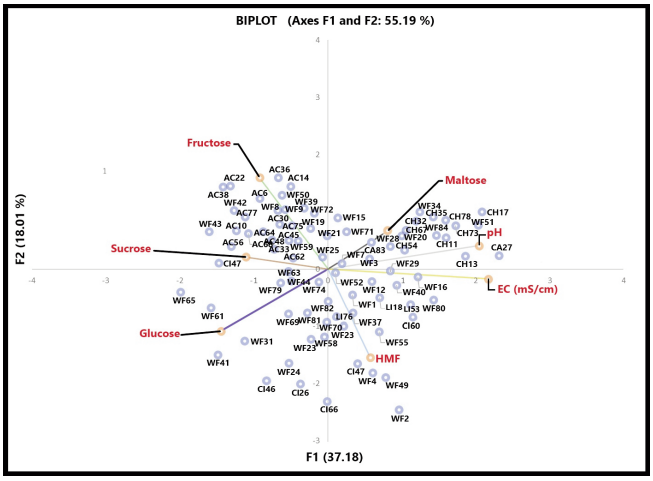

Fig. 2 - Biplot from Factor analysis. Relationships between 84 honey samples and different physicochemical parameters. AC = acacia, CI = citrus fruits, CH = chestnut, WF = wildflower, LI = linden.

to a specific physico-chemical characteristic of the honey. In particular, the wildflowers are concentrated on the HMF vector, and, in a diametrically opposite position, the samples of acacia honey are grouped very close along the fructose axis. This is in accordance with the fact that HMF is formed through the degradation of fructose, thus establishing a negative correlation between these two parameters. Citrus and Linden honeys are situated in the lower region of the graph, indicating slightly higher glucose values for citrus honey and higher HMF values for both honeys. The pH values are also significantly higher in chestnut samples, although maltose seems to somehow influence its distribution.

PTR-ToF-MS results

Data on the emissions of volatile organic compounds (VOCs) from the five honey groups - Multifloral, Acacia, Chestnut, Citrus, and Linden- are presented in Table 4. All signals have been separated according to their respective molecular weights and are expressed as the mean concentration in parts per billion by volume (ppbV). The Table shown a subset of 33 compounds obtained upon filtering the data (were eliminated all signals with an average concentration below to 1 ppbV). From our study on 84 different honey samples a total of 37 different compounds with an average value higher than 1ppbv were found. Among these, the peaks with the higher emission were detected at 33.034, 45.033, 47.010, 59.049, which corresponding to the following compounds methanol, acetaldehyde, formic acid and acetone. Similar results were obtained from other studies on honey samples from different botanical origins (Kuś and van Ruth, 2015; Schuhfried *et al.*, 2016). The average of total emission recorded for each honey botanical origin varies from a minimum value of 265.2 ppbv for Acacia and 336.1 for Linden honey to a maximum value of 1971.8 for citrus honey (Table 4). Acacia and Linden honey showed a rather similar volatile profile characterized by both a lower level of emission and a lower number of signals (30 and 28 respectively) compared to the other botanical origins. Citrus honey emerged both for a higher emission of methanol, acetaldehyde and acetone compounds as well for a higher emission of terpene compounds (mz 111.101, 121.101, 135.116, 137.132) compared to the other botanical origins. Chestnut samples are characterized by large signals of compounds detected at m/z: 69.033 ($C_4H_5O^+$, Tentatively Identified as Furan), 83.086 ($C_6H_{11}^+$, TI as C6 compounds) and 105.069 ($C_5H_{13}S^+$, TI pen-

Table 4 - Number of signals detected, chemical formula, tentative identification, VIP score and average amount (ppbV) of each compound detected from different honey samples

N° of compounds	mz	Chemical Compound	Tentative Identification*	Multifloral (n=49)	Acacia (n=16)	Chestnut (n=11)	Citrus (n=5)	Linden (n=3)
1	27022	$C2H3^+$	Acetylene	33.49	10.80	30.91	99.50	11.85
2	33033	$CH5O^+$	Methanol	189.78	61.30	61.15	243.54	49.04
3	41038	$C3H5^+$	Alkyl fragment	22.58	5.39	11.16	50.39	19.03
4	43018	$C2H3O^+$	aceton or acetate fragments	44.33	14.86	47.49	87.52	23.39
5	45033	$C2H5O^+$	Acetaldehyde**	369.53	88.24	506.01	884.92	106.64
6	47010	$CH3O2^+$	Formic acid/formats**	25.21	7.05	41.97	180.30	7.90
7	49011	$CH5S^+$	S compound (Methanethiol)	3.23	2.10	2.93	8.96	2.00
8	53038	$C4H5^+$	Cyclobutadiene	2.48	2.03	2.21	2.79	2.00
9	55054	$C4H7^+$	Alkyl fragment	6.43	4.09	5.93	18.59	3.68
10	57069	$C4H9^+$	Alcohol fragments	13.25	5.02	3.02	6.33	2.59
11	59049	$C3H7O^+$	Acetone**	119.70	42.56	210.57	284.23	100.21
12	61028	$C2H5O2^+$	Acetic acid	12.25	7.25	11.14	15.98	5.94
13	63033	$C2H7S^+$	S compound (Dimethyl sulfide)	13.07	4.07	17.29	25.27	2.15
14	65.00	$C5H5^+$	S compound	2.59	Tr	2.35	3.63	Tr
15	67050	$C5H7^+$	3-Penten-1-yne/Terpene fragment	Tr	Tr	Tr	2.65	Tr
16	69033	$C4H5O^+$	Furan	5.33	2.93	16.02	5.84	Tr
17	71086	$C5H11^+$	Alcohol compounds	2.24	2.77	2.24	1.10	1.10
18	73054	$C4H9O^+$	Butan-2-one	13.60	5.54	10.70	20.21	3.48
19	75044	$C3H7O2^+$	Isobutanol	2.67	2.71	2.58	2.64	2.33
20	77038	$C6H5^+$	Alkyl fragment	2.11	2.03	2.06	2.14	1.01
21	79049	$C6H7^+$	Benzene/terpene fragment	2.88	2.72	2.52	3.34	0.00
22	83086	$C6H11^+$	C6 compounds	2.35	2.02	6.92	2.87	1.10
23	85059	$C5H9O^+$	(E)-2-Pentenal	3.14	2.17	2.14	3.21	0.00
24	87044	$C4H7O2^+$	2,3-Butanedione	7.59	3.65	10.10	15.91	3.81
25	93069	$C7H9^+$	Terpene fragments	Tr	Tr	Tr	3.23	Tr
26	95011	$C2H7O2S^+$	dimethyl sulfone	7.34	4.53	3.52	6.44	3.82
27	97033	$C5H5O2^+$	Furfural	6.09	3.26	4.30	8.49	2.63
28	99044	$C5H7O2^+$	Furfuryl alcohol	Tr	Tr	Tr	Tr	Tr
29	101060	$C5H9O2^+$	Dihydro-methyl-furanone	Tr	Tr	0.00	0.00	0.00
30	103080	$C5H11O2^+$	2-/3-methylbutyric acid	Tr	0.00	0.00	2.71	0.00
31	105069	$C5H13S^+$	Pentanethiol**	Tr	0.00	4.26	1.89	Tr
32	107086	$C8H1\ 1^+$	1,3-Dimethylbenzene/Terpenes fragments	Tr	Tr	1.87	1.58	0.00
33	109070	$C7H9O^+$	Benzyl alcohol	Tr	0.00	Tr	1.12	1.41
34	111101	$C8H15^+$	Terpenes fragments**	Tr	0.00	Tr	1.88	0.00
35	121101	$C9H1\ 3^+$	Terpenes fragments	Tr	0.00	1.12	2.54	0.00
36	135116	$C10H15^+$	Terpenes**	Tr	0.00	Tr	2.10	0.00
37	137132	$C10H17^+$	Terpenes	Tr	0.00	0.00	2.08	0.00
Total emission average				889.30	265.28	996.51	1971.86	332.26
Total signals detected				37	30	34	36	28

* Each value has been tentatively assigned to compounds, based on PTR-honey literature data (Kuś and van Ruth, 2015; Schuhfried *et al.*, 2016; Ballabio *et al*., 2018).

** compounds with the highest VIP score.

Tr means trace and these compounds have been identified in at least 1 sample per group but with an overall average value below 1 ppbV.

tanethiol) in agreement with Ballabbio *et al*. (2018). As can be seen from the data shown in the table 4, the average volatile profile of multifloral honey showed an average emission for many signals and trace compounds in large numbers (identified only in some samples).

No significant differences were observed between the volatile organic compound (VOC) emissions of commercial honey and those produced by beekeepers.

PLS-DA analysis

With the aim to get an overview of the VOC data collected, a PLS-DA analysis was applied on the whole dataset obtained from 37 different VOCs data collected from 84 samples. It emerges that the honey samples distance themselves from each other according to their botanical origin. Multifloral honey seem to show a variable trend probably linked to its botanical origin. To provide a more detailed characterization of the VOCs emitted by different honey samples, VIP scores higher than 1 and their possible identification on the basis of literature data were reported in Table 4 (marked by two asterisks). The volatile compounds with higher VIP value could be good candidates for the honey species identification. In particular, the chemical species with the higher significance were detected at mz 45.033(TI Acetaldehyde), 47.01 (TI Formic acid/formates), 59.049 (TI Acetone), 105.069 (TI Pentanethiol), 111.10 (TI Terpenes fragments), 135.116 (TI Terpenes).

PLS-DA approach was applied to find VOCs able to discriminate among species. By applying the model developed by the PLS-DA on honey samples of different botanical origin, a correct distinction of the taxonomic category of two/five different groups was achieved. Indeed, the multifloral honey could be obtained by honeybees from the nectar of different flowers. Score plot from the PLS-DA model is shown in figure 3. The global quality of the model, evaluated by its performances indicators (Table 5), resulted robust enough to discriminate the botanical origin of the citrus and chestnut samples compared to the others in the calibration/validation data set, and in the independent test set. Indeed, the PLS-DA three-component model successfully classified 100% of

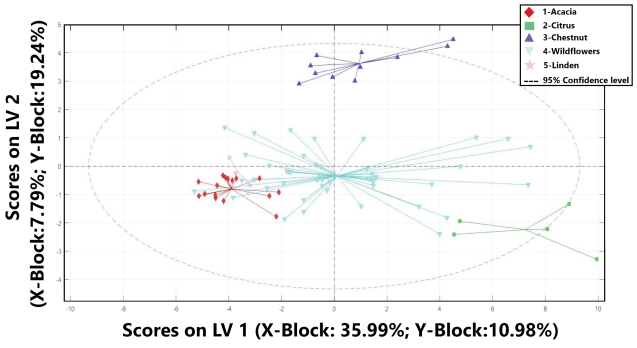

Fig. 3 - Score plot (LV1, LV2) of the PLS-DA model. Samples of different botanical origin are highlighted. Red = acacia; green = citrus fruits; blue = chestnut; light blue = wildflower; lilac = linden.

Table 5 - PLS-DA statistics for the honey samples for the five botanical origins: 1 = acacia, 2 = citrus fruits, 3 = chestnut, 4 = wildflower, 5 = linden. Sensitivity (SE), Specificity (SP), Class. Error, RMSEC, RMSECV, RMSEP, for Calibration (Cal), Cross Validation (CV), and Prediction (Pred), respectively

Statistics	Y-BLOCKS				
	Class 1 - acacia	Class 2 – citrus fruits	Class 3 - chestnut	Class 4 - wildflower	Class 5 - linden
Sensitivity (SE) (Cal)	1.000	1.000	1.000	0.721	1.000
Specificity (SP) (Cal)	0.800	1.000	1.000	0.724	0.643
Sensitivity (SE) (CV)	0.833	1.000	1.000	0.744	1.000
Specificity (SP) (CV)	0.800	0.970	1.000	0.552	0.643
Sensitivity (SE) (P)	1.000	0.000	0.000	0.000	1.000
Specificity (SP) (P)	0.875	1.000	0.833	0.182	0.583
Class. error (Cal)	0.100	0.000	0.000	0.277	0.178
Class. error (CV)	0.183	0.015	0.000	0.352	0.178
Class. error (Pred)	0.062	0.500	0.583	0.909	0.208
RMSEC	0.319	0.104	0.169	0.414	0.161
RMSECV	0.328	0.159	0.199	0.464	0.166
RMSEP	0.387	0.296	0.719	0.674	0.044

honey samples from classes 2-chestnut and 3-citrus into their respective taxonomic categories during fitting, cross-validation (internal validation), and prediction (external validation), while the acacia and linden honey samples are confused with the multifloral samples.

4. Conclusions

In this study, a comprehensive analysis of various physico-chemical properties and volatile organic compounds (VOCs) present in Italian honeys was conducted. It was revealed that the quality of the honey sold is excellent, as legal limits were adhered to for all samples (except for few EC values). The electrical conductivity (EC) values demonstrated significant variability, with chestnut and linden honeys standing out due to their high and relatively high EC values, respectively. The observed pH variations among different honey types were consistent with their botanical origins. Furthermore, parameters such as sucrose content and the fructose-to-glucose ratio, indicative of potential adulterations, remained within legal limits for both commercial and beekeeper honey.

Moreover, within this context, the honey varieties were discerned based on their distinctive characteristics.

Aged honey, as expected, was characterized by a high HMF level; however, it remained within legal thresholds. Acacia honey, characterized by its high fructose content, exhibited low pH and EC values. Interestingly, in VOC analysis, it displayed similarities with linden honey due to their shared low emissions of volatile compounds. Chestnut honey, which had high pH and EC values, was easy to differentiate from other types of honey using both conventional metrics and PTR-ToF-MS-based VOC analyses. Its distinctive profile made it simple to classify. Citrus honey displayed physicochemical characteristics similar to linden and multifloral honeys, but its distinctive VOC emissions allowed for a more accurate identification. Conversely, linden and multifloral honeys shared close resemblances in chemical and physical analyses, and the significant variability in the multifloral variety's composition due to its diverse floral sources hindered differentiation through VOCs.

Factor Analysis provided insights into the relationships between different sugars, HMF, pH, and electrical conductivity, highlighting distinct groupings of honey samples. Finally, VOCs analysis revealed a diverse range of compounds, with noticeable variations attributed to the botanical origin of the honey. Partial Least Squares-Discriminant Analysis (PLS-DA) facilitated discrimination among different honey types based on their VOC profiles highlighting the potential for this method in distinguishing honey types based on VOC profiles. The analysis of VOCs has the advantage of being a faster alternative to pollen analysis, providing an efficient means of differentiating between honey samples with varying botanical sources. For this reason, PTR-ToF-MS-based VOC analysis serves as a valuable tool to complement or even replace melissopalynological analysis, as demonstrated by the effective combination of VOC analysis and PLS-DA for distinguishing honeys of different botanical origins.

In conclusion, the collective application of physico-chemical and VOC analyses yielded a comprehensive means of effectively characterizing honey varieties. This integrated approach underscores the robustness of employing multiple techniques for a thorough understanding of honey attributes.

Acknowledgements

This research is part of the BEEWIN project: BEEkeepers Weather indexed INsurance project funded by MIPAAF (Ministero delle Politiche Agricole Alimentari e Forestali).

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