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CONTENTS

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Effect of harvest maturity stage and ripening remediation agents on the shelf life and biochemical quality attributes of tomato (*Solanum lycopersicum* L.) fruits

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Key words: 1-MCP, $KMnO_4$, postharvest, RRA, Zeolite.

Abstract: Tomato fruit is highly perishable because of the characteristic high rate of ethylene production and respiration during ripening. Delayed ripening could be achieved through the use of ripening remediation agents (RRA) that either absorb or block ethylene binding to the fruit receptor. The effects of ripening remediation agents on shelf life and biochemical quality attributes were evaluated on tomato fruits harvested at three maturity stages (breaker, turning and full-ripe). In 2018 and 2019, harvested fruits were stored under seven ripening remediation treatments: 0.1 µL/L 1-MCP, 0.3 µL/L 1-MCP, 0.5 µL/L 1-MCP, 5% $KMnO_4$, 10% $KMnO_4$, 10 g of Zeolite and 20 g of Zeolite and an open shelf condition as the control. At the end of the storage period, fruits were assessed for shelf life as well as total soluble solids (TSS), titratable acids (TA), ascorbic acid, and lycopene contents. There was significant (p≤0.05/0.01) influence of ripening remediation treatments on fruits for all the measured parameters. Fruits stored with RRAs consistently out-performed those stored in the open shelf. RRAs 0.3 µL/L1-MCP, 0.5 µL/L1-MCP and 5% $KMnO_4$ solution media had longer shelf life and higher values of total soluble solids, titratable acidity, lycopene and ascorbic acid contents. The use of 1-MCP and 5% $KMnO_4$ is recommended as effective scavenger of ethylene for extending the shelf life and maintaining some quality attributes of stored tomato fruits.

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important fruit vegetables crops in the world. It plays an important role in human diet, being mostly used as a vegetable in the preparation of soup, salad, pickles, ketchup, puree, sauces and in many other ways. It is also a rich source of phytochemicals and vitamins that provide protection against chronic diseases, different types of cancers, cardiac vascular diseases and age-related ailments because of its anti-oxidant, anti-carcinogenic and anti-

mutagenic properties (Chaudhary *et al*., 2018).

Regardless of the health and nutritional relevance of tomato, its production and value chain potentials realization is constrained by post-harvest losses of quantity and quality of produce available to consumers. One of the impacts of post-harvest losses results in reduction of food that is accessible for human consumption, which is worsened by increasing demand for food (Kikulwe *et al.,* 2018). The tomato fruit is highly perishable, and a climacteric rise in respiration takes place during ripening, which is considered a turning point in the life of the fruits as regards quality. Being a climacteric fruit, a marked increase of respiration rate and ethylene production during ripening process occurs that reduces the shelf life of the fruit, which may constitute a major challenge in the value chain (Arah *et al.,* 2015). The presence of this gas accelerates fruit ripening and quality deterioration by shortening the shelf life of the fruit. Ripening is a natural phenomenon that involves a series of biochemical changes that are responsible for the textural changes, starch breakdown, change of color, pigment formation, volatile and aroma development and finally abscission of fruits (Maduwanthi and Marapana, 2019). In tomato, ripening involves different dramatic biochemical and physiological changes of the fruit which are characterized by lycopene accumulation, chlorophyll loss, softening, and changes in aroma and other compositional properties. The regulation of these changes, thus, has been a major corcern for research aimed towards improving fruit quality and shelf life (Yasuhiro, 2016).

Increasing the postharvest life of tomato is an important aspect in view of its huge postharvest losses. The onset of ripening in tomato is governed by an increase in ethylene production and it is highly dependent on continuous presence of ethylene and ethylene-mediated actions (Zhao *et al*., 2021), therefore, the need to prevent the build-up of the gas around the produce. This has been found effective in delaying ripening in bananas (Zewter *et al*., 2012) and was achieved through the use of substances that either absorb or block ethylene binding to its receptor and these substances could be termed Ripening Remediation Agents (RRA).

Because of the important role of ethylene and ethylene-mediated actions in the onset and progression of ripening in tomato (Paul *et al*., 2002), preventing the buildup of the gas around the produce had been used to delay ripening in bananas. This has been achieved through the use of Ripening Remediation Agents (RRAs) which either absorb or block ethylene binding to its receptor. Concerning the latter, some RRAs inhibits ethylene's role in ripening by their presence at the ethylene-binding sites so that ethylene would not be able to bind and cause subsequent signal translation and transduction in the ripening process (Zewter *et al*., 2012). Others act by removing unwanted ethylene gas through the oxidation process, converting it to carbon dioxide and water, thereby halting the ripening process and ensuring the quality of freshness of the product in the packaged environment (Sen *et al*., 2012). Also some act as ethylene adsorbers as they have great potential in the agro-industry to remove ethylene due to their cation exchange capacity, high porosity and surface area of uptakes (Yin *et al*., 2020).

Tomato, being a perishable crop due to its high moisture content, has a short shelf life under tropical conditions (Arah *et al*., 2015). This makes it important to develop strategies for the development of handling technologies that reduce or remove the ethylene production of the storage environment, while at the same time sustaining the quality. In view of these, the present study was carried out to compare the effects of different ripening remediation agents on shelf life and some biochemical quality attributes of stored tomato fruits.

2. Materials and Methods

Plant materials

The tomato fruits (var. Beske) used for the experiment were obtained from the experimental field at the Teaching and Research Farm, Directorate of University Farms, Federal University of Agriculture, Abeokuta (FUNAAB), Ogun State, Nigeria in 2018 and 2019 where they were grown under field conditions. Harvesting was done at three harvest maturity indices following the USDA Tomato Colour Chart. Harvested fruits were taken to the Laboratory of the Department of Horticulture and Landscape Management, College of Plant Science and Crop Production, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria for storage. The fruit samples were sorted and graded according to uniformity of size. Thereafter, fruits were carefully visually observed and only those that were wholesome were finally used in the experiment while those with defects or signs of diseases were discarded.

*Treatments a*nd *experimental design*

Tomato fruits were harvested at three maturity stages identified by the USDA tomato colour chart, viz breaker, turning, full ripe (USDA, 2005), and were washed in distilled water to reduce microbial population and remove adhering dirt and dust. Thereafter, 500 g of fruits in the different maturity stages were exposed to seven (7) RRAs and an open shelf (that is, without any RRA, serving as the 'control'). Seven RRAs were employed which were in three categories: ethylene absorber [Potassium permanganate ($KMnO_4$)], ethylene absorber (Zeolites) and ethylene inhibitor [1-Methylcyclopropene (1-MCP)]. The amounts of RRAs used were: 0.1 µl/L, 0.3 µl/L and 0.5 µl/L for 1-MCP; 5% and 10% for $KMnO_4$ solution; 10 g and 20 g of Zeolite. Thus, 24 treatments were composed by combining three harvest maturity indices and and eight exposures (RRAs and control). Gaseous 1-MCP was prepared from SmartFreshTM (AgroFresh Inc.) commercial powder (0.14% of active ingredient). The application was done in air-tight plastic containers (capacity of 1 m^3) applying 0.1, 0.3 and 0.5 µl/L of 1-MCP for 24 h, at a temperature of 25°C and 85-90% RH. Potassium permanganate ($KMnO_4$) solutions of 5% and 10% concentrations were prepared by dissolving 5 g and 10 g $KMnO_4$ powder in 100 ml of distilled water and put into small containers to be placed beside fruit samples. Also, 10 g and 20 g of Zeolite, in granular form, were also put into small containers to be placed beside fruit samples. Fruits with the treatments applied were stored at room temperature. Control treatment fruits were also stored at room temperature, but without any RRA.

Each treatment had 500 g of whole and healthy tomato fruits stored in perforated plastic containers. Untreated fruits (control) were kept in similar containers and placed in an open shelf. The experiment was laid out in Completely Randomized Design with three replications. After the storage, five fruits were sampled randomly per treatment in each replication and were evaluated for shelf life and some quality parameters, viz total soluble solids, ascorbic acid, titratable acidity and lycopene.

Assessment of shelf life and some fruit quality attributes

Tomato fruits were stored at an average temperature between 30-32°C and a relative humidity of 78-80% in both years. The shelf life (days) of the tomato fruits was determined by visually observing the incidence and extent of spoilage with respect to storage days. This was determined from the time they were stored to the time they became unsuitable for consumption. For the analysis of total soluble solids content (TSS) and titratable acidity (TA) of each sample, tissue sap was squeezed out from fresh fruit materials with a press. In this juice, TSS were determined with an Atago Handheld Refractometer in Brixº. Titratable acid (TA) content was determined by titrating method and calculating the result as grams of malic acid per 100 g fresh weight (%). Ascorbic acid content of the samples was determined according to the recommended method of AOAC (2000) using 2, 6-dichlorophenol indophenol and expressed as mg kg^{-1}. Lycopene content was estimated using a Spectrometer by extraction with hexane and absorbance measurement at 503 nm and expressed in mg kg^{-1}. Fruit firmness was determined using handpi model GY-series penetrometer. Fruit firmness in a sample was measured by pushing the central probe against the equatorial plane of the fruit until the central probe flattened. The flattening of the probe caused the needle in the instrument to deflect and the number where the needle stopped was recorded as the value for the fruit firmness (Kitinoja and Hussein, 2005).

Statistical analysis

Data were subjected to 2-way analysis of variance (ANOVA) and significantly different means were separated at 5% probability level. Correlation between all pair-wise traits were estimated for measured traits. All analyses were performed using the Statistical Analysis System, SAS, version 9.3 (SAS Institute, 2012).

3. Results

Harvest maturity index significantly ($p \leq 0.05$) affected the shelf life of stored tomato fruits, and followed the same trend in both years of the study. In 2018 and 2019 respectively, fruits harvested at breaker stage had the longest shelf life of 37 and 40 days, followed by those harvested at turning stage with 36 and 35 days shelf life while fruits harvested at full ripe stage had the shortest shelf life of 35 and 32 days (Table 1).

The shelf life of stored tomato fruits was significantly ($p \leq 0.05$) affected by the Ripening remediation treatments and this has comparable trends in both years of the study (Table 2). In 2018, fruits exposed to 0.3 µL/L had the longest shelf life of 45 days, immediately followed by fruits stored with 0.5

Table 1 - Effect of harvest maturity index on shelf life of tomato fruits in years 2018 and 2019

Harvest index	Shelf life (Days)	
	2018	2019
Breaker	37.99 a	40.70 a
Turning	35.73 b	34.70 b
Full ripe	31.08 c	32.18 c

Means followed by the same letters in the same column are not significantly different at 5% probability level of DMRT.

Table 2 - Effect of ripening remediation agent on shelf life of tomato fruits in years 2018 and 2019

Ripening remediation agent	Shelf lfe (Days)	
	2018	2019
0.1 µL/L 1-MCP	36.63 b	36.75 ab
0.3 µL/L 1-MCP	43.25 a	42.75 ab
0.5 µL/L 1-MCP	41.88 a	37.88 ab
5% KMnO4	44.63 a	45.74 a
10% KMnO4	36.38 b	32.10 b
10 g of Zeolite	27.75 c	30.25 cb
20 g ofZeolite	35.50 b	32.40 b
Open shelf (control)	18.25 d	17.88 c

Means followed by the same letters in the same column are not significantly different at 5% probability level of DMRT.

µ L/L 1-MCP and 5% $KMnO_4$ solution media with 43 and 42 days shelf life respectively. Furthermore, fruits stored with 0.1 µL/L 1-MCP had a shelf life of 37 days while those stored in 10% $KMnO_4$ solution and 20 g of Zeolite media had the same shelf life of 36 days. Untreated fruits however, had the shortest shelf life of 18 days.

In the second year of the experiment, a similar trend was observed in the effect of RRAs on the shelf life of the stored fruits. Fruits exposed to 0.3 µL/L and 0.5 µL/L 1-MCP and those stored in 5% $KMnO_4$ solution medium had shelf life of 46, 43 and 39 days respectively, followed by fruits under 0.1 µL/L 1-MCP with a shelf life of 37 days. Fruits stored in 10% $KMnO_4$ solution and 20 g of Zeolite medium had comparable shelf life of 32 days while fruits left in the open shelf had the shortest shelf life of 18 days. It was noted that 0.3 µL/L1-MCP, 0.5µL/L1-MCP and 5% $KMnO_4$ solution were the most effective in extending the shelf life of tomato.

As displayed in Table 3, total titratable acidity (TTA) was significantly ($p \leq 0.05$) affected by the ripening remediation treatments in both 2018 and 2019. In years 2018 and 2019, the highest TTA of 0.43 and 0.42 g/l respectively were recorded for fruits treated with 0.3 µl/L and 0.5 µl/L 1- MCP and those in 5% $KMnO_4$ solution medium while fruits left on the open shelf had the lowest average TTA concentration of 0.37 g/l.

The lycopene content of the fruits was significantly affected by the ripening remediation treatments (Table 3). In both years of the experiment, fruits kept in open shelf condition recorded the highest lycopene contents of 401.40 µg/100 g (in 2018) and 392.53 µg/100 g (in 2019) which were comparable with the values obtained for fruits treated with 10% $KMnO_4$ solution (405.55 µg/100 g in 2018), 10 g Zeolite medium having lycopene content of 403.20 and 392.45 µg/100 g in 2018 and 2019 respectively. The lowest lycopene contents of 389 µg/100 g in 2018 and 365.19 µg/100 g in 2019 were observed for 5% $KMnO_4$ and 0.5 µl/L 1-MCP respectively. Further-more, ascorbic acid content of the fruits was significantly ($p \leq 0.05$) influenced by the ripening

Table 3 - Effect of ripening remediation substances on some nutritive traits of tomato fruits in 2018 and 2019

Ethylene remediation treatments	TTA (g/l)		Lycopene (µg/100 g)		Ascorbic acid (mg/100 g)		TSS (%)	
	2018	2019	2018	2019	2018	2019	2018	2019
0.1 µL/L 1-MCP	0.42 a	0.40 ab	390.79 ab	381.41 ab	18.26 a	18.55 a	5.73 ab	5.78 ab
0.3 µL/L 1-MCP	0.43 a	0.42 a	392.21 ab	372.06 b	17.71 ab	19.15 a	5.89 a	5.85 a
0.5 µL/L 1-MCP	0.43 a	0.42 a	392.99 bc	365.19 b	17.79 ab	18.80 a	5.80 a	5.89 a
5% KMnO4	0.43 a	0.42 a	389.00 b	375.36 b	17.78 ab	18.07 a	5.80 a	5.85 a
10% KMnO4	0.39 b	0.40 ab	405.55 a	384.29 ab	17.34 ab	18.09 a	5.50 b	5.61 b
10 g Zeolite	0.38 b	0.40 ab	403.20 a	392.45 a	16.69 b	16.74 b	5.50 b	5.49 b
20 g Zeolite	0.41 ab	0.38 b	398.29 ab	387.28 ab	17.01 ab	17.29 ab	5.50 b	5.52 b
Open shelf (control)	0.37 b	0.37 b	407.40 a	392.53 a	16.31 b	17.51 ab	5.25 bc	5.22 bc

Means followed by the same letters in the same column are not significantly different at 5% probability level of DMRT.

remediation treatments in both years (Table 3). In 2018, the ascorbic acid content ranged from 16.31 mg/100 g for fruits stored in the open shelf to 18.26 mg/100 g for fruits stored with 0.1 µl/L 1-MCP. Substantial amounts of ascorbic acid were also observed in fruits with 0.5 µl/L 1-MCP, 5% $KMnO_4$, 0.3 µl/L 1-MCP, 10% $KMnO_4$, and 20 g Zeolite in decreasing order. In 2019, ascorbic acid content of stored fruits ranged from 16.74 mg/100 g for 10 g Zeolite to 19.15 mg/100 g for 0.3 µl/L 1-MCP. Substantial Ascorbic acid contents were also observed for 0.1 µl/L 1-MCP, 0.5 µl/L 1-MCP, 10% $KMnO_4$, 5% $KMnO_4$, open shelf and 20 g Zeolite, in decreasing order. The total soluble solids (TSS) of stored fruits was also significantly ($p \leq 0.05$) affected by the ripening remediation treatments (Table 3). Fruits kept in the open shelf condition, however, recorded the highest TSS in both years of the study. In 2018, the TSS content recorded in fruits exposed to 0.1 µl/L and 0.5 µl/L 1-MCP and 5% $KMnO_4$ solution medium was lower compared to those stored in Zeolite medium and those kept in the open shelf. In 2019, fruits stored in 10 g and 20 g of Zeolite medium had higher TSS compared to those exposed to 1-MCP concentrations and those stored in 5% and 10% $KMnO_4$ solution (Table 3).

As reported before, tomato fruits harvested at the breaker and turning stages recorded significantly longer shelf life than fruits harvested at the full ripe stage except for fruits harvested at the turning stage and stored with Zeolite (Table 1). Comparing fruits from the same stage of maturity in both 2018 and 2019, fruits stored with 0.3 µl/L 1-MCP had the longest shelf life for fruits harvested at the breaker stage, while 5% $KMnO_4$ effected the longest shelf life for fruits picked at the turning stage while full ripe fruits had the longest shelf life when stored with 0.3 µL/L 1-MCP and 5% $KMnO_4$ (Table 4).

There was a general decrease in TTA for all the treatments. However, fruits harvested at breaker and turning stages recorded higher TTA contents with exposure to 0.1 µl/L, 0.3 µl/L and 0.5 µl/L 1- MCP and those stored in 5% $KMnO_4$ solution medium when compared to other treatments (Table 5). Lycopene content was higher for fruits harvested at full ripe kept in the open shelf while there was low lycopene content for those harvested at breaker stage with stored with 1-MCP and 5% $KMnO_4$ solution medium.

In the same vein, fruits harvested at breaker and turning stages had higher ascorbic acid contents when exposed to 1-MCP and 5% $KMnO_4$ solution medium while those kept in the open shelf had lower ascorbic acid content comparable with those stored with 10% $KMnO_4$ solution and Zeolite. On the other hand, fruits harvested at the breaker stage and exposed to 1-MCP or $KMnO_4$ solution media recorded significantly lower TSS than the full-ripe fruits kept in the open shelf as shown in Table 5.

Significant ($p \leq 0.05/0.01$) levels of association, comparable for both 2018 and 2019 experiments, were observed in the relationship among shelf life and measured biochemical parameters of tomato

Table 4 - Harvest maturity index and ripening remediation agents on shelf life of tomato fruit in years 2018 and 2019

	Shelf life (days)							
	0.1 µL/L 1-MCP	0.3 µL/L 1-MCP	0.5 µL/L 1-MCP	5% KMnO4	10% KMnO4	10 g Zeolite	20 g Zeolite	Open shelf (control)
Breaker								
2018	37 ab	46 a	37 ab	42 a	40 a	27 b	40 a	19 c
2019	31 ab	42 a	36 ab	45 a	36 ab	36 ab	36 ab	18 c
mean	34	44	36.5	43.5	38	31.5	38	18.5
Turning								
2018	36 ab	37 ab	33 ab	41 a	40 a	25 b	36 ab	19 c
2019	33 ab	40 a	37 ab	42 a	33 ab	28 b	36 ab	19 c
mean	34.5	38.5	35	41.5	36.5	26.5	36	19
Full-ripe								
2018	34 ab	40 a	31 ab	40 a	37 ab	31 ab	39 a	16 c
2019	37 ab	39 ab	34 ab	39 ab	28 b	33 ab	37 ab	13 c
mean	35.5	39.5	32.5	39.5	32.5	32	38	14.5

Means followed by the same letters in the same column are not significantly different at 5% probability level of DMRT.

Table 5 - Interaction of harvest maturity index and ripening remediation agents on biochemical quality attributes of tomato fruits in years 2018 and 2019

Harvest maturity index	Ripening remediation treatments	TTA (g/l)		Lycopene (µg/100g)		Vitamin C (mg/100g)		TSS (%)	
		2018	2019	2018	2019	2018	2019	2018	2019
Breaker	0.1 µL/L 1-MCP	0.41 a	0.42 a	382.26 ab	381.38 ab	16.67 a	16.87 a	5.59 b	5.57 b
	0.3 µL/L 1-MCP	0.41 a	0.42 a	384.93 ab	371.42 b	16.43 a	16.39 a	5.56 b	5.52 b
	0.5 µL/L 1-MCP	0.41 a	0.42 a	382.18 ab	378.93 b	16.78 a	16.36 a	5.54 b	5.52 b
	5% $KMnO_4$	0.42 a	0.42 a	388.67 ab	384.94 ab	16.21 a	16.11 a	5.53 b	5.54 b
	10% $KMnO_4$	0.36 b	0.40 a	399.10 ab	396.71 ab	15.21 ab	15.22 ab	5.67 b	5.62 b
	10 g Zeolite	0.37 b	0.38 b	397.89 ab	411.30 a	15.83 ab	15.93 ab	5.75 ab	5.75 ab
	20 g Zeolite	0.38 ab	0.38 b	396.71 ab	411.23 a	15.38 ab	15.01 ab	5.75 ab	5.75 ab
	open shelf (control)	0.35 b	0.35 b	412.73 a	407.36 a	15.91 ab	15.96 ab	5.95 a	5.93 a
Turning	0.1 µL/L 1-MCP	0.39 ab	0.39 ab	394.77 ab	391.22 ab	16.91 a	16.91 a	5.64 b	5.69 b
	0.3 µL/L 1-MCP	0.39 ab	0.40 a	386.42 ab	389.41 ab	16.71 a	16.86 a	5.69 b	5.66 b
	0.5 µL/L 1-MCP	0.39 ab	0.40 a	386.99 ab	388.50 ab	16.82 a	16.50 a	5.66 b	5.64 b
	5% $KMnO_4$	0.40 a	0.40 a	389.63 ab	389.71 ab	16.38 a	16.31 a	5.66 b	5.63 b
	10% $KMnO_4$	0.37 ab	0.38 ab	400.51 a	402.38 a	15.98 ab	15.92 ab	5.72 ab	5.77 ab
	10 g Zeolite	0.37 ab	0.38 ab	396.18 ab	403.78 ab	15.78 ab	15.61 ab	5.75 ab	5.79 ab
	2 0g Zeolite	0.37 ab	0.39 ab	389.28 ab	391.18 a	15.86 ab	15.89 ab	5.71 ab	5.71 ab
	open shelf (control)	0.34 b	0.34 b	410.91 a	413.67 a	14.71 b	14.62 b	5.99 a	5.98 a
Full-ripe	0.1 µL/L 1-MCP	0.38 ab	0.38 ab	392.51 ab	392.73 ab	15.43 ab	15.93 ab	5.77 ab	5.78 ab
	0.3 µL/L 1-MCP	0.39 ab	0.39 ab	389.42 ab	392.11 ab	15.56 ab	15.45 b	5.72 ab	5.78 ab
	0.5 µL/L 1-MCP	0.39 ab	0.39 ab	384.66 ab	389.81 ab	15.77 ab	15.86 ab	5.72 ab	5.78 ab
	5% $KMnO_4$	0.39 ab	0.39 ab	387.48 ab	389.82 ab	15.39 ab	15.93 b	5.74 ab	5.74 ab
	10% $KMnO_4$	0.40 a	0.37 ab	402.38 a	404.86 a	14.73 b	14.78 b	5.82 a	5.89 a
	10 g Zeolite	0.37 b	0.37 ab	403.56 a	405.36 a	14.48 b	14.14 b	5.94 a	5.92 a
	20 g Zeolite	0.37 b	0.37 ab	399.83 ab	396.74 ab	14.97 b	14.76 b	5.95 a	5.95 a
	open shelf (control)	0.34 b	0.37 ab	413.56 a	411.86 a	13.92 b	13.82 b	5.99 a	5.99 a

Means followed by the same letters in the same column are not significantly different at 5% probability level of DMRT.

fruits in this study (Table 6). In 2018 and 2019, titratable acidity had positive and significant correlation with ascorbic acid (r = 0.64 and 0.63 respectively) and shelf life (r= 0.70 and 0.74 respectively) but shared negative and significant correlation with lycopene (r = -0.86 and -0.83 respectively) and total soluble sugars (r= -0.94 and -0.82 respectively). In 2018 and 2019, lycopene content had positive and significant association with total soluble sugars (r = 0.80 and 0.85 respectively) and shared negative and significant association with ascorbic acid (r= -0.70 and -0.71 respectively) and shelf life (r= -0.65 and -0.68 respectively). Furthermore, there was negative and significant correlation between ascorbic acid and total soluble sugars with r= -0.76 and -0.80 while sharing positive and significant association with shelf life with r= 0.51 and r 0.42 for 2018 and 2019 respectively. Total soluble sugars also had negative and significant correlation with shelf life (r= -0.70 and r= -

Table 6 - Pearson correlation coefficients of the relationship among shelf life and measured biochemical quality components of tomato fruits stored with ripening remediation agents in 2018 (lower diagonal) and 2019 (upper diagonal)

Parameter measured	Titratable acidity	Lycopene	Ascorbic acid	Total soluble sugars	Shelf life
Titratable acidity	1	-0.86 **	0.64 **	-0.94 **	0.70 **
Lycopene	-0.83 **	1	-0.70 **	0.80 **	-0.65 **
Ascorbic acid	0.63 **	-0.71 **	1	-0.76 **	0.51 *
Total soluble sugars	-0.82 **	0.85 **	-0.80 **	1	-0.70 **
Shelf life	0.74 **	-0.68 **	0.42 *	-0.69 **	1

*, ** significant at 5 and 1% probabilities, respectively.

0.69 in 2018 and 2019 respectively).

4. Discussion and Conclusions

The ripening remediation treatments considerably affected the shelf life of stored tomato in both years of the study and the extended shelf life could have been as a result of the efficacy of these treatments to delay the conversion of starch to sugars thus reducing the ethylene production and peroxidase activity of the fruits. Similar results of delay in conversion of starch to sugars for extended shelf life of tomato were observed with the use of gibberellic acid as reported by Srividya *et al*. (2014). The identified RRAs could also have been able to extend the shelf life of the fruits due to their ability to control respiratory metabolism, thus maintaining the produce for a longer period as suggested by Nath *et al*. (2015).

During storage, acidity decreased with ripening as the organic (malic and citric) acids in the fruits got metabolized. The loss of TTA during storage period could be related to higher respiration rate as ripening advances, where organic acids are used as substrate in the respiration process. Exposure of the tomato fruits to the RRAs in this study delayed the consumption of the TTA, with 1-MCP and 5% $KMnO_4$ being the most reliable in achieving this. Regassa *et al*. (2012) reported the sequential disappearance of malic and citric acids in ripening tomato fruits leading to reduction in the amount of TTA.

Lycopene content of tomato fruits were differently affected by RRA but exposure to 1-MCP concentrations and 5% $KMnO_4$ solution treatments delayed the accumulation of lycopene in the fruits for both years. This might be due to decrease in respiratory rate, inhibiting ethylene activity, consequently reducing metabolism of the fruit (Nath *et al*., 2015). The delay in lycopene development in this study could have been as a result of the efficacy of the ripening remediation treatments in suppressing the production of ethylene in fruits thus delaying lycopene accumulation. The restrictive effect of 1-MCP on lycopene accumulation in this study supports the previous reports of Taye *et al*. (2019).

The treatment with 1-MCP concentrations and $KMnO_4$ had comparable patterns of effect on ascorbic acid content. Generally, fruits treated with 1-MCP and 5% $KMnO_4$ had higher ascorbic acid contents compared to other treatments. The efficacy of 1-MCP concentrations in this study corroborates the observations of Sabir *et al*. (2012) that 1-MCP had significant effect on ascorbic acid content by decreasing ethylene content of tomato fruit thereby increasing ascorbic acid content. Generally, this study indicated that there was a decrease in ascorbic acid content of tomato fruits which showed significant decrease during storage as reported by Ahmed *et al*. (2018).

Generally, as reported by Tilahun *et al*. (2019), tomato fruits harvested at the matured green and breaker stages had lower TSS level, while fruits harvested at light-red stage of full-ripe had the highest TSS. However, in this study, the efficacy of the ripening remediation agents was evident in slowing down the breakdown of carbohydrates into soluble sugars (fructose and glucose) or excessive moisture loss that aids the hydrolysis of cell wall polysaccharides. The fact that fruits kept in the open shelf recorded the highest TSS in both years can be attributed to faster advancement in ripening than those treated with RRAs as previously specified by Ahmed *et al*. (2018). The increase as influenced by the ripening remediation treatments may have occurred as a result of breakdown of carbohydrates into soluble sugars, or excessive moisture loss that aids the hydrolysis of cell wall polysaccharides. However, Beckles (2012) earlier noted that 1-MCP may increase, reduce or leave unchanged, the development of TSS depending on fruit species.

1-MCP at 0.3 µl/L concentration and 5% $KMnO_4$ were the most effective in extending the shelf life of fruits harvested however, those left in the open shelf consistently had the shortest shelf life implying that RRA application was effective in extending the marketable life of the fruits. The longer shelf life recorded by the fruits at the breaker and turning stages may be attributed to the ability of the ripening remediation agents to control respiratory metabolism, thus maintaining the produce for a longer period as suggested by Nath *et al*. (2015). Harvesting fruits at the proper maturity stage has a great influence on the nutrient content as well as shelf life of any fruit. However, in this study, the ripening remediation agents had great influence in slowing down the action of the ripening hormone that could accelerate the decline in the ascorbic acid content of the tomato fruit.

It is cumbersome to consider multiple traits in a selection scheme. Information on the relationship among various traits with shelf life would thus be beneficial to designing an efficient storage system. The significant correlation of shelf life with all the measured nutritional quality attributes coupled with

the interrelationship among the attributes presents the possibility of extending the shelf life of tomato fruits through designing an effective storage system that focuses on manipulating the production and/or accumulation of titratable acids, total soluble solids, lycopene and ascorbic acid. Comparable findings in the relationship among nutritional quality traits of tomato, including titratable acids, total soluble solids, and lycopene have earlier been reported by Singh *et al.* (2018) and Shobo *et al.* (2020).

The use of RRAs was effective in increasing the shelf life and maintaining the nutritional properties of tomato fruits in storage. However, the RRAs differed in their effectiveness in both capacities. RRAs 0.3 µL/L1-MCP, 0.5µL/L1-MCP and 5% $KMnO_4$ solution media had longer shelf life and higher values of total soluble solids, titratable acidity, lycopene and ascorbic acid contents. The use of 1-MCP and 5% $KMnO_4$ is recommended as effective scavenger of ethylene for extending the shelf life and maintaining some quality attributes of stored tomato fruits.

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AHS
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Arbuscular mycorrhizal fungi potentiate the root system and the quality of goldenberry fruits

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Abstract: The lack of information on the horticultural performance of goldenberry (*Physalis peruviana* L.) is one of the factors that limits the expansion of the crop. Still, aiming to establish a sustainable management for this culture, inoculation with arbuscular mycorrhizal fungi (AMF) can be adopted. Therefore, the objective of the research was to investigate whether goldenberry plants in the absence and presence of inoculation with AMF differ in terms of horticultural performance. The four treatments studied were the absence (control) and the presence of three inoculants based on AMF (mycorrhizal community, *Glomus intraradices* and *Rhizophagus clarus*), arranged in a randomized block design, with five replications. Goldenberry plants produced in substrate enriched with AMF had a more voluminous root system and a greater amount of fine roots. Additionally, the fruits were sweeter and more flavorful when produced by plants inoculated with the mycorrhizal community and with *R. clarus*. It is concluded that mycorrhization has no effect on fruit production. However, goldenberry plants submitted to mycorrhizal biotechnology enhance the chemical quality of fruits and present a more profuse root system. *G. intraradices* is most effective in colonizing the roots of the plant host.

1. Introduction

Goldenberry (*Physalis peruviana* L., Solanaceae) is a horticultural crop native to the Andean highlands that has attracted worldwide attention due to its bioactive compounds such as carotenoids, physalins and polyphenols (Ramadan, 2011). In addition to promoting the health of consumers, these biomolecules present in fruit extracts have antifungal action against phytopathogens, such as *Botrytis cinerea* Pers., and therefore can be widely used in agriculture as a bioinput (Filippi *et al.,* 2020). Still, goldenberry stands out for its potential for intensive cultivation

(Etzbach *et al.,* 2018). A single plant can produce 300 fruits and the productivity of this horticultural crop can reach from 20 to 33 tons per hectare (Yildiz *et al.,* 2015). Despite the traditional establishment of crops in the open field (Muniz *et al.,* 2014), the goldenberry cultivation in greenhouse is increasing (Aguilar-Carpio *et al.,* 2018). This is because greenhouse cultivation can help to avoid inconveniences such as pests, diseases, rain, strong winds, hail and frost (Costa *et al.,* 2016).

Similar to the traditional cultivation of other Solanaceae, such as tomato and pepper, in order to obtain an optimal productive yield of goldenberry, producers need to use a large amount of chemical inputs, which can contaminate the agroecosystem of cultivation (Chiomento *et al.,* 2020 a). There is no doubt, therefore, that the establishment of agroecological agriculture is an important tool for sustainable food production, with environmental and socioeconomic benefits (Llano *et al.,* 2018). Thus, an alternative to minimize the inconveniences in the cultivation of goldenberry and start the establishment of sustainable management in this horticultural culture corresponds to the use of inoculants based on arbuscular mycorrhizal fungi (AMF). As there is a limitation regarding the availability of commercial AMF-based inoculants available in Brazil (Trentin *et al.,* 2022), this reduces the use of mycorrhizal biotechnology in the production of goldenberry due to the lack of knowledge of this bioinput by the producers. The limitation regarding the availability of commercial inoculants is mainly due to the high cost linked to the production technology of this bioinput.

AMF (phylum Glomeromycota), a ubiquitous group of soil microorganisms, establish symbiotic associations with more than 70% of vascular plants (Brundrett and Tedersoo, 2018). The literature reporting the association between mycorrhiza and goldenberry is scarce. For example, under saline conditions, AMF increased fruit growth rate (Miranda *et al.,* 2011) and improved berry unsaturated fatty acid concentration in response to heavy metal stress (Hristozkova *et al.,* 2017). Under water stress, arbuscular mycorrhiza increased root dry matter accumulation and improved attributes related to plant gas exchange (Reyes *et al.,* 2019). In non-stressful environments, it was found that goldenberry plants subjected to mycorrhizal biotechnology produced less acidic and tastier fruits (Chiomento *et al.,* 2020 a). This scarcity of information demands more research to fill the existing gaps regarding the morpho-horticultural performance of goldenberry and their interactive effects with mycorrhizas.

In Brazil there is only one commercial AMF inoculant available to farmers. The commercial scale production of this bioinput has high costs linked to the inoculum production technology, such as the establishment of cultures of AMF species and transport, handling and development of the carrier substrate (Schlemper and Stürmer, 2014). To avoid some of these costs, *on-farm* production of inoculants is used, with indigenous or exotic AMF isolates, in which the technology can be easily transferred to farmers (Douds Junior *et al.,* 2012). The process of obtaining the *on-farm* inoculant can be started using AMF infective propagules, such as spores, hyphae and parts of colonized roots (Douds Junior *et al.,* 2010).

Therefore, based on the hypothesis that mycorrhizal biotechnology enhances plant host growth and improves fruit chemical quality, here we investigate whether the horticultural performance of goldenberry is influenced by the use of AMF-based *on-farm* inoculants.

2. Materials and Methods

Plant material

The research was carried out in Passo Fundo (28° 15’ 46” S, 52° 24’ 24” W), Rio Grande do Sul (RS), Brazil, in greenhouses, from August (winter) 2018 to July (winter) 2019 .

A commercial tray with goldenberry fruits at maturation stage 5 was purchased (ICONTEC, 1998). In August 2018, seeds from three randomly chosen fruits were selected, transferred to paper towels and kept at room temperature until dry. Subsequently, these seeds were germinated in plastic gerbox boxes containing blotting paper and 0.1 molar (M) potassium nitrate (KNO_3) solution. The boxes were stored in a biochemical oxygen demand (BOD) oven, at 25°C±1°C, until the plants were obtained for the production of seedlings, which constituted the plant material for the research. The steps for obtaining the plants are shown in figure 1.

Experimental design

The four treatments studied were the absence (control) and the presence of three inoculants based on AMF [mycorrhizal community, *Glomus intraradices* N.C. Schenck & G.S. Mr. and *Rhizophagus clarus* (T.H. Nicolson & N.C. Schenck) C.

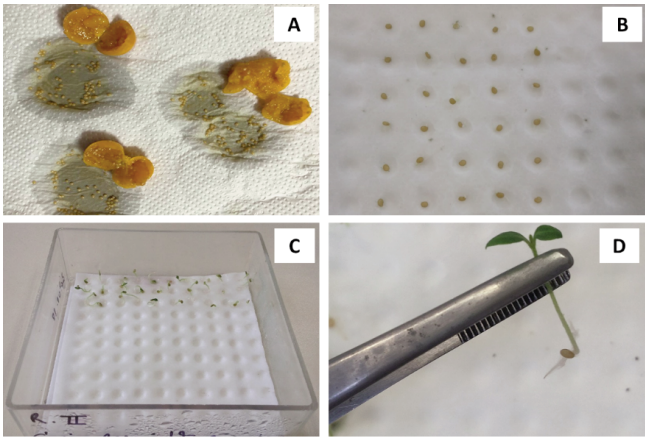

Fig. 1 - Obtaining goldenberry plants. (A) Separation of seeds from fruits. (B) Selection of seeds. (C) Germination in gerbox. (D) Plants produced.

Walker & A. Schüßler], arranged in a randomized block design with five replications. Each plot consisted of three goldenberry plants.

The AMF community used came from the croptrap of agricultural soil collected at a reference site for strawberry cultivation in the municipality of São José do Hortêncio (29° 29' 33" S, 51° 12' 24" W), Rio Grande do Sul State, Brazil (Chiomento *et al.,* 2019 a), composed of ten fungal species according to the classification of Glomeromycota proposed by Redecker *et al.* (2013): *Acaulospora foveata* Trappe & Janos, *Claroideoglomus* aff. *luteum*, *Claroideoglomus claroideum* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler, *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler, *Funneliformis* aff. *geosporum*, *Funneliformis* aff. *mosseae*, *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler, *Glomus* aff. *versiforme*, *Glomus* sp. (caesaris like) and *Glomus* sp2. The isolate *G. intraradices* came from the commercial product MYKE® PRO and the isolate *R. clarus* was obtained from the International Collection of Glomeromycota Culture (CICG).

Cultivation procedures

We applied the treatments (AMF) in two stages: 1) in the acclimatization of the seedlings; 2) in transplanting to the place of cultivation. Thus, of the total amount of mycorrhizal inoculant used (10 g), we applied 5 g in the acclimatization of the seedlings and 5 g at the time of transplanting.

In September (spring) of 2018, thirty days after the germination of goldenberry seeds, the plants obtained (Fig. 1D) were acclimatized in 72-cell polystyrene trays, filled with the sterilized Horta 2® substrate (120°C for 20 minutes) and with treatments related to mycorrhization (1/2 of the total amount), with the purpose of seedling production. Horta 2® is composed of pine bark, vermiculite, acidity correctors and fertilizers (nitrogen, phosphorus and potassium) in amounts not supplied by the manufacturer. A 500 g sample of the substrate was analyzed to obtain its physical (Brazil, 2007) and chemical (MAPA, 2014) attributes (Table 1).

The trays were kept on metal benches, 1.2 m from the ground surface, in a greenhouse (90 m^2), installed in the northeast-southeast direction, with a semicircular roof. The galvanized steel structure is covered with a low-density polyethylene film with anti-ultraviolet additive (150 micron thickness) and the sides are covered with an anti-aphid screen. The irrigation used during acclimatization was with sprinklers (1.8 $L.min^{-1}$ per unit), in the mechanized system. The irrigation regime consisted of activating the sprinklers seven times a day, with total wetness of 14 minutes. The water depth supplied to the seedlings was 7.8 $mm.day^{-1}$.

In December (summer) 2018, after three months of acclimatization, the seedlings were transplanted

Table 1 - Physical and chemical properties of the Horta 2® substrate

Substrate	Physical properties						
	D (Kg m^{-3})	TP ($m^3.m^{-3}$)	AE ($m^3.m^{-3}$)	RAW ($m^3.m^{-3}$)	BW ($m^3.m^{-3}$)	RW ($m^3.m^{-3}$)	
Horta 2 ®	241	0.837	0.303	0.149	0.020	0.365	
	Chemical properties						
	N % ($m.m^{-1}$)	P_2O_5 ($m.m^{-1}$)	K_2O % ($m.m^{-1}$)	OC % ($m.m^{-1}$)	pH	EC % ($mS.cm^{-1}$)	CEC ($mmol_c.kg^{-1}$)
	0.36	0.39	0.00	12.60	6.1	0.45	278.60

(z) D= density; TP= total porosity; AE= aeration space; RAW= easily available water; BW= buffer water; RW= remaining water.

(w) N= nitrogen; P_2O_5 = phosphorus pentoxide; K_2O= potassium oxide; OC= organic carbon; pH= hydrogen potential; EC= electric conductivity; CEC= cation exchange capacity.

into pots (3.6 L), filled with sterilized Horta 2® (120°C for 20 minutes) and complemented with the other part of the treatments related to mycorrhization (1/2 of the total amount). The pots were kept in beds covered with mulching, in a greenhouse (430 m^2), with a semicircular roof, installed in the northeast-southeast direction. The galvanized steel structure was covered with a low-density polyethylene film (150 microns thick) and with an anti-ultraviolet additive.

Localized irrigation was carried out using drip rods (2.4 $L.h^{-1}$ per unit), in the mechanized system. The irrigation regime consisted of activating the dripping rods six times a day, with total wetting for six minutes. The nutrient solutions supplied to the plants, fortnightly, were made according to Furlani and Fernandes Júnior (2004), but with a 50% reduction in phosphorus supply. Through a mini meteorological station, we verified that the average general temperature recorded during the experiment was 25.66°C.

The plants were conducted with three stems and were tutored with the aid of wires. No biocides were used during the crop cycle. The evaluations started after the fruiting of the plants, in February (summer) of 2019. We evaluated the root system morphology and the productive yield (number and weight) and quality of fruits.

Root system morphology

At the end of the experiment, in July 2019, the plants roots were washed in water to eliminate substrate fragments. The roots were digitized by a scanner and the images obtained were analyzed by the WinRHIZO® software. The attributes evaluated were total length (TL, cm), surface area (SA, cm^2) and volume (V, cm^3). The roots were grouped by the software into different diameter classes in relation to their total length (Böhm, 1979): very thin (VT, Ø<0.5 mm), thin (TH, Ø from 0.5 to 2 mm) and thick (TK, Ø>2 mm).

To verify the infective capacity of AMF, root portions of mycorrhizal plants were prepared according to Phillips and Hayman (1970) and their percentage of mycorrhizal colonization (MC) was determined according to Trouvelot *et al.* (1986), by the equation:

$$MC(\%) = \frac{(\text{total number of fragments with mycorrhizal roots})}{(\text{total number of fragments})} \times 100 \quad (1)$$

Fruit production

From fruiting, in February 2019, the total number of fruits (TNF, number per plant) and the total production of berries (TP, grams per plant) were evaluated. In addition, the average fresh fruit mass (AFFM, grams) was evaluated. The fruits were harvested when they were in the stages of maturation between 4 and 6 (ICONTEC, 1998). The fruits were weighed on an electronic digital scale.

Chemical fruit quality

The analysis of fruit quality was performed at the end of the experiment, in July 2019. The chemical characteristics of the fruits were evaluated regarding the content of total soluble solids (TSS, %) and total titratable acidity (TTA, % of citric acid), from 20 fruits of each treatment for each repetition. The TSS content was determined in an analog refractometer, and the TTA was performed according to the norms of the Adolfo Lutz Institute (Zenebon *et al.,* 2008). To evaluate the flavor of the fruits, the TSS/TTA ratio was determined.

Data analysis

The data obtained were submitted to analysis of variance (Anova) and the averages of the treatments were compared by the Tukey test, at 5% error probability, with the aid of the Costat® program (Cohort Software, 2003).

3. Results

Root system morphology

We verified a significant effect of mycorrhizal inoculants only for the attributes MC, V and TH. *G. intraradices* had a greater ability to infect plant roots than the mycorrhizal community and *R. clarus* (Fig. 2A). The fungal structures identified inside the roots of goldenberry plants were hyphae, vesicles and arbuscules. In addition, plants inoculated with *R. clarus* had 41% and 42% greater root volume than non-mycorrhizal plants and those inoculated with the AMF community, respectively (Fig. 2B). Also, plants inoculated with the mycorrhizal community had a greater amount of fine roots (+47%) compared to the control (Fig. 2C).

Fruit production

We observed a positive effect of treatments only for the AFFM attribute. Non-mycorrhizal plants produced fruits with higher average fresh mass (+29%) compared to plants inoculated with the mycorrhizal

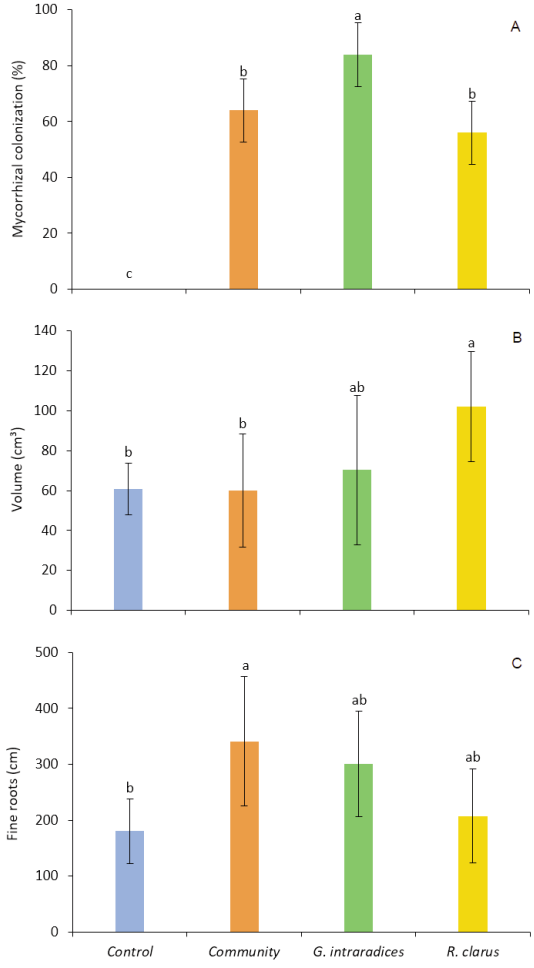

Fig. 2 - Root system morphology of goldenberry plants in the presence and absence of AMF inoculation. (A) Mycorrhizal colonization (%). (B) Root volume (cm³). (C) Amount of fine roots (cm). Data presented as mean ± standard deviation. Means followed by the same letter in the column did not differ significantly by the Tukey test (p≤0.05).

community, but did not differ from plants mycorrhizal with *G. intraradices* and *R. clarus* (Fig. 3).

Chemical fruit quality

Mycorrhizal inoculants influenced TSS and TSS/TTA attributes (Fig. 4). Sweeter (Fig. 4A) and tastier (Fig. 4B) fruits were produced by plants inoculated with the mycorrhizal community and with the isolate *R. clarus*.

4. Discussion and Conclusions

Here, we show that goldenberry plants in the absence and presence of AMF inoculation differed in horticultural performance. In the first productive

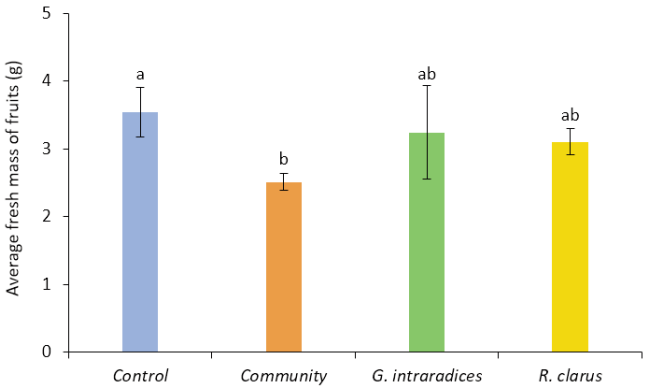

Fig. 3 - Average fresh fruit mass (grams) of goldenberry plants in the presence and absence of AMF inoculation. Data presented as mean ± standard deviation. Means followed by the same letter in the column did not differ significantly by the Tukey test (p≤0.05).

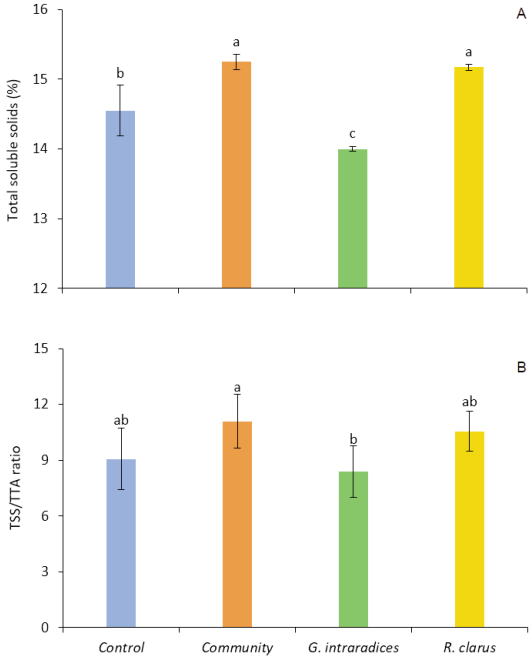

Fig. 4 - Chemical quality of goldenberry fruits in the presence and absence of AMF inoculation. (A) Total soluble solids (%). (B) Fruit flavor. Data presented as mean ± standard deviation. Means followed by the same letter in the column did not differ significantly by the Tukey test (p≤0.05).

cycle of the plants, we did not observe the effect of mycorrhization on the production of berries. We believe that in goldenberry the fungal species used require a period of more than one year to benefit the fruit yield, as occurs in strawberry (*Fragaria* X *ananassa* Duch.) cultivation (Robinson-Boyer *et al.*, 2016). However, goldenberry plants inoculated with AMF showed a more profuse root system.

Furthermore, the use of the mycorrhizal community and *R. clarus* allowed to harvest fruits with better chemical quality. This indicates that the fungal species present in these two inoculants have an affinity for this horticultural crop. The use of AMF compatible with the host commonly provides more satisfactory results (Chiomento *et al.,* 2022). *G. intraradices* was more effective in colonizing the roots of the plant host. However, the mycorrhizal community tested in this study stood out in relation to the fungal isolates for generally improving the horticultural performance of goldenberry.

We verified that the root system of the mycorrhizal plants was more profuse, with greater volume (Fig. 2B) and with a greater amount of fine roots (Fig. 2C). This benefit to the roots has already been reported by Reyes *et al.* (2019), who demonstrated that mycorrhization in goldenberry under water stress increased the accumulation of root dry matter. Due to the plasticity of the roots, their characteristics can be modulated by several factors, including AMF (Hodge *et al.,* 2009). During the establishment of the association between host and fungus, many molecular signals are initiated, including an AMF (lipo-chitooligosaccharides) diffusible factor called "Myc factor", which stimulates the formation of finer roots (Oláh *et al.,* 2005), altering the morphology of the root system of plants. In addition, root modifications under mycorrhization may be related to the allocation of sugars to roots (Wu *et al.,* 2011) and hormonal regulation (Zou *et al.,* 2017), independent of symbiotic signaling (Gutjahr, 2014). The more fine roots there are in mycorrhizal plants, the better their acquisition of water and nutrients (Chiomento *et al.,* 2021), as these roots are the ones that most acquire and use the available resources in the plant growth medium (Costa *et al.,* 2019).

Differently from what was expected, the productive performance of goldenberry was higher when the plants were not mycorrhized (Fig. 3). This suggests that AMF initially demand carbon from the host for their maintenance and only later repay this benefit to the plant symbiont. These results, however, contradict the literature. For example, Miranda *et al.* (2011) reported benefits of AMF in the production of goldenberry, under limiting conditions to plants, which were subjected to abiotic stresses, which did not happen in our study. There is not always a high relationship between fungal infectivity and efficiency in promoting crop growth due to the time required to establish bidirectional flow between symbionts (Abbott and Robson, 1981), which limits the plant's response to mycorrhization (Lambais and Cardoso, 1990). Although mycorrhizal colonization is important, the percentage of root infectivity is not always correlated with the efficiency of symbiosis (Konvalinková and Jansa, 2016).

This lack of relationship between infectivity and AMF efficiency in improving crop growth may be related to the time required for the establishment of root colonization (Abbott and Robson, 1981). Under long-term, arbuscular mycorrhiza promotes more benefits to the plant host (Ortas, 2012), mainly by increasing the acquisition of water and minerals to the plant. The major function of AMF is suggested to be nutrient acquisition (Zhang *et al.,* 2019) and thus, under high nutrient conditions, AMF can shift from a net benefit to a cost for the host (Johnson *et al.,* 2015). In our study, the nutrients supplied during cultivation were not limited to goldenberry; therefore, the inoculation effect may not have been potentiated in terms of fruit yield.

However, we proved the benefit of mycorrhization on the chemical quality of the fruits through the increase in the sugar content (Fig. 4A) and the better berry flavor (Fig. 4B), as already reported for zucchini (*Cucurbita pepo* L.) (Rouphael *et al.,* 2015), strawberry (Costa *et al.,* 2020) and tomato (*Solanum lycopersicum* L.) (Sellitto *et al.,* 2019). Plants grown with AMF showed a more developed root system (Fig. 2), which allows extrarradicial hyphae to extend beyond the rhizosphere, making water acquisition more efficient (Xu *et al.,* 2017). Due to greater water availability, these plants have greater stomatal opening, which increases the rate of transpiration and, thus, there is a greater supply of carbon dioxide for photosynthesis (Vicente-Sánchez *et al.,* 2014). As a result, there is a greater production of sugars, which are the primary source of photosynthesis, and this explains the increase in the fruit sugar content and, consequently, the best flavor of the berries (Fig. 4).

The lack of effect of *G. intraradices* on berries quality can be attributed to the low effectiveness of this fungal species on goldenberry. Various factors modulate the arbuscular mycorrhiza effect on the performance of their associated plants and this includes the traits of the host and the fungi themselves (Chiomento *et al.,* 2019 a). Cultivated plants vary in their responsiveness to AMF due to their morphology (Chiomento *et al.,* 2019 b) and AMF differ in the benefits provided to the plant (Werner and Kiers, 2015).

The two genera that made up the monospecific inoculants, *Glomus* and *Rhizophagus*, have already been reported in studies of AMF diversity in goldenberry (Ramírez-Gómez *et al.,* 2019) and have also been used in applicability studies (*Rhizophagus*) in this horticultural crop (Miranda *et al.,* 2011). However, in our study, the mycorrhizal community, representing a multispecific inoculant, stood out in relation to the fungal isolates for generally improving the horticultural performance of goldenberry, mainly by increasing the amount of fine roots produced by the plants (Fig. 2C) and for benefiting fruit quality (Fig. 4). Inoculation with AMF populations, such as the fungal community used in this study, generally provides more satisfactory results due to greater compatibilities at the fungus-host interface and by increasing mutualistic effects with two or more symbionts instead of just one (Chiomento *et al.,* 2019 b). This mycorrhizal community was obtained through the trap culture technique, that is, produced *on-farm*.

A mycorrhizal inoculant rich in propagules and produced *on-farm* may be a suitable solution for large-scale inoculation of crops (agroecosystems), seedlings (nurseries) and in potting media for vegetable growers (soilless cultivation) (Douds Junior *et al.,* 2006). The use of non-sterilized growth medium (soil and/or substrate) as a component of the on-farm inoculant represents a source of propagules for other AMF present in this growth medium, which results in an inoculant with greater taxonomic diversity (Schlemper and Stürmer, 2014). This is strongly desired due to the functional diversity exhibited by mycorrhizal species for the promotion of plant growth, for example (Chiomento *et al.,* 2020 b). Furthermore, a diversified inoculant potentiates a combination between fungal isolates and an eventual host (Douds Junior *et al.,* 2006).

Therefore, the results of our research confirmed the potential of applying mycorrhizal biotechnology to goldenberry, as the work demonstrated that AMF can be a valuable tool for the cultivation of this vegetable. The complex roles of AMF in agroecosystems are just beginning to be understood (Chiomento *et al.,* 2022). In this way, a greater understanding of the application and benefits of AMF can enable their use in the sustainable production of vegetables (Trentin *et al.,* 2022).

We conclude that in the first production cycle, there is no effect of mycorrhization on the total number of fruits and total production of berries. We believe that for the goldenberry cultivation the fungal species used require a period of more than one year to benefit the fruit yield. On the other hand, goldenberry plants submitted to mycorrhizal biotechnology have a more profuse root system and produce fruits with better chemical quality. *G. intraradices* is most effective in colonizing the plant host roots. However, the mycorrhizal community stands out in relation to the fungal isolates for generally improving the horticultural performance of goldenberry. Thus, the application of this biotechnological tool in the goldenberry culture can be an alternative to spread and promote its sustainable cultivation.

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AHS
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Combining humic acid with NPK fertilizer improved growth and yield of chili pepper in dry season

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Abstract: This research aimed to study the effect of humic acid and NPK fertilizer (15:15:15) on growth and yield of red chili, and to obtain the most suitable composition of humic acid and NPK fertilizer which gave the best growth and yield. The study used a randomized block design with five replications. The treatments tested were the composition of humic acid and NPK fertilizer: 100% humic acid; 75% humic acid + 25% NPK; 50% humic acid + 50% NPK; 25% humic acid + 75% NPK; and 100% NPK. Data on plant growth and yield were processed by Analysis of Variance, and means were compared using Fisher's Least Significant Difference test. In addition, data on plant biochemical and soil chemical parameters were determined compositely by mixing leaves taken from sample plants or soil samples into one homogenous sample. Results showed that there was no significant difference in growth and yield of plants treated with 100% humic acid in comparison with those plants treated with 100% NPK. However, in comparison with 100% humic acid, the application of different ratios of humic acid/NPK increased plant chlorophyll contents by 65% - 82% and total sugar by 28% - 71%. The application of humic acid/NPK increased soil fertility by improving soil pH as well as N, P and K. In the combination of humic acid/NPK, the best growth and yield were obtained with the application of 25% humic acid + 75% NPK fertilizers. Therefore, for the sustainability of chili cultivation, the use of humic acid needs to be accompanied with NPK fertilizers at a reduced amount, along with the increase in the dose of humic acid.

1. Introduction

In Indonesia Chili (*Capsicum annuum* L.) is one of vegetable crops with high economic value due to its large domestic and export demaind and, from time to time, price fluctuations may cause consumer unrest. The national inflation rate is significantly influenced by the increase in chili prices in certain seasons. To control the price fluctuations the Indonesian government is increasing the planting area in rainy season, and controlling the planting area as well as production during dry season.

Increasing production in the dry season normally is managed by application of inorganic fertilizers and use of chemicals to control pests and diseases. However, this practice has a negative impact on agricultural ecosystem and the environment. Additionally, implementing principles of sustainable agriculture such as the application of humic acid, a soil conditioner, has the potential to improve this conventional agricultural practice. Humic acid has a complex molecular structure and capability to stimulate and activate biological and physiological processes in soil organisms.

The study of Abdellatif *et al.* (2017) showed that the application of 14.4 kg ha^{-1} humic acid to chili plants grown under heat stress could increase plant's average height, number of flower buds, number of flowers, number of fruits per plant, as well as fruit weight. The application of 7.5-12 mL of humic acid into 10 kg of sterile soil increased the population of soil microorganisms *Azotobacter beijerinckii* and *Aspergillus niger*. Foliar spray of 50 mL L^{-1} humic acid significantly increased leaf area, yield and total soluble solids in grapes (Popescu and Popescu, 2018). Onion plants sprayed with 1000 mg L^{-1} humic acid produced the highest growth rate and yield (Al-Fraihat *et al.*, 2018).

In addition to the improvement in cultivation, chili production can be increased by growing them during the dry season. Whilst a lot of land is available for cropping during the dry season the production is constrained by the limited availability of water. Application of humic acid as a soil conditioner could remediate water shortage in plants due to increasing leaf water content, increasing photosynthesis, antioxidant metabolism and enzyme activity; thus improving plant tolerance to stress (Al-Shareef *et al.*, 2018).

Previous studies have demonstrated that humic acid improved plant growth and yield in a number of plant species. Hermanto *et al.* (2013) reported that application of 20 kg ha^{-1} humic acid along with 100% dose of NPK fertilizer resulted in maximum growth and yield as well as increased the availability and uptake of N, P, K, Zn and Fe in maize. Furthermore, Moraditochaee (2012) showed that the application of humic acid at 40 mg L^{-1} and 75 kg ha^{-1} N produced the highest harvest index in peanut.

Sustainable production of plants can be achieved by reducing the use of inorganic fertilizers. Application of humic acid could help achieve this important goal. Whilst humic acid has been widely applied to various plant species; the search of literature showed that no research has focused on the effect of humic acid in combination with NPK fertilizer on growth and yield of chili pepper. Therefore, this study aimed at assessing the effect of humic acid in combination with NPK fertilizer on growth and yield of red chili, and to obtain the most suitable ratio of humic acid/NPK for best growth and yield in chili plants grown in dry season in Indonesia.

2. Materials and Methods

The trial was conducted at the Teaching and Research Farm within the Faculty of Agriculture at the University of Jambi in Indonesia during the 2020 dry season. The study used commercially available humic acid (AH-90, with 90% humic acid content) and inorganic NPK fertilizer (15:15:15). There were 5 combinations tested: 100% humic acid; 75% humic acid + 25% NPK; 50% humic acid + 50% NPK; 25% humic acid + 75% NPK and 100% NPK. A randomized block design with 5 replications was used in this study. Humic acid was applied at the recommended dose (5 kg ha^{-1}) and so was NPK fertilizer (700 kg ha^{-1}). The humic acid and NPK were applied one week before planting in between rows.

Seeds were sown on a standard seedling medium, then transferred to nursery at 7 days after sowing and left in the nursery for 21 days. The medium used during the nursery growth was a mixture of soil, compost, and sand (2:1:1). Seedlings were sprayed every 7 days with foliar fertilizer Bayfolan-D at 2 mL L^{-1} and insecticide (active ingredient Amabektin and Mesurol) at 2 mL L^{-1}.

The one-month-old seedlings were transplanted in the field onto the growing media supplemented with humic acid and NPK fertilizer at different rates as per treatments described above. A silvercoated black plastic was placed on the beds before transplanting the seedlings. Prior to planting, a fungicide (Carbofuran) was applied into planting holes at 1.5 g per plant. The planting space was 50 cm x 50 cm. A liquid NPK fertilizer (15:15:15) was applied at a concentration of 2 g L^{-1} with a volume of 100 mL per plant at 6, 8 and 10 weeks after transplanting.

Harvesting was carried out during the first flowering period (10 to 13 weeks after transplanting), with the criterion that 80% of fruits becoming red. Observation of vegetative growth was carried out during fruit enlargement period (9 weeks after trans-

planting) on the following parameters: plant height, number of productive branches, leaf area, and plant total dry weight. The leaf area was measured using gravimetric method with the following formulae (Sitompul and Guritno, 1995):

$$\text{Leaf area (cm}^2\text{)} = (x/y)\cdot z$$

where x = total leaf dry weight, y = sub sample leaf dry weight, and z = sub sample leaf area.

Observation of plant biochemical measurements were made on chlorophyll content, total sugar, and relative leaf water content (RLWC). Composite leaf samples were made by physically mixing individual leaves taken from 3 sample plants of 3 replicates into one homogenous sample. Compositing reduced the number of analyses to be performed and was designed to provide a representative sample of the treatment. Ten youngest mature leaves on main stem were collected at 9 weeks after transplanting (WAT). Clean and dry leaf samples were placed in a sample bag prior to laboratory analyses. Chlorophyll analysis was carried out using Acetone method (Arnon, 1949), and total sugar was analyzed using Anthrone method (Yoshida *et al.*, 1976). Relative Leaf Water Content (RLWC) was determined according to González and González-Vilar (2001) method.

Data on yields in terms of number of the fruits and fruit weight per plant were collected at harvest time. In addition, soil chemical analyses were performed before transplanting and after the last harvest. Soil analyses included pH, moisture content, organic C based on Walkley-Black method (Walkley and Black, 1934), total N using Kjeldahl method (Kjeldahl, 1883), available P applying Bray-I method (Bray and Kurtz, 1945) and exchangeable K (NH_4OAc 1N extraction at pH 7) using method as described in (Warncke and Brown, 1998).

The data were scrutinized employing an analysis of variance (ANOVA) from Microsoft Excel application (Version 16.63.1, 2022, Microsoft Corporation, USA). Fisher's Least Significant Difference (FLSD) at 5% level of probability was used to compare means of treatments.

3. Results

Analysis of variance showed that the application of humic acid (HA) and NPK fertilizer did not significantly affect plant height, number of branches, total leaf area, and dry weight. However, application of 25% HA in conjunction with 75% NPK fertilizer resulted in enhanced growth compared to other treatments (Fig. 1). In addition, the application of 25% HA + 75% NPK improved growth compared with 100% HA application whereby plant height, number of branches, leaf area and plant dry matter increased by 29.46%, 21.96%, 23.59% and 28.42% respectively.

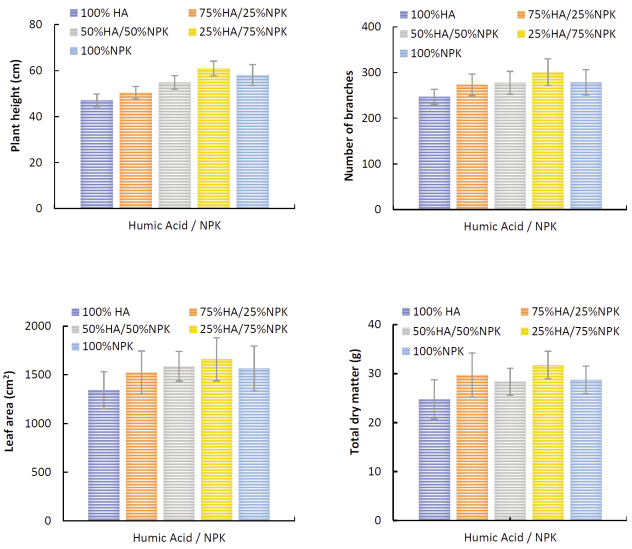

Fig. 1 - Growth of chili on various ratios of humic acid (HA)/NPK at 9 weeks after transplantation (A = plant height, B = number of branches, C = leaf area, D = total dry weight). Error bars indicate the Standard Error (n=5).

The measurement of biochemical parameters showed that the application of 100% HA resulted in the lowest chlorophyll content (2.40 mg L^{-1}) and total sugar (0.07%). Furthermore, the lowest relative leaf water content (RLWC) was found on 100% NPK application (Table 1).

Table 1 - Effect of different ratios of humic acid) HA / NPK on chlorophyll content (*), total sugar, and relative leaf water content (RLWC) at 9 weeks after transplantation

Humic acid/NPK	Chlorophyll content (mg·L^{-1})	Total sugar (%)	RLWC (%)
100% HA	2.40	0.07	92.6
75% HA/25% NPK	3.97	0.12	93.3
50% HA/50% NPK	4.15	0.12	93.7
25% HA/75% NPK	4.37	0.09	93.0
100% NPK	4.30	0.11	91.3

(*) Chlorophyll content and total sugar were determined compositely by physically mixing individual leaves taken from each 3 sample plants of 3 replicates into one homogenous sample.

Analysis of variance showed that the application of different ratios of HA/NPK had no significant effect on the number and weight of fruits per plant. However, among the combinations tested the 25% HA + 75% NPK resulted in the highest number of fruits, and the 50% HA + 50% NPK produced the largest fruit weight (Fig. 2).

The results of soil chemical analyses (Table 2) showed that the application of various ratios of HA/NPK had altered soil chemical properties such as pH, organic C, total N, P, K, and soil water content. Generally, the application of HA along with NPK fertilizer improved N, P and K content. Whereas soil pH and organic carbon are highest when 100% HA is applied (Table 2).

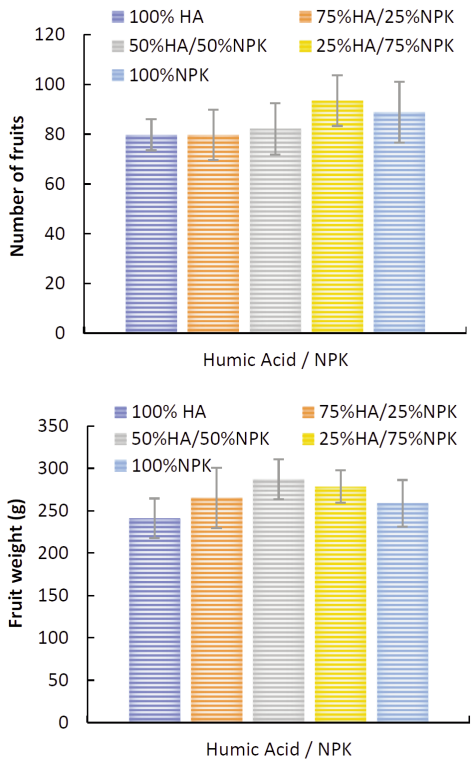

Fig. 2 - Effect of different ratios of humic acid (HA)/NPK on number of fruits (A) and fruit weight (B). Error bars indicate the Standard Error (n=5).

4. Discussion and Conclusions

The application of different humic acid/NPK ratios had no significant effect on growth and yield of chili as measured by plant height, total leaf area, number of branches and total dry weight, as well as number of fruits and average fruit weight. This means that humic acid can substitute NPK fertilizer. However, when compared to the use of only humic acid or NPK fertilizer, the application of different ratios of humic acid/NPK improved plant height, total leaf area, number of branches, total dry weight, fruit numbers and fruit weight.

NPK is widely used to improve plant growth and yield of many horticultural crops (Nafiu *et al.*, 2011; Agbede *et al.*, 2017; Achiri *et al.*, 2018; Adekiya *et al.*, 2019; Nwokwu *et al.*, 2020). However, the use of inorganic fertilizers such as NPK to help increase plant growth and yield need to be carefully considered, due to their detrimental impact on soil structure and soil biota. According to Adekiya *et al.* (2019) NPK fertilizer did not increase soil organic matter, nor reduced soil bulk density. Krestini *et al.* (2020) claimed that NPK fertilizer did not provide carbon compounds that contributed to the improvement of soil physical and biological properties. In addition, Agbede *et al.* (2017) reported that continued application of inorganic fertilizers can increase soil acidity and soil degradation because inorganic fertilizers release nutrients more quickly. Therefore, balanced fertilization is recommended to increase the efficiency of fertilizer use and help improve physical, chemical, and biological properties of soil.

The results obtained here show that application of 100% humic acid provided less growth and yield in

Table 2 - The effect of different ratios of humic acid (HA)/NPK on soil chemical properties

Humic acid/NPK	pH	C-organic (%)	N-total (%)	P (ppm)	K ($cmol^+/kg$)	Water content (%)
100% HA	6.09	1.55	0.22	104.50	0.06	7.70
75% HA/25% NPK	6.02	1.45	0.29	124.80	0.19	7.56
50% HA/50% NPK	5.47	1.28	0.28	152.30	0.47	7.61
25% HA/75% NPK	5.52	1.20	0.24	144.70	0.62	7.67
100% NPK	5.32	1.22	0.23	227.30	1.07	7.50

chili plants compared with mixture of humic acid/NPK during the one growth season of this research. However in a longitudinal experiment on peanut Li *et al.* (2019) demonstrated that significant increase in N, P and K content of soil was found after three years continuous application of humic acid.

The reason for this increase is due to humic compounds high stability rate, and therefore the mineralization process can occur very slowly. As a consequence, humic acid cannot be used as a source of plant nutrition (Canellas *et al.*, 2015). In research reported here the chemical analyses indicated soil that was treated with 100% humic acid was lower in N, P and K content, but higher in organic carbon and soil moisture content in comparison with those treated with different humic acid/NPK ratios (Table 2). This was in accordance with (Li *et al.*, 2019; 2020) findings that the application of humic acid-based fertilizer could increase soil nutrient contents, including the total soil nitrogen, phosphorus, and potassium.

Increase in soil water content was aligned with increase in relative leaf water content (RLWC) in chili plants (Table 1). Al-Shareef *et al.* (2018) reported that the application of humic acid as a soil enhancer could overcome water shortage in plants by increasing RLWC, thereby increasing plant tolerance to stress. Further, Xu *et al.* (2015) stated that the application of soil enhancers such as humic acid could increase plant growth by retaining available water and nutrients, therefore reducing the impact of drought stress and nutrient loss.

The application of 100% humic acid also increased soil pH from 4.43 to 6.09, resulting in better plant growth and development due to improved availability of various nutrients in the soil at pH of 6.09. Wahyudi (2007) reported that the application of humic acid might reduce the amount of exchangeable aluminum and increase chelating aluminum in soil, thereby increasing soil pH. Hasanudin (2003) suggested that the reduction in exchangeable aluminum was due to the formation of complex organometal compound (Al-chelate). Increase in soil pH will increase the availability of soil nutrients needed by plants (Gardner *et al.*, 1985). In peanut, Li *et al.* (2019) found that compared with control, humic acid significantly increased the yield and quality of peanut in continuous cropping. Their results showed that application of humid acid significantly increased the soil total nitrogen, total phosphorus, total potassium, available N, available P, available K, and organic matter from second year, with maximum effect displayed in third year.

Chili plants treated with different humic acid/NPK ratios showed better growth and yields in comparison to those plants treated with humic acid or NPK alone. The composition of 25% humic acid + 75% NPK fertilizer produced the highest growth (plant height, leaf area, number of branches and plant dry matter), as well as the highest yield (number of fruits) compared to other compositions. Furthermore, application of 50% humic acid + 50% NPK fertilizer resulted in the highest fruit weight. This result is in line with the increase in plant chlorophyll content, where the composition of 25% humic acid + 75% NPK fertilizer caused higher plant chlorophyll content. An increase in chlorophyll content intensifies the ability of plants to carry out photosynthesis, thereby increasing plant growth (Ferrara and Brunetti, 2010).

Increase in soil K content due to humic acid / NPK application caused better K uptake, increasing plant growth and yield in potato (Xu *et al.*, 2015). Potassium is crucial in plant photosynthetic activity (Marschner, 2012) and assimilate translocation efficiency (Sawan, 2018). This is in line with our results where application of humic acid and NPK (75% HA and 25% NPK or 50% HA and 50% NPK) increased sugar content in plant tissue (Table 1).

The composition of 50% humic acid + 50% NPK and 25% humic acid + 75% NPK in this study provided better growth and yield of chili plants than 100% humic acid or 100% NPK applications. This indicates a positive effect of the use of humic acid in partially replacing the role of NPK fertilizer. The ability of humic acid to replace NPK fertilizer is probably due to the ability of humic acid to modify the plant root system architecture as well as improving the capacity of soil to bind water, thus enabling plants to acquire water and nutrients more easily (Suwahyono, 2011; Canellas and Olivares, 2014), reducing plant stress in dry land cultivation during periods of drought (Xu *et al.*, 2015).

The analysis of soil water content for different ratios of humic acid/NPK showed medium treated with humic acid has a better water content than those treated with 100% NPK (7.56-7.7 versus 7.5). In addition, the relative leaf water content (RLWC) at various ratios of humic acid / NPK was higher than that of 100% NPK application (Table 1).

Our findings are consistent with a number of previous studies indicating that the application of humic acid needs to be accompanied by NPK fertilizer to help improve plant productivity (For example in

peanut (Moraditochaee, 2012), tomato (Sarno *et al.*, 2015) and cocoa (Santi, 2016)). The slow rate of humic acid mineralization needs to be followed by the application of NPK fertilizers. This is aimed to increase the availability of nutrients for plants, especially in relation to the relatively short lifespan of the chili plant which is only 3 - 4 months. In addition, it is also necessary to consider the amount of humic acid and the time of application, so that the use of NPK fertilizer as a source of plant nutrition can be further reduced.

Data presented here indicate that the application of different ratios of humic acid / NPK could greatly increase the growth and yield of chili pepper grown in dry land cultivating, where the composition of 25% humic acid + 75% NPK fertilizer resulted in improved plant growth and yield. It was found that the application of humic acid could reduce the use of NPK fertilizers by up to 50%. Thus, for sustainable chili production in dry land cultivation, application of humic acid in conjunction with NPK fertilizer is recommended at a reduced amount.

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Biological effects of some *Colchicum autumnale* L. extracts on tissue development of two varieties of *Ocimum basilicum* L.

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Key words: Colchicine, basil, bulbs, flowers, meadow saffron.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: *Colchicum autumnale* L. is a perennial herb from the Colchicaceae family with an unusual life cycle, and it is characterized by an underground corm and hypogynous flowers that appear in autumn. Its medicinal importance is represented by its primary alkaloid, colchicine, which has been studied for its anti-inflammatory and antimitotic properties and used in the treatment of some diseases and artificial polyploidy induction in plants. This study aims to determine and evaluate the biological effects induced by treatment with *C. autumnale* extracts on tissue development in test plants, represented by two *Ocimum basilicum* L. varieties: 'Italiano Classico' and 'Aromat de Buzău'. Morpho-anatomical observations and measurements and photosynthetic pigments analyses were employed. Results show unusual shapes of leaves, differences in stomata size and density, and heteromorphic cells in leaves and epicotyl's structure in both studied varieties of basil treated with *C. autumnale* extracts.

1. Introduction

Colchicum autumnale L., generally known as meadow saffron, naked lady, or wild saffron (Nagesh *et al*., 2011), is a colchicine-containing species widespread throughout Europe. It belongs to the Colchicaceae family, which comprises perennial herbs characterized by an underground corm and hypogynous flowers with six tepals (Bowles, 1924; Nordenstam, 1998, in Jung *et al*., 2011). The biochemical profile of this species reveals the presence of alkaloids, phenolics, terpenoids, glycosides, and other bioactive compounds (Davoodi *et al*., 2021; Hailu *et al*., 2021). The alkaloid concentration in this species varies depending on the plant organ; it was reported that it varies in seeds (0.5-1.2%), fresh flowers (1.2-2%), fresh leaves (0.15-0.4%), and fresh bulbs (0.1-0.6%); among all alkaloids, colchicine constitutes 50-70% of the total alkaloid content, followed by some small amounts of colchicoside, demecolcine, and other tropolone derivatives (Kupper *et al*., 2010). These variations are due to the fact that

secondary metabolites biosynthesis and accumulation are influenced by genetic, morphogenetic, and environmental factors (Yang *et al*., 2018).

Despite its toxicity, colchicine has been used in the treatment of gout, Familial Mediterranean Fever (FMF), and other diseases (Nagesh *et al*., 2011), and its antimitotic properties have been exploited in plant breeding for the production of polyploid crops (Roberts and Wink, 1998, in Jung *et al.*, 2011) and ornamental species (Manzoor *et al*., 2019).

To extract colchicine from the plant source, the most commonly used method is Soxhlet, and the best choice of solvent for this method has been reported to be methanol for *C. autumnale* and other species of the Colchicaceae family (Finnie and Van Staden, 1991; Pandey and Banik, 2012, in Çankaya *et al*., 2019). Colchicine binds to tubulin dimers and prevents microtubule assembly, inducing microtubule depolymerization and preventing mitotic spindle formation (Caperta *et al*., 2006). It was shown that colchicine treatment affects specific morphological (stomatal size), physiological (photosynthetic rate), and biochemical (chlorophyll content) indices in various plant species (Cao *et al*., 2018; Trojak-Goluch *et al*., 2021), but the effects of colchicine derivatives or *C. autumnale* extracts in plants are unknown.

This study aims to evaluate the effects of *C. autumnale* methanolic extracts on seed germination and plantlet tissue development by measuring several morpho-anatomical and photosynthetic indices to determine the allelopathic effect and the impact of the extracts of this plant species on other plants. For this purpose, *Ocimum basilicum* was chosen as a test species for its culinary, ornamental, medicinal, and economic importance and specific morpho-anatomical characteristics: four-edged stem (rectangular-quadrangular shaped) with four ribs and ovate-lanceolate opposite leaves with attenuate serrate edges, uniseriate pluricellular tector hairs, peltate (four-celled head) and capitate (one or two-celled head) glandular hairs that appear on the surface of the stem and leaves (Zamfirache *et al*., 2008; Nassar *et al.*, 2014), that make potential abnormalities easy to spot.

2. Materials and Methods

Plant material

The seeds of *O. basilicum* 'Italiano Classico' and 'Aromat de Buzău' were purchased from commercial sources (Unisem S.A., Iași, and S.C.D.L. Buzău). A total of 32 individual plants of *C. autumnale* were collected from a pasture in Voroneț, Suceava County, lat. 47.58889° N, long. 25.90861°E, alt. 576.68 m, in October 2019. The colchicine standard was provided by the Institute of Biological Research, Iași, Romania.

The bulbs and flowers of *C. autumnale* were kept in an oven at 65°C for 12 hours to stop enzymatic reactions and then dried at room temperature (21±1°C) for 7 days, away from any source of light. Subsequently, the dry material was ground in an electric grinder, placed in glass jars wrapped in aluminum foil, and stored in the refrigerator until used in extract preparation.

Extraction and quantification of colchicine content in extracts

The extraction was processed in a Soxhlet apparatus in methanol (Sigma Aldrich, Germany), using 5 grams of powder from each organ (bulb and flower), according to the method of Franz and Koehler (1992, in Alali *et al*., 2004). The extraction of colchicine from the dried plant material was performed until the solvent in the extraction chamber was clear, in the following way: 5-6 cycles (1-1.5 cycles/hour) for bulbs and 17-18 cycles (2-3 cycles/hour) for flowers. After extraction, the methanol was evaporated in a rotary evaporator (IKA RV3 Eco, Germany). Each dry extract was weighed and then dissolved in 50 ml of 70% methanol. Two colchicine-containing extracts were obtained, one from the bulbs and abbreviated BE (bulb extract) and one from flowers - abbreviated FE (flower extract).

The quantification of the colchicine in the extracts was performed by RP-HPLC, according to Alali *et al*. (2004). The separation was performed on a Shimadzu Prominence HPLC system (2x LC20AD pumps, SIL20AC autosampler, CT20AC oven, SPD M20A DAD detector) using a Zorbax Eclipse XDB - C18 (250 mm length, 3 micron particle size) column, with acetonitrile as mobile phase A, and 3% acetic acid (Sigma Aldrich, Germany) as mobile phase B. Elution was performed at a flow rate of 1 ml/min using the following program: 0-3 min 90% B isocratic, 3-11 min 90-40% B gradient, 11-12 min 40% B isocratic, 12-13 min 40-90%B gradient, 13-20 min 90% B isocratic. A volume of 20µl of colchicine standard and 5 µl of each extract were injected. Colchicine was detected at 245 nm and eluted at 13.4±0.08 minutes. Chromatographic data were acquired using the Shimadzu LC solution Software and manually inter-

preted. For calibration, a range of 0.2 to 5 µg colchicine was used.

Experimental design

The two extracts were used in treatments, pure or diluted with distilled water (1:1) on the seeds and on the cauline apexes of *O. basilicum* 'Italiano Classico' and 'Aromat de Buzău' potted plantlets (Fig. 1). Three controls (C_0 = distilled H_2O, C_1 = 35% MeOH, C_2 = 70% MeOH) were prepared according to the methanol concentration in extracts and dilutions.

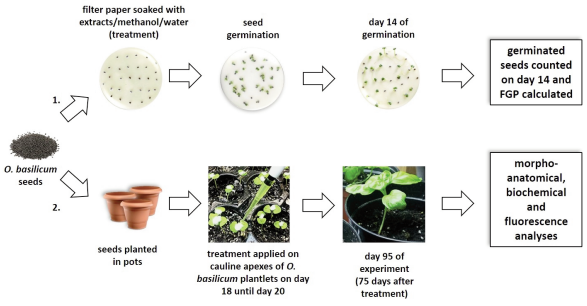

Fig. 1 - Treatment application strategies.

The method of treatment consisted of applying extracts on *O. basilicum* seeds distributed in Petri dishes. The seeds were previously sterilized with 3% H_2O_2 (5 minutes) and 5% NaClO (5 minutes). The treatment was applied by soaking the filter paper with 2 ml of pure or diluted extracts (20 seeds/Petri dish x 3 replicates for each variant of treatment). The seeds were kept in a thermostat at a temperature of 20±1°C and a 17h/7h light-dark cycle per day for 14 days and were watered once every two days. The experiment was performed in triplicate. Final Germination Percentage (FGP) was calculated according to the formula by Bezini *et al*. (2019):

$$FGP = n/N \times 100$$

where n represents the number of germinated seeds at the end of the germination test and N is the total number of seeds.

The other treatment application strategy was based on some methods used for polyploidy induction by colchicine treatment (Suzuki *et al*., 2005; Ye *et al*., 2010; Kushwah *et al*., 2018) and was carried out by applying the treatment on basil plantlets on day 18 after planting, by soaking cotton balls applied on the cauline apexes with 100 µl of extract per day for three consecutive days. The plantlets were kept in pots with Compo Sana soil (peat + perlite) at 21±2°C and 17 hours of light per day and were watered once every three days. The experiment lasted for 95 days (18 days of pre-cultivation and 75 days of monitoring of plant development) and the treated plantlets were analyzed at the end of the experiment (day 75 after treatment).

Morphological observations of treated plantlets

The macromorphological differences in leaves' shape and stem development that were observed between variants of treated *O. basilicum* plantlets of both varieties were photographed.

For micromorphology analysis, the plant material consisting of the first leaves was prepared in the following way: for each variant 2 first node leaves from 3 different plants were cut (1 cm^2) and transferred in successive acetone baths, critical-point dried with CO_2, and covered with a 10 nm gold (Au) layer using an EMS 550x sputter coater. Leaf samples of each surface were analyzed using scanning electron microscopy (Tescan Vega II SBH electron microscope from the Faculty of Biology, ''Alexandru Ioan Cuza" University of Iași, Romania) with VegaTC software. Stomata size (on both leaf surfaces) measurements were made using the ImageJ software for 5 stomata on 6 leaves. Stomata, tector, and glandular hairs densities were determined by counting their occurrences on 1 mm^2 of leaf surface on 6 leaves.

Anatomy analysis of treated plantlets

Epicotyl fragments from 3 plantlets of each treatment were selected for sectioning and examination. The plant material, previously kept in 70% ethanol was sectioned using a hand microtome and botanical razor. The sections were stained through the double staining method (with ruthenium red and iodine green), then placed on slides, observed through an optical microscope (Euromex bScope BS.1153-Pli) using a 10x (0.25) lens, and photographed using Xiaomi Mi A1 camera (12 MP, f/2.2, 26 mm (wide), ½.9", 1.25 µm, PDAF). Epicotyl circumference was measured using ImageJ and compared to control variants.

Photosynthetic pigments content assay of treated plantlets

The content of photosynthetic pigments in both *O. basilicum* varieties was analyzed according to Sumanta *et al.* (2014) using ethanol (Chemical Company S.A., Iași, Romania) as a solvent for leaf extracts (fresh leaves from 3 plants of each variant of treatment were weighted and milled with quartz sand, then dissolved in ethanol and filtered). The contents of chlorophyll a, chlorophyll b, and

carotenoid pigments were calculated with formulas given in the reference article. The data was processed in GraphPad Prism version 9.3.0.

Chlorophyll fluorescence measurement of treated plantlets

For the measurement and evaluation of chlorophyll fluorescence (indirect measurement of photochemical efficiency of photosystem II = ΦPSII and electron transport rate = ETR), 3 leaves were selected from different individuals from each treatment (from plantlets that were pre-exposed to dark conditions), and were analyzed using the Hansatech Ltd. PAM Fluorometer. The data was imported from the data system of the Hansatech device to Parview32 software and analyzed in GraphPad Prism version 9.3.0.

Statistical analyses

Statistical calculations and comparations were performed in GraphPad Prism version 9.3.0., using Two-way ANOVA and Tukey's multiple comparisons test for all morphological: stomata size (n= 3), stomata, tector and glandular hairs density (n= 3) on both leaf surfaces, anatomical: epicotyl circumference (n= 3), biochemical: chlorophyll a, b and carotenoids content (n= 3), and physiological indices: final germination percentage for treated seeds (n= 20) and photosystem II efficiency and electron transport rate (n = 3) for treated plantlets. Values in graphs were represented as means ± SEM, and for Tukey's multiple comparison tests, statistical significance is marked on graphs in the following way: **** = p<0.0001, *** = p<0.001, ** = p<0.01, * = p<0.05. Where no asterisk is present, results are not statistically significant.

3. Results

Colchicine content quantification in extracts

The detected colchicine (Fig. 2a) concentrations were 0.119±0.007 mg/ml colchicine in the bulb extract (Fig. 2b) and 0.286±0.015 mg/ml colchicine in the flower extract (Fig. 2c).

Germination test

The final germination percentage recorded on seeds treated with extracts revealed that the bulb extract and a concentration of 70% methanol impede the germination of all seeds, regardless of the variety of basil. *O. basilicum* 'Italiano Classico' seeds had a higher germination percentage than the 'Aromat de Buzău' cultivar under treatment, but the latter

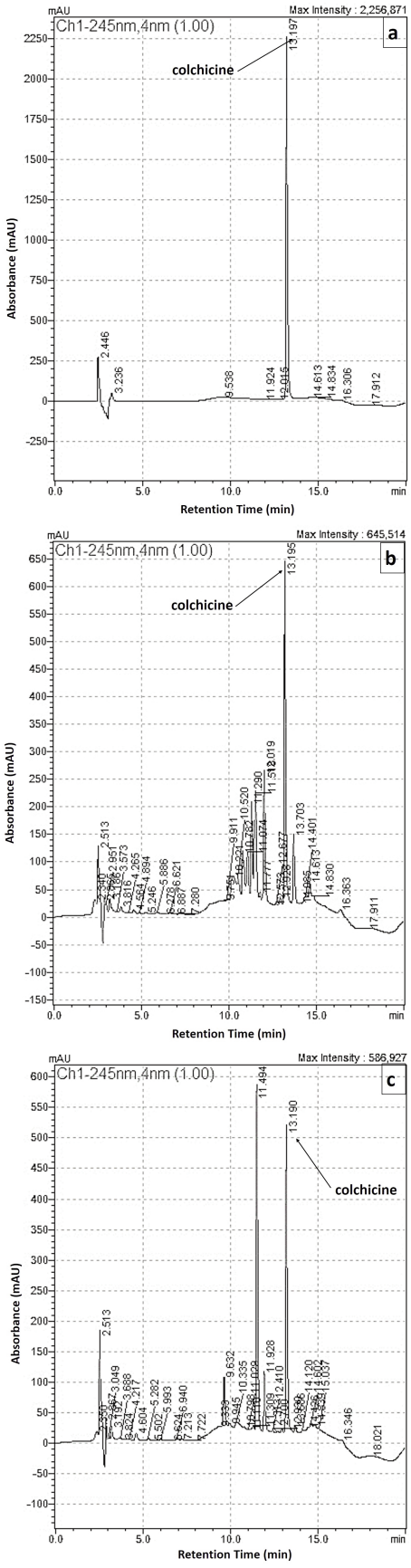

Fig. 2 - Chromatograms for colchicine standard (a) and for bulb (b) and flower (c) extracts.

showed a better germination percentage than its control variant when treated with diluted flower extracts (Table 1). The inhibitory effects of treatments (with extracts and methanol) effects were statistically significant (**** = p<0.0001) compared to C_0. Effects of *C. autumnale* extracts were also significant (p<0.0001), compared to their corresponding methanol control (1:1 BEt and 1:1 FEt to C_1; BEt and FEt to C_2). Other statistical comparisons are presented in supplementary materials (SM) Tables S1-S6.

Morphological observations of treated plantlets

Plantlet morphology. In 'Italiano Classico' basil plantlets, the epicotyl's growth was inhibited; the plantlets remained short (mainly when treated with bulb extracts), and their epicotyls had very short internodes (Fig. 3). The upper leaves were darker in color compared to the control and displayed unusual shapes: elongated or bifurcate, with straight or incomplete margins and diverted nervures (Fig. 4 c, d, e).

Treated 'Aromat de Buzău' plantlets in different stages of growth and development, with various stem heights and ramifications are reported in figure 5. In some plantlets, the main epicotyl's growth was either inhibited or necrotic, which strongly stimulated the ramifications, mostly when treated with pure

Table 1 - Final germination percentage of *O. basilicum* seeds under treatment with *C. autumnale* extracts

Test variant	Final germination percentage (%)	
	'Italiano Classico'	'Aromat de Buzău'
C_0 (H_2O)	80±1.92	68.89±7.78
C_1 (MeOH 35%)	70±11.55 ****	23.33±4.41 ****
1:1 BEt	0±0 ****	0±0 ****
1:1 FEt	40±18.93 ****	33.33±6.01 ****
C_2 (MeOH 70%)	0±0 ****	0±0 ****
BEt	0±0 ****	0±0 ****
FEt	0±0 ****	0±0 ****

BE= bulb extract; FE= flower extract; t= treatment; ****= p <0.0001.

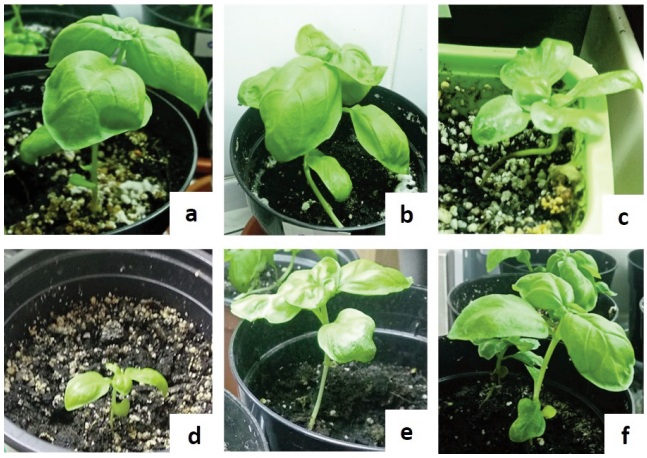

Fig. 3 - Morphology of *O. basilicum* 'Italiano Classico' plantlets treated with *C. autumnale* extracts. a) H_2O Control; b) MeOH; c) diluted (1:1) bulb extract; d) pure bulb extract; e) diluted (1:1) flower extract; f) flower extract.

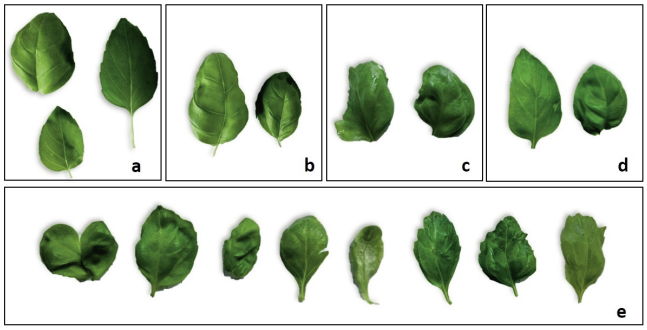

Fig. 4 - Morphology of *O. basilicum* 'Italiano Classico' leaves from the basal node of plantlets treated with *C. autumnale* extracts. a) H_2O Control; b) MeOH; c) diluted bulb extract; d) diluted flower extract; e) pure flower extract; leaves from plantlets treated with pure bulb extract could not be photographed because of insufficient plant material.

Fig. 5 - Morphology of *O. basilicum* 'Aromat de Buzău' plantlets treated with C. autumnale extracts. a) MeOH and H_2O Control; b) diluted (1:1) bulb extract; c) pure bulb extract; d) diluted (1:1) flower extract; e) pure flower extract.

flower extract. The upper leaves were much smaller than those from the control variant and displayed unusual shapes (Fig. 6c, d, e), with incomplete margins and deviated or bifurcated nervures (Fig. 6f).

Leaf micromorphology observations of treated plantlets. A typical basil leaf (of a plant treated with H_2O = C_0) observed through scanning electron microscopy has diacytic and anomocytic stomata, glandular and tector hairs (tector hairs appeared only

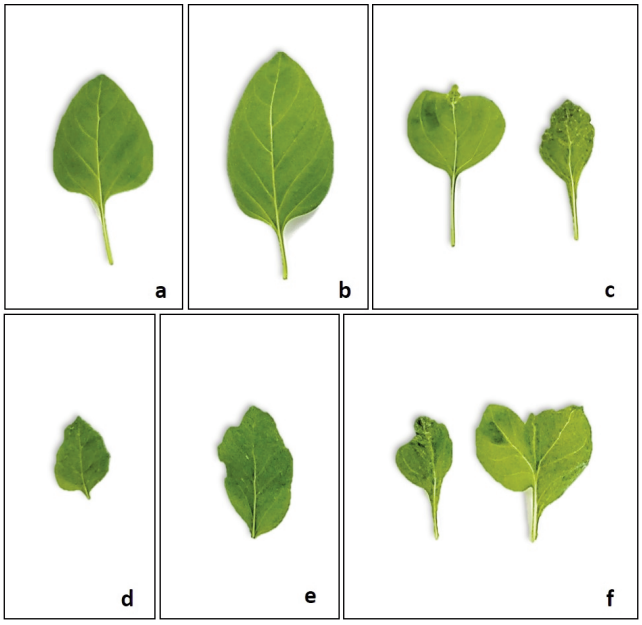

Fig. 6 - Morphology of *O. basilicum* 'Aromat de Buzău' leaves from the basal node of plantlets treated with *C. autumnale* extracts. a) H_2O Control; b) MeOH; c) diluted bulb extract; d) pure bulb extract; e) diluted flower extract; f) pure flower extract.

on the adaxial leaf surface), and epidermal cells with wavy sidewalls (Fig. 7a). The leaf epidermis of plantlets treated with methanol appears dehydrated and uneven (Fig. 7b), tector hair morphogenesis and elongation are stimulated, and glandular hairs with an altered shape of the glandular cells occur (Fig. 7c).

Leaves from 'Italiano Classico' plantlets treated with flower extracts showed heteromorphic or twin stomata (Fig. 7d), accentuated corrugations of the epidermal cell sidewalls (Fig. 7e), and abnormalities of the glandular hairs' shapes (Fig. 7f, g). Bulb extracts treatments only stimulated tector hairs morphogenesis.

The aspect of a typical leaf surface of 'Aromat de Buzău' basil plantlets is similar to 'Italiano Classico', but when treated with bulb extracts, epidermal cells appear elongated, the shape of the sidewalls is

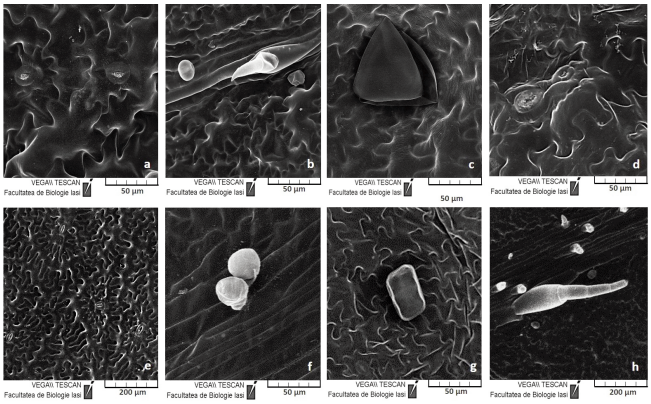

Fig. 7 - Abnormalities observed on *O. basilicum* leaf surfaces after treatment with methanol or *C. autumnale* extracts. a) typical aspect of an abaxial leaf surface; b) typical aspect of an adaxial leaf surface; c) glandular hair with an altered shape; d) twin stomata; e) epidermal cells with very wavy sidewalls; f) twin glandular hairs; g) glandular hair with a modified shape; h) tector hair morphogenesis and an elongated pluricellular tector hair.

altered, and the morphogenesis and elongation of tector hairs occur following the treatment with methanolic flower extracts (Fig. 7h).

Micromorphology measurements of treated plantlets. Stomata area and density. Stomata size significantly increased on both leaf surfaces of 'Italiano Classico' plantlets treated with diluted bulb extract ($p<0.05$ when compared to C_0, $p<0.0001$ when compared to C_1 on the inferior leaf surface, and $p<0.0001$ when compared to C_0 and C_1 on the superior leaf surface) and pure flower extract ($p<0.001$ when compared to C_0, $p<0.0001$ when compared to C_2 on the inferior leaf surface, and $p<0.0001$ when compared to C_0 and C_2 on the superior leaf surface) and only on the abaxial leaf surface when treated with diluted flower extract ($p<0.0001$ when compared to C_0 and C_1)(Fig. 8a), and their density increased in plantlets treated with pure bulb extract on the inferior leaf surface ($p<0.01$ when compared to C_0 and $p<0.05$ when compared to C_2) (Fig. 8c, d). The density of tector hairs increased significantly when plants were treated with 70% MeOH on the inferior leaf surface as a response to chemical stress.

Stomata size increased on both leaf surfaces of 'Aromat de Buzău' basil plantlets treated with pure bulb extract ($p<0.0001$ when compared to C_0 and C_2) and only on the adaxial leaf surface when the plantlets were treated with diluted flower extract ($p<0.0001$ when compared to C_0 and C_1) (Fig. 8a, b). Stomata density increased on both leaf surfaces of

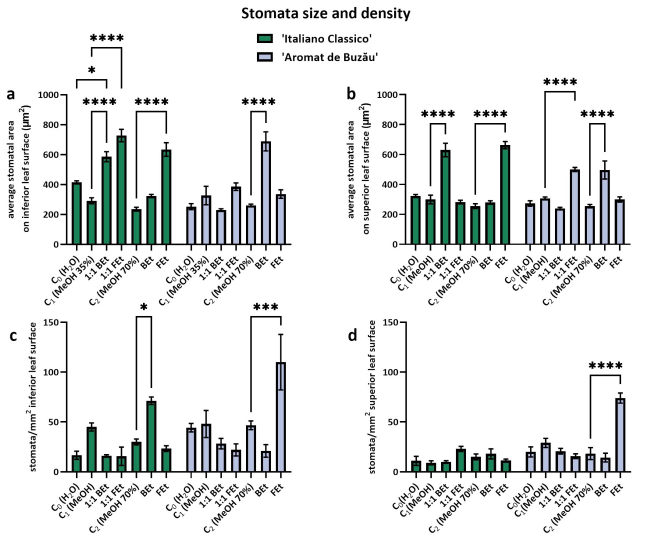

Fig. 8 - Average stomatal area on abaxial (a) and adaxial (b) leaf surfaces and density on abaxial (c) and adaxial (d) leaf surfaces of treated *O. basilicum* plantlets (BE = bulb extract; FE = flower extract; t = treatment; **** = p<0.0001; *** = p<0.001; ** = p<0.01; * = p<0.05).

plantlets treated with pure flower extract (p<0.001 when compared to C_0 and C_2) (Fig. 8c, d).

Glandular and tector hairs density. The density of tector hairs on the abaxial leaf surface increased in all 'Italiano Classico' basil plantlets, being highest in those treated with pure bulb extract and in the 'Aromat de Buzău' plantlets treated with flower extracts and 70% methanol, compared to C_0, where they did not appear. The treatment with extracts did not significantly impact either basil variety on the adaxial leaf surface. On the other hand, glandular hairs density did not change on either leaf surface of 'Italiano Classico' basil plantlets, but it was significantly higher on the adaxial leaf surface of 'Aromat de Buzău' plantlets treated with 70% methanol (SM - Fig. S1).

Epicotyl anatomy of treated plantlets

By examining the epicotyl anatomic structure of plantlets treated with *C. autumnale* extracts, some changes in the cross-section's outline, an uneven lignification of the xylem vessels, and heteromorphic cells in the cortical parenchyma and marrow, and an increase in the size and density of tector hairs were observed.

The contour of the cross-section through the epicotyl of a typical basil plantlet (control) is square, with 4 prominent ribs, which is a characteristic aspect of the species from the Lamiaceae family. The single-layered epidermis has numerous single-celled, long tector hairs and glandular hairs. The layers below the epidermis are differentiated into two subzones: the angular collenchyma and the parenchymal cortex, with 4-6 layers of rounded cells that leave small air gaps between them. The central cylinder comprises conductive tissues in which elements of the primary structure (generated from procambium) and the secondary structure (generated from cambium) are observed. Secondary xylem and libriform (sclerenchyma wood) fibers can be observed in approximately equal proportions. The marrow is parenchymal-meatic (Fig. 9, 10).

'Italiano Classico' basil plantlets treated with diluted bulb extract showed a weaker lignification of the xylem bundles and an elongated, rectangular contour of the epicotyl cross-section. The asymmetry of the epicotyl's shape in plantlets treated with flower extract is correlated with an increased number of cell layers on one side. Many cell layers indicate individuals with thicker epicotyls than the control. Also, more multicellular tector hairs were observed compared to the control (Fig. 9).

'Aromat de Buzău' basil plantlets treated with diluted bulb extract displayed a thinner epicotyl than the control variant, shorter tector hairs, and an aeriferous cavity in the pith, whereas the epicotyl of the plantlets treated with undiluted bulb extract displayed some heteromorphic cells in the parenchymal cortex, which led to an abnormal outline of the cross-

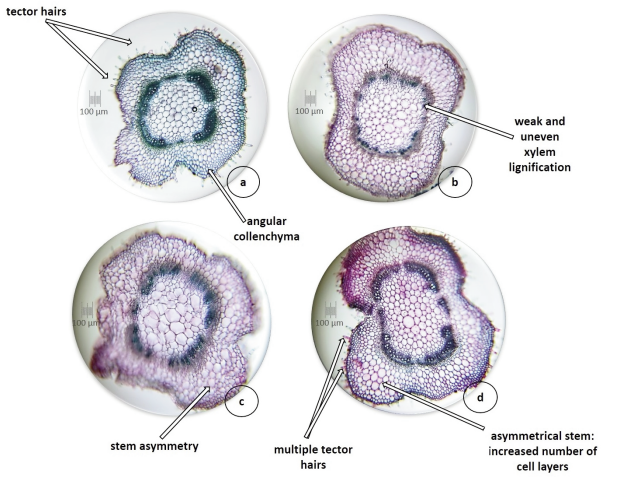

Fig. 9 - Epicotyl cross-section from *O. basilicum* 'Italiano Classico' plantlets, after treatment with *C. autumnale* diluted bulb (b), flower (c), and pure flower (d) extracts, compared to the control (a) (epicotyls of plantlets treated with pure bulb extract could not be analyzed because of insufficient plant material).

section. Similar modified characteristics were observed in 'Aromat de Buzău' basil plantlets treated with flower extract: when treated with a diluted flower extract, the epicotyl displayed an increase in the number of tector hairs, in contrast with undiluted flower extract treatment, where it decreased. In addition, a weak lignification of xylem vessels, heteromorphic parenchymal cells, and an overall outline thinning of the epicotyl were possible consequences of the treatment (Fig. 10).

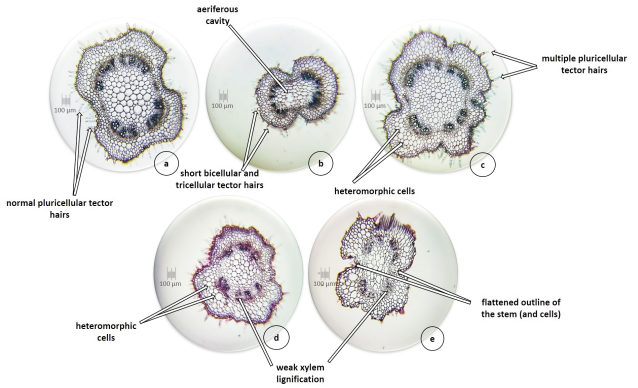

Fig. 10 - Epicotyl cross-section from *O. basilicum* 'Aromat de Buzău' plantlets, after treatment with *C. autumnale* diluted bulb (b), flower (c), and pure bulb (d) and flower (e) extracts, compared to the control (a).

Epicotyl circumference increased in 'Italiano Classico' basil plantlets when treated with pure flower extract ($p<0.01$ when compared to C_0 and $p<0.0001$ when compared to C_2). The plantlets treated with the bulb extract could not be analyzed due to the fragility of the plant material and some anomalies of the epicotyl (very short internodes). In contrast, 'Aromat de Buzău' basil had a much lower epicotyl circumference when treated with diluted bulb extract ($p<0.0001$ when compared to C_1 but not significant when compared to C_0) but higher in plantlets treated with pure bulb extract ($p<0.0001$ when compared to C_0 and C_2) (Fig. 11).

Photosynthetic pigments content of treated plantlets

The applied treatments did not affect chlorophyll a and carotenoid pigments content in both basil varieties. Chlorophyll b content was highest in plantlets treated with 70% methanol (SM Fig. S2).

Photosystem II efficiency and electron transport rate of treated plantlets

The photosystem II efficiency of both varieties and the electron transport rate (ETR) of 'Aromat de Buzău' basil plantlets were not significantly affected by the applied treatment. In 'Italiano Classico' basil, the electron transport rate was very high when plantlets were treated with 70% MeOH (C_2) (SM Fig. S3).

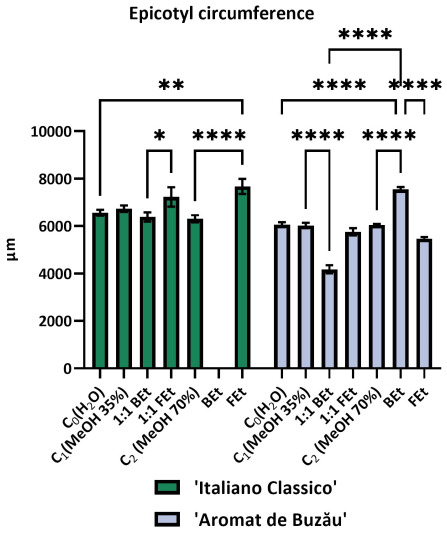

Fig. 11 - Epicotyl circumference of *O. basilicum* plantlets after treatment with *C. autumnale* extracts. BE= bulb extract; FE= flower extract; t= treatment; **** = $p<0.0001$; ** = $p<0.01$; * = $p<0.05$; epicotyls of plantlets treated with pure bulb extract could not be analyzed because of insufficient plant material.

4. Discussion and Conclusions

C. autumnale pure extracts inhibited the germination of seeds of both 'Italiano Classico' and 'Aromat de Buzău' basil varieties, probably because of the high concentration of methanol, but they could also have an inhibitory effect on germination and growth of weeds in plant cultures due to their allelopathic properties. More seeds of the 'Italiano Classico' basil variety germinated than 'Aromat de Buzău', but the latter withstood the treatment on cauline apex better than the Italian basil.

Morphological observations showed that the treatments have similar effects on leaf morphogenesis of both basil varieties, at macroscopical and microscopical levels, with some differences in the response of a particular basil variety to flower or bulb extract treatments. The epidermis dehydration, alterations in glandular hair shapes, and stimulated morphogenesis and elongation of tector hairs could appear because of the solvent used in treatments

rather than due to the applied treatments with *Colchicum autumnale* extracts. The increase in the number of tector hairs observed on the epicotyl epidermis, or their occurrence where they wouldn't usually appear (on the abaxial leaf surface), might indicate a defensive response to the chemical stress induced by methanol. In contrast, an increased density of secretory hairs on the leaf adaxial surface of 'Aromat de Buzău' plantlets treated with methanol might indicate a better capacity to protect themselves, as glandular hairs' main role is to protect the plant against external factors: herbivores and pathogens, extreme temperatures, excessive loss of water, and competing plants by secreting lipophilic substances that act as repellents or poisons (Hazzoumi *et al*., 2020). The observations on the anatomy of the plantlet epicotyl confirmed the information from the literature that colchicine affects xylem differentiation and thickening because of the disappearance of wall microtubules (Pickett-Heaps, 1967), which might lead to abnormal morphogenesis of the mature plants' stem that develops in a plagiotropic position. Another interesting observation is that heteromorphic cells induced by the treatment with *C. autumnale* extracts that appear in the leaf structure and in the epicotyl might indicate the presence of mixoploid tissues, which could also explain the abnormal leaf shapes and the epicotyl development anomalies.

The treatment with *C. autumnale* extracts did not affect biochemical and physiological indices of the photosynthetic apparatus of treated basil plantlets. Only treatment with methanol might influence photosynthesis due to its impact on chlorophyll b content and electron transport rate.

These indices might be suitable for potential polyploid evaluation and selection from plants subjected to similar treatments. If its toxicity levels are adequately managed and exploited, *Colchicum autumnale* could have a great potential for becoming a cost-effective source of allelopathic compounds for crop pest control or herbicide production, besides its importance as a source of anti-mitotic agents for artificial polyploidy induction in plants.

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Biological effects of some *Colchicum autumnale* L. extracts on tissue development of two varieties of *Ocimum basilicum* L.

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Abstract: *Colchicum autumnale* L. is a perennial herb from the Colchicaceae family with an unusual life cycle, and it is characterized by an underground corm and hypogynous flowers that appear in autumn. Its medicinal importance is represented by its primary alkaloid, colchicine, which has been studied for its anti-inflammatory and antimitotic properties and used in the treatment of some diseases and artificial polyploidy induction in plants. This study aims to determine and evaluate the biological effects induced by treatment with *C. autumnale* extracts on tissue development in test plants, represented by two *Ocimum basilicum* L. varieties: 'Italiano Classico' and 'Aromat de Buzău'. Morpho-anatomical observations and measurements and photosynthetic pigments analyses were employed. Results show unusual shapes of leaves, differences in stomata size and density, and heteromorphic cells in leaves and epicotyl's structure in both studied varieties of basil treated with *C. autumnale* extracts.

Table S1 - Tukey's multiple comparisons test results for *O. basilicum* seed germination (FGP)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
C_0 (H_2O): 'Genovese' vs. C_0 (H_2O): 'Aromat de Buzău'	11.11	7.051 to 15.17	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. C_1 (MeOH 35%): 'Genovese'	10	5.941 to 14.06	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. C_1 (MeOH 35%): 'Aromat de Buzău'	56.67	52.61 to 60.73	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. 1:1 BEt: 'Genovese'	80	75.94 to 84.06	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. 1:1 BEt: 'Aromat de Buzău'	80	75.94 to 84.06	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. 1:1 FEt: 'Genovese'	40	35.94 to 44.06	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. 1:1 FEt: 'Aromat de Buzău'	46.67	42.61 to 50.73	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. C_2 (MeOH 70%): 'Genovese'	80	75.94 to 84.06	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. C_2 (MeOH 70%): 'Aromat de Buzău'	80	75.94 to 84.06	Yes	****	<0.0001
C_0 (H_2O):'Genovese' vs. BEt: 'Genovese'	80	75.94 to 84.06	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. BEt: 'Aromat de Buzău'	80	75.94 to 84.06	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. FEt: 'Genovese'	80	75.94 to 84.06	Yes	****	<0.0001
C_0 (H_2O): 'Genovese' vs. FEt: 'Aromat de Buzău'	80	75.94 to 84.06	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. C_1 (MeOH 35%):' Genovese'	-1.11	-5.169 to 2.949	No	NS	0.9997
C_0 (H_2O): 'Aromat de Buzău' vs. C_1 (MeOH 35%): 'Aromat de Buzău'	45.56	41.50 to 49.62	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. 1:1 BEt: 'Genovese'	68.89	64.83 to 72.95	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. 1:1 BEt: 'Aromat de Buzău'	68.89	64.83 to 72.95	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. 1:1 FEt: 'Genovese'	28.89	24.83 to 32.95	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. 1:1 FEt: 'Aromat de Buzău'	35.56	31.50 to 39.62	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. C_2 (MeOH 70%): 'Genovese'	68.89	64.83 to 72.95	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. C_2 (MeOH 70%): 'Aromat de Buzău'	68.89	64.83 to 72.95	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. BEt: 'Genovese'	68.89	64.83 to 72.95	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. BEt: 'Aromat de Buzău'	68.89	64.83 to 72.95	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. FEt: 'Genovese'	68.89	64.83 to 72.95	Yes	****	<0.0001
C_0 (H_2O): 'Aromat de Buzău' vs. FEt: 'Aromat de Buzău'	68.89	64.83 to 72.95	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. C_1 (MeOH 35%): 'Aromat de Buzău'	46.67	42.61 to 50.73	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. 1:1 BEt: 'Genovese'	70	65.94 to 74.06	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. 1:1 BEt: 'Aromat de Buzău'	70	65.94 to 74.06	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. 1:1 FEt: 'Genovese'	30	25.94 to 34.06	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. 1:1 FEt: 'Aromat de Buzău'	36.67	32.61 to 40.73	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. C_2 (MeOH 70%): 'Genovese'	70	65.94 to 74.06	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. C_2 (MeOH 70%): 'Aromat de Buzău'	70	65.94 to 74.06	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. BEt: 'Genovese'	70	65.94 to 74.06	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. BEt: 'Aromat de Buzău'	70	65.94 to 74.06	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. FEt: 'Genovese'	70	65.94 to 74.06	Yes	****	<0.0001
C_1 (MeOH 35%): 'Genovese' vs. FEt: 'Aromat de Buzău'	70	65.94 to 74.06	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. 1:1 BEt: 'Genovese'	23.33	19.27 to 27.39	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. 1:1 BEt: 'Aromat de Buzău'	23.33	19.27 to 27.39	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. 1:1 FEt: 'Genovese'	-16.67	-20.73 to -12.61	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. 1:1 FEt: 'Aromat de Buzău'	-10	-14.06 to -5.941	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. C_2 (MeOH 70%): 'Genovese'	23.33	19.27 to 27.39	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. C_2 (MeOH 70%): 'Aromat de	23.33	19.27 to 27.39	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. BEt: 'Genovese'	23.33	19.27 to 27.39	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. BEt: 'Aromat de Buzău'	23.33	19.27 to 27.39	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. FEt: 'Genovese'	23.33	19.27 to 27.39	Yes	****	<0.0001
C_1 (MeOH 35%): 'Aromat de Buzău' vs. FEt: 'Aromat de Buzău'	23.33	19.27 to 27.39	Yes	****	<0.0001

BE= bulb extract; FE= flower extract; t= treatment.

To be continued....

Table S1 - Tukey's multiple comparisons test results for *O. basilicum* seed germination (FGP)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
1:1 BEt: 'Genovese' vs. 1:1 BEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Genovese' vs. 1:1 FEt: 'Genovese'	-40	-44.06 to -35.94	Yes	****	<0.0001
1:1 BEt: 'Genovese' vs. 1:1 FEt: 'Aromat de Buzău'	-33.33	-37.39 to -29.27	Yes	****	<0.0001
1:1 BEt: 'Genovese' vs. C_2 (MeOH 70%): 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Genovese' vs. C_2 (MeOH 70%): 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Genovese' vs. BEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Genovese' vs. BEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Genovese' vs. FEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Genovese' vs. FEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Aromat de Buzău' vs. 1:1 FEt: 'Genovese'	-40	-44.06 to -35.94	Yes	****	<0.0001
1:1 BEt: 'Aromat de Buzău' vs. 1:1 FEt: 'Aromat de Buzău'	-33.33	-37.39 to -29.27	Yes	****	<0.0001
1:1 BEt: 'Aromat de Buzău' vs. C_2 (MeOH 70%): 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Aromat de Buzău' vs. C_2 (MeOH 70%): 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Aromat de Buzău' vs. BEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Aromat de Buzău' vs. BEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Aromat de Buzău' vs. FEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 BEt: 'Aromat de Buzău' vs. FEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
1:1 FEt: 'Genovese' vs. 1:1 FEt: 'Aromat de Buzău'	6.67	2.611 to 10.73	Yes	****	<0.0001
1:1 FEt: 'Genovese' vs. C_2 (MeOH 70%): 'Genovese'	40	35.94 to 44.06	Yes	****	<0.0001
1:1 FEt: 'Genovese' vs. C_2 (MeOH 70%): 'Aromat de Buzău'	40	35.94 to 44.06	Yes	****	<0.0001
1:1 FEt: 'Genovese' vs. BEt: 'Genovese'	40	35.94 to 44.06	Yes	****	<0.0001
1:1 FEt: 'Genovese' vs. BEt: 'Aromat de Buzău'	40	35.94 to 44.06	Yes	****	<0.0001
1:1 FEt: 'Genovese' vs. FEt: 'Genovese'	40	35.94 to 44.06	Yes	****	<0.0001
1:1 FEt: 'Genovese' vs. FEt: 'Aromat de Buzău'	40	35.94 to 44.06	Yes	****	<0.0001
1:1 FEt: 'Aromat de Buzău' vs. C_2 (MeOH 70%): 'Genovese'	33.33	29.27 to 37.39	Yes	****	<0.0001
1:1 FEt: 'Aromat de Buzău' vs. C_2 (MeOH 70%): 'Aromat de Buzău'	33.33	29.27 to 37.39	Yes	****	<0.0001
1:1 FEt: 'Aromat de Buzău' vs. BEt: 'Genovese'	33.33	29.27 to 37.39	Yes	****	<0.0001
1:1 FEt: 'Aromat de Buzău' vs. BEt: 'Aromat de Buzău'	33.33	29.27 to 37.39	Yes	****	<0.0001
1:1 FEt: 'Aromat de Buzău' vs. FEt: 'Genovese'	33.33	29.27 to 37.39	Yes	****	<0.0001
1:1 FEt: 'Aromat de Buzău' vs. FEt: 'Aromat de Buzău'	33.33	29.27 to 37.39	Yes	****	<0.0001
C_2 (MeOH 70%): 'Genovese' vs. C_2 (MeOH 70%): 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
C_2 (MeOH 70%): 'Genovese' vs. BEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
C_2 (MeOH 70%): 'Genovese' vs. BEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
C_2 (MeOH 70%): 'Genovese' vs. FEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
C_2 (MeOH 70%): 'Genovese' vs. FEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
C_2 (MeOH 70%): 'Aromat de Buzău' vs. BEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
C_2 (MeOH 70%): 'Aromat de Buzău' vs. BEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
C_2 (MeOH 70%): 'Aromat de Buzău' vs. FEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
C_2 (MeOH 70%): 'Aromat de Buzău' vs. FEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
BEt: 'Genovese' vs. BEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
BEt: 'Genovese' vs. FEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
BEt: 'Genovese' vs. FEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
BEt:' Aromat de Buzău' vs. FEt: 'Genovese'	0	-4.059 to 4.059	No	NS	>0.9999
BEt: 'Aromat de Buzău' vs. FEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999
FEt: 'Genovese' vs. FEt: 'Aromat de Buzău'	0	-4.059 to 4.059	No	NS	>0.9999

BE= bulb extract; FE= flower extract; t= treatment.

Table S2 - Tukey's multiple comparisons test results for epicotyl circumference measurement of *O. basilicum* plantlets

Treatment	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
'Italiano Classico'					
$C_0(H_2O)$ vs. C_1(MeOH 35%)	-176.3	-922.7 to 570.0	No	NS	0.9868
$C_0(H_2O)$ vs. 1:1 BEt	179.7	-566.7 to 926.0	No	NS	0.9855
$C_0(H_2O)$ vs. 1:1 FEt	-673.3	-1420 to 72.99	No	NS	0.0962
$C_0(H_2O)$ vs. C_2 (MeOH 70%)	259.7	-486.7 to 1006	No	NS	0.9165
$C_0(H_2O)$ vs. BEt	6567	5820 to 7313	Yes	****	<0.0001
$C_0(H_2O)$ vs. FEt	-1114	-1860 to -367.3	Yes	**	0.0012
C_1(MeOH 35%) vs. 1:1 BEt	356	-390.3 to 1102	No	NS	0.7239
C_1(MeOH 35%) vs. 1:1 FEt	-497	-1243 to 249.3	No	NS	0.3635
C_1(MeOH 35%) vs. C_2 (MeOH 70%)	436	-310.3 to 1182	No	NS	0.5139
1:1 BEt vs. 1:1 FEt	-853	-1599 to -106.7	Yes	*	0.0179
1:1 BEt vs. BEt	6387	5641 to 7133	Yes	****	<0.0001
1:1 BEt vs. FEt	-1293	-2040 to -547.0	Yes	***	0.0002
1:1 FEt vs. BEt	7240	6494 to 7986	Yes	****	<0.0001
1:1 FEt vs. FEt	-440.3	-1187 to 306.0	No	NS	0.5025
C_2 (MeOH 70%) vs. BEt	6307	5561 to 7053	Yes	****	<0.0001
C_2 (MeOH 70%) vs. FEt	-1373	-2120 to -627.0	Yes	****	<0.0001
BEt vs. FEt	-7680	-8427 to -6934	Yes	****	<0.0001
'Aromat de Buzău'					
$C_0(H_2O)$ vs. C_1(MeOH 35%)	35.67	-710.7 to 782.0	No	NS	>0.9999
$C_0(H_2O)$ vs. 1:1 BEt	1879	1132 to 2625	Yes	****	<0.0001
$C_0(H_2O)$ vs. 1:1 FEt	302.7	-443.7 to 1049	No	NS	0.8443
$C_0(H_2O)$ vs. C_2 (MeOH 70%)	17.67	-728.7 to 764.0	No	NS	>0.9999
$C_0(H_2O)$ vs. BEt	-1497	-2243 to -750.3	Yes	****	<0.0001
$C_0(H_2O)$ vs. FEt	586.7	-159.7 to 1333	No	NS	0.1949
C_1(MeOH 35%) vs. 1:1 BEt	1843	1097 to 2589	Yes	****	<0.0001
C_1(MeOH 35%) vs. 1:1 FEt	267	-479.3 to 1013	No	NS	0.906
C_1(MeOH 35%) vs. C_2 (MeOH 70%)	-18	-764.3 to 728.3	No	NS	>0.9999
1:1 BEt vs. 1:1 FEt	-1576	-2322 to -829.7	Yes	****	<0.0001
1:1 BEt vs. BEt	-3375	-4122 to -2629	Yes	****	<0.0001
1:1 BEt vs. FEt	-1292	-2038 to -545.7	Yes	***	0.0002
1:1 FEt vs. BEt	-1799	-2546 to -1053	Yes	****	<0.0001
1:1 FEt vs. FEt	284	-462.3 to 1030	No	NS	0.8787
C_2 (MeOH 70%) vs. BEt	-1514	-2261 to -768.0	Yes	****	<0.0001
C_2 (MeOH 70%) vs. FEt	569	-177.3 to 1315	No	NS	0.2225
BEt vs. FEt	2083	1337 to 2830	Yes	****	<0.0001

BE= bulb extract; FE= flower extract; t= treatment.

Table S3 - Tukey's multiple comparisons test results for the stomatal area measurement in treated O. basilicum plantlets

Treatments	Inferior leaf surface					Superior leaf surface				
	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
'Italiano Classico'										
C_0 (H_2O) vs. C_1 (MeOH 35%)	122.8	-24.77 to 270.3	No	NS	0.1615	22.84	-85.71 to 131.4	No	NS	0.9947
C_0 (H_2O) vs. 1:1 BEt	-171.6	-319.1 to -24.03	Yes	*	0.0132	-307.7	-416.3 to -199.2	Yes	****	<0.0001
C_0 (H_2O) vs. 1:1 FEt	-313.2	-460.7 to -165.6	Yes	****	<0.0001	40.38	-68.17 to 148.9	No	NS	0.9107
C_0 (H_2O) vs. C_2 (MeOH 70%)	179.3	31.77 to 326.9	Yes	**	0.0083	67.22	-41.33 to 175.8	No	NS	0.4869
C_0 (H_2O) vs. BEt	91.26	-56.29 to 238.8	No	NS	0.4884	43.12	-65.43 to 151.7	No	NS	0.882
C_0 (H_2O) vs. FEt	-219.9	-367.4 to -72.35	Yes	***	0.0006	-340.2	-448.8 to -231.7	Yes	****	<0.0001
C_1 (MeOH 35%) vs. 1:1 BEt	-294.4	-441.9 to -146.8	Yes	****	<0.0001	-330.6	-439.1 to -222.0	Yes	****	<0.0001
C_1 (MeOH 35%) vs. 1:1 FEt	-435.9	-583.5 to -288.4	Yes	****	<0.0001	17.54	-91.01 to 126.1	No	NS	0.9988
C_1 (MeOH 35%) vs. C_2 (MeOH 70%)	56.54	-91.01 to 204.1	No	NS	0.8986	44.38	-64.17 to 152.9	No	NS	0.8672
1:1 BEt vs. 1:1 FEt	-141.6	-289.1 to 5.967	No	NS	0.0677	348.1	239.6 to 456.7	Yes	****	<0.0001
1:1 BEt vs. BEt	262.8	115.3 to 410.4	Yes	****	<0.0001	350.9	242.3 to 459.4	Yes	****	<0.0001
1:1 BEt vs. FEt	-48.32	-195.9 to 99.23	No	NS	0.9498	-32.48	-141.0 to 76.07	No	NS	0.9674
1:1 FEt vs. BEt	404.4	256.9 to 552.0	Yes	****	<0.0001	2.74	-105.8 to 111.3	No	NS	>0.9999
1:1 FEt vs. FEt	93.26	-54.29 to 240.8	No	NS	0.4621	-380.6	-489.1 to -272.1	Yes	****	<0.0001
C_2 (MeOH 70%) vs. BEt	-88.06	-235.6 to 59.49	No	NS	0.5311	-24.1	-132.6 to 84.45	No	NS	0.993
C_2 (MeOH 70%) vs. FEt	-399.2	-546.8 to -251.7	Yes	****	<0.0001	-407.4	-516.0 to -298.9	Yes	****	<0.0001
BEt vs. FEt	-311.2	-458.7 to -163.6	Yes	****	<0.0001	-383.3	-491.9 to -274.8	Yes	****	<0.0001
'Aromat de Buzău'										
C_0 (H_2O) vs. C_1 (MeOH 35%)	-74.12	-221.7 to 73.43	No	NS	0.7163	-33.9	-142.4 to 74.65	No	NS	0.9599
C_0 (H_2O) vs. 1:1 BEt	22.12	-125.4 to 169.7	No	NS	0.9992	33.08	-75.47 to 141.6	No	NS	0.9644
C_0 (H_2O) vs. 1:1 FEt	-133.5	-281.0 to 14.05	No	NS	0.1	-227	-335.5 to -118.4	Yes	****	<0.0001
C_0 (H_2O) vs. C_2 (MeOH 70%)	-8.54	-156.1 to 139.0	No	NS	>0.9999	18.12	-90.43 to 126.7	No	NS	0.9985
C_0 (H_2O) vs. BEt	-436.3	-583.8 to -288.7	Yes	****	<0.0001	-223.8	-332.3 to -115.2	Yes	****	<0.0001
C_0 (H_2O) vs. FEt	-84.14	-231.7 to 63.41	No	NS	0.5841	-26.7	-135.2 to 81.85	No	NS	0.9879
C_1 (MeOH 35%) vs. 1:1 BEt	96.24	-51.31 to 243.8	No	NS	0.4239	66.98	-41.57 to 175.5	No	NS	0.4912
C_1 (MeOH 35%) vs. 1:1 FEt	-59.38	-206.9 to 88.17	No	NS	0.8755	-193.1	-301.6 to -84.53	Yes	****	<0.0001
C_1 (MeOH 35%) vs. C_2 (MeOH 70%)	65.58	-81.97 to 213.1	No	NS	0.8159	52.02	-56.53 to 160.6	No	NS	0.7581
1:1 BEt vs. 1:1 FEt	-155.6	-303.2 to -8.073	Yes	*	0.0326	-260.1	-368.6 to -151.5	Yes	****	<0.0001
1:1 BEt vs. BEt	-458.4	-605.9 to -310.9	Yes	****	<0.0001	-256.8	-365.4 to -148.3	Yes	****	<0.0001
1:1 BEt vs. FEt	-106.3	-253.8 to 41.29	No	NS	0.3065	-59.78	-168.3 to 48.77	No	NS	0.623
1:1 FEt vs. BEt	-302.8	-450.3 to -155.2	Yes	****	<0.0001	3.22	-105.3 to 111.8	No	NS	>0.9999
1:1 FEt vs. FEt	49.36	-98.19 to 196.9	No	NS	0.9446	200.3	91.73 to 308.8	Yes	****	<0.0001
C_2 (MeOH 70%) vs. BEt	-427.7	-575.3 to -280.2	Yes	****	<0.0001	-241.9	-350.4 to -133.3	Yes	****	<0.0001
C_2 (MeOH 70%) vs. FEt	-75.6	-223.1 to 71.95	No	NS	0.6975	-44.82	-153.4 to 63.73	No	NS	0.8619
BEt vs. FEt	352.1	204.6 to 499.7	Yes	****	<0.0001	197.1	88.51 to 305.6	Yes	****	<0.0001

BE= bulb extract; FE= flower extract; t= treatment.

Table S4 - Tukey's multiple comparisons test results for the stomatal area measurement in treated *O. basilicum* plantlets

Treatmemts	Inferior leaf surface					Superior leaf surface				
	Mean Diff.	95.00% CI of diff.	Below threshold?	Sum-mary	Adjusted P Value	Mean Diff.	95.00% CI of diff.	Below threshold?	Sum-mary	Adjusted P Value
Italiano Classico'										
C_0 (H2O) vs. C_1 (MeOH 35%)	-28.33	-68.90 to 12.24	No	NS	0.311	2	-12.31 to 16.31	No	NS	0.9992
C_0 (H_2O) vs. 1:1 BEt	0.6667	-39.90 to 41.24	No	NS	>0.9999	1	-13.31 to 15.31	No	NS	>0.9999
C_0 (H_2O) vs. 1:1 FEt	1	-39.57 to 41.57	No	NS	>0.9999	-12	-26.31 to 2.313	No	NS	0.1434
C_0 (H_2O) vs. C_2 (MeOH 70%)	-13.33	-53.90 to 27.24	No	NS	0.935	-4	-18.31 to 10.31	No	NS	0.9695
C_0 (H_2O) vs. BEt	-54.67	-95.24 to -	Yes	**	0.0038	-7	-21.31 to 7.313	No	NS	0.7012
C_0 (H_2O) vs. FEt	-6.667	-47.24 to 33.90	No	NS	0.9981	-0.3333	-14.65 to 13.98	No	NS	>0.9999
C_1 (MeOH 35%) vs. 1:1 BEt	29	-11.57 to 69.57	No	NS	0.2863	-1	-15.31 to 13.31	No	NS	>0.9999
C_1 (MeOH 35%) vs. 1:1 FEt	29.33	-11.24 to 69.90	No	NS	0.2745	-14	-28.31 to	No	NS	0.0581
C_1 (MeOH 35%) vs. C2 (MeOH 70%)	15	-25.57 to 55.57	No	NS	0.8921	-6	-20.31 to 8.313	No	NS	0.8236
1:1 BEt vs. 1:1 FEt	0.3333	-40.24 to 40.90	No	NS	>0.9999	-13	-27.31 to 1.313	No	NS	0.0925
1:1 BEt vs. BEt	-55.33	-95.90 to -	Yes	**	0.0033	-8	-22.31 to 6.313	No	NS	0.5638
1:1 BEt vs. FEt	-7.333	-47.90 to 33.24	No	NS	0.9968	-1.333	-15.65 to 12.98	No	NS	>0.9999
1:1 FEt vs. BEt	-55.67	-96.24 to -	Yes	**	0.0031	5	-9.313 to 19.31	No	NS	0.9151
1:1 FEt vs. FEt	-7.667	-48.24 to 32.90	No	NS	0.996	11.67	-2.647 to 25.98	No	NS	0.1648
C_2 (MeOH 70%) vs. BEt	-41.33	-81.90 to -	Yes	*	0.0438	-3	-17.31 to 11.31	No	NS	0.993
C_2 (MeOH 70%) vs. FEt	6.667	-33.90 to 47.24	No	NS	0.9981	3.667	-10.65 to 17.98	No	NS	0.9801
BEt vs. FEt	48	7.432 to 88.57	Yes	*	0.0132	6.667	-7.647 to 20.98	No	NS	0.7446
'Aromat de Buzău'										
C_0 (H2O) vs. C_1 (MeOH 35%)	-3.667	-44.24 to 36.90	No	NS	>0.9999	-9	-23.31 to 5.313	No	NS	0.4291
C_0 (H_2O) vs. 1:1 BEt	16	-24.57 to 56.57	No	NS	0.8602	-0.6667	-14.98 to 13.65	No	NS	>0.9999
C_0 (H_2O) vs. 1:1 FEt	22.33	-18.24 to 62.90	No	NS	0.5805	4.333	-9.980 to 18.65	No	NS	0.9553
C_0 (H_2O) vs. C_2 (MeOH 70%)	-2.333	-42.90 to 38.24	No	NS	>0.9999	1.667	-12.65 to 15.98	No	NS	0.9997
C_0 (H_2O) vs. BEt	23.33	-17.24 to 63.90	No	NS	0.5316	5.667	-8.647 to 19.98	No	NS	0.8581
C_0 (H_2O) vs. FEt	-65.67	-106.2 to -	Yes	***	0.0004	-54	-68.31 to -	Yes	****	<0.0001
C_1 (MeOH 35%) vs. 1:1 BEt	19.67	-20.90 to 60.24	No	NS	0.7093	8.333	-5.980 to 22.65	No	NS	0.5178
C_1 (MeOH 35%) vs. 1:1 FEt	26	-14.57 to 66.57	No	NS	0.4071	13.33	-0.9800 to	No	NS	0.0795
C_1(MeOH 35%) vs. C_2 (MeOH 70%)	1.333	-39.24 to 41.90	No	NS	>0.9999	10.67	-3.647 to 24.98	No	NS	0.2442
1:1 BEt vs. 1:1 FEt	6.333	-34.24 to 46.90	No	NS	0.9986	5	-9.313 to 19.31	No	NS	0.9151
1:1 BEt vs. BEt	7.333	-33.24 to 47.90	No	NS	0.9968	6.333	-7.980 to 20.65	No	NS	0.7856
1:1 BEt vs. FEt	-81.67	-122.2 to -	Yes	****	<0.0001	-53.33	-67.65 to -	Yes	****	<0.0001
1:1 FEt vs. BEt	1	-39.57 to 41.57	No	NS	>0.9999	1.333	-12.98 to 15.65	No	NS	>0.9999
1:1 FEt vs. FEt	-88	-128.6 to -	Yes	****	<0.0001	-58.33	-72.65 to -	Yes	****	<0.0001
C_2 (MeOH 70%) vs. BEt	25.67	-14.90 to 66.24	No	NS	0.4219	4	-10.31 to 18.31	No	NS	0.9695
C_2 (MeOH 70%) vs. FEt	-63.33	-103.9 to -	Yes	***	0.0007	-55.67	-69.98 to -	Yes	****	<0.0001
BEt vs. FEt	-89	-129.6 to -	Yes	****	<0.0001	-59.67	-73.98 to -	Yes	****	<0.0001

BE= bulb extract; FE= flower extract; t= treatment.

Table S5 - Tukey's multiple comparisons test results for tector hairs density analysis on the leaf surfaces of treated *O. basilicum* plantlets

Treatments	Inferior leaf surface					Superior leaf surface				
	Mean Diff.	95.00% CI of diff.	Below threshold?	Sum-mary	Adjusted P Value	Mean Diff.	95.00% CI of diff.	Below threshold?	Sum-mary	Adjusted P Value
'Italiano Classico'										
$C_0(H_2O)$ vs. C_1(MeOH 35%)	-1.667	-4.861 to 1.527	No	NS	0.6376	5	-6.340 to 16.34	No	NS	0.7882
$C_0(H_2O)$ vs. 1:1 BEt	-1.667	-4.861 to 1.527	No	NS	0.6376	6	-5.340 to 17.34	No	NS	0.6231
$C_0(H_2O)$ vs. 1:1 FEt	-2	-5.194 to 1.194	No	NS	0.4339	6	-5.340 to 17.34	No	NS	0.6231
$C_0(H_2O)$ vs. C_2 (MeOH 70%)	-0.6667	-3.861 to 2.527	No	NS	0.9931	4.667	-6.673 to 16.01	No	NS	0.8354
$C_0(H_2O)$ vs. BEt	-4	-7.194 to -	Yes	**	0.0078	2	-9.340 to 13.34	No	NS	0.9972
$C_0(H_2O)$ vs. FEt	-0.6667	-3.861 to 2.527	No	NS	0.9931	-0.3333	-11.67 to 11.01	No	NS	>0.9999
C_1(MeOH 35%) vs. 1:1 BEt	0	-3.194 to 3.194	No	NS	>0.9999	1	-10.34 to 12.34	No	NS	>0.9999
C_1(MeOH 35%) vs. 1:1 FEt	-0.3333	-3.527 to 2.861	No	NS	0.9999	1	-10.34 to 12.34	No	NS	>0.9999
C_1(MeOH 35%) vs. C_2 (MeOH 70%)	1	-2.194 to 4.194	No	NS	0.9478	-0.3333	-11.67 to 11.01	No	NS	>0.9999
1:1 BEt vs. 1:1 FEt	-0.3333	-3.527 to 2.861	No	NS	0.9999	0	-11.34 to 11.34	No	NS	>0.9999
1:1 BEt vs. BEt	-2.333	-5.527 to	No	NS	0.264	-4	-15.34 to 7.340	No	NS	0.9115
1:1 BEt vs. FEt	1	-2.194 to 4.194	No	NS	0.9478	-6.333	-17.67 to 5.007	No	NS	0.5646
1:1 FEt vs. BEt	-2	-5.194 to 1.194	No	NS	0.4339	-4	-15.34 to 7.340	No	NS	0.9115
1:1 FEt vs. FEt	1.333	-1.861 to 4.527	No	NS	0.8263	-6.333	-17.67 to 5.007	No	NS	0.5646
C_2 (MeOH 70%) vs. BEt	-3.333	-6.527 to -	Yes	*	0.0368	-2.667	-14.01 to 8.673	No	NS	0.9871
C_2 (MeOH 70%) vs. FEt	4.441e-	-3.194 to 3.194	No	NS	>0.9999	-5	-16.34 to 6.340	No	NS	0.7882
BEt vs. FEt	3.333	0.1392 to	Yes	*	0.0368	-2.333	-13.67 to 9.007	No	NS	0.9936
'Aromat de Buzău'										
$C_0(H_2O)$ vs. C_1(MeOH 35%)	0	-3.194 to 3.194	No	NS	>0.9999	-12.33	-23.67 to -	Yes	*	0.0269
$C_0(H_2O)$ vs. 1:1 BEt	0	-3.194 to 3.194	No	NS	>0.9999	-7.333	-18.67 to 4.007	No	NS	0.3968
$C_0(H_2O)$ vs. 1:1 FEt	-0.6667	-3.861 to 2.527	No	NS	0.9931	-6.333	-17.67 to 5.007	No	NS	0.5646
$C_0(H_2O)$ vs. C_2 (MeOH 70%)	-0.3333	-3.527 to 2.861	No	NS	0.9999	-6	-17.34 to 5.340	No	NS	0.6231
$C_0(H_2O)$ vs. BEt	0	-3.194 to 3.194	No	NS	>0.9999	-2	-13.34 to 9.340	No	NS	0.9972
$C_0(H_2O)$ vs. FEt	-0.6667	-3.861 to 2.527	No	NS	0.9931	-1.333	-12.67 to 10.01	No	NS	0.9997
C_1(MeOH 35%) vs. 1:1 BEt	0	-3.194 to 3.194	No	NS	>0.9999	5	-6.340 to 16.34	No	NS	0.7882
C_1(MeOH 35%) vs. 1:1 FEt	-0.6667	-3.861 to 2.527	No	NS	0.9931	6	-5.340 to 17.34	No	NS	0.6231
C_1(MeOH 35%) vs. C_2 (MeOH 70%)	-0.3333	-3.527 to 2.861	No	NS	0.9999	6.333	-5.007 to 17.67	No	NS	0.5646
1:1 BEt vs. 1:1 FEt	-0.6667	-3.861 to 2.527	No	NS	0.9931	1	-10.34 to 12.34	No	NS	>0.9999
1:1 BEt vs. BEt	0	-3.194 to 3.194	No	NS	>0.9999	5.333	-6.007 to 16.67	No	NS	0.7363
1:1 BEt vs. FEt	-0.6667	-3.861 to 2.527	No	NS	0.9931	6	-5.340 to 17.34	No	NS	0.6231
1:1 FEt vs. BEt	0.6667	-2.527 to 3.861	No	NS	0.9931	4.333	-7.007 to 15.67	No	NS	0.8767
1:1 FEt vs. FEt	0	-3.194 to 3.194	No	NS	>0.9999	5	-6.340 to 16.34	No	NS	0.7882
C_2 (MeOH 70%) vs. BEt	0.3333	-2.861 to 3.527	No	NS	0.9999	4	-7.340 to 15.34	No	NS	0.9115
C_2 (MeOH 70%) vs. FEt	-0.3333	-3.527 to 2.861	No	NS	0.9999	4.667	-6.673 to 16.01	No	NS	0.8354
BEt vs. FEt	-0.6667	-3.861 to 2.527	No	NS	0.9931	0.6667	-10.67 to 12.01	No	NS	>0.9999

BE= bulb extract; FE= flower extract; t= treatment.

Table S6 - Tukey's multiple comparisons test results for secretory hairs density analysis on the leaf surfaces of treated *O. basilicum* plantlets

Treatments	Inferior leaf surface					Superior leaf surface				
	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
'Italiano Classico'										
$C_0(H_2O)$ vs. C_1(MeOH 35%)	1	-5.715 to 7.715	No	NS	0.9989	0	-8.627 to 8.627	No	NS	>0.9999
$C_0(H_2O)$ vs. 1:1 BEt	3.333	-3.382 to 10.05	No	NS	0.6872	-0.6667	-9.293 to 7.960	No	NS	>0.9999
$C_0(H_2O)$ vs. 1:1 FEt	0	-6.715 to 6.715	No	NS	>0.9999	0	-8.627 to 8.627	No	NS	>0.9999
$C_0(H_2O)$ vs. C_2 (MeOH 70%)	2.333	-4.382 to 9.049	No	NS	0.917	-4	-12.63 to 4.627	No	NS	0.7484
$C_0(H_2O)$ vs. BEt	0.3333	-6.382 to 7.049	No	NS	>0.9999	-1.333	-9.960 to 7.293	No	NS	0.9987
$C_0(H_2O)$ vs. FEt	1.667	-5.049 to 8.382	No	NS	0.983	-2	-10.63 to 6.627	No	NS	0.988
C_1(MeOH 35%) vs. 1:1 BEt	2.333	-4.382 to 9.049	No	NS	0.917	-0.6667	-9.293 to 7.960	No	NS	>0.9999
C_1(MeOH 35%) vs. 1:1 FEt	-1	-7.715 to 5.715	No	NS	0.9989	0	-8.627 to 8.627	No	NS	>0.9999
C_1(MeOH 35%) vs. C_2 (MeOH 70%)	1.333	-5.382 to 8.049	No	NS	0.9947	-4	-12.63 to 4.627	No	NS	0.7484
1:1 BEt vs. 1:1 FEt	-3.333	-10.05 to 3.382	No	NS	0.6872	0.6667	-7.960 to 9.293	No	NS	>0.9999
1:1 BEt vs. BEt	-3	-9.715 to 3.715	No	NS	0.7783	-0.6667	-9.293 to 7.960	No	NS	>0.9999
1:1 BEt vs. FEt	-1.667	-8.382 to 5.049	No	NS	0.983	-1.333	-9.960 to 7.293	No	NS	0.9987
1:1 FEt vs. BEt	0.3333	-6.382 to 7.049	No	NS	>0.9999	-1.333	-9.960 to 7.293	No	NS	0.9987
1:1 FEt vs. FEt	1.667	-5.049 to 8.382	No	NS	0.983	-2	-10.63 to 6.627	No	NS	0.988
C_2 (MeOH 70%) vs. BEt	-2	-8.715 to 4.715	No	NS	0.9586	2.667	-5.960 to 11.29	No	NS	0.9508
C_2 (MeOH 70%) vs. FEt	-0.6667	-7.382 to 6.049	No	NS	0.9999	2	-6.627 to 10.63	No	NS	0.988
BEt vs. FEt	1.333	-5.382 to 8.049	No	NS	0.9947	-0.6667	-9.293 to 7.960	No	NS	>0.9999
'Aromat de Buzău'										
$C_0(H_2O)$ vs. C_1(MeOH 35%)	0.6667	-6.049 to 7.382	No	NS	0.9999	-1.333	-9.960 to 7.293	No	NS	0.9987
$C_0(H_2O)$ vs. 1:1 BEt	0.3333	-6.382 to 7.049	No	NS	>0.9999	-4.667	-13.29 to 3.960	No	NS	0.5995
$C_0(H_2O)$ vs. 1:1 FEt	0.6667	-6.049 to 7.382	No	NS	0.9999	-1	-9.627 to 7.627	No	NS	0.9997
$C_0(H_2O)$ vs. C_2 (MeOH 70%)	-3	-9.715 to 3.715	No	NS	0.7783	-14	-22.63 to -	Yes	***	0.0004
$C_0(H_2O)$ vs. BEt	-1	-7.715 to 5.715	No	NS	0.9989	-2	-10.63 to 6.627	No	NS	0.988
$C_0(H_2O)$ vs. FEt	-5.333	-12.05 to 1.382	No	NS	0.1861	-7.333	-15.96 to 1.293	No	NS	0.1335
C_1(MeOH 35%) vs. 1:1 BEt	-0.3333	-7.049 to 6.382	No	NS	>0.9999	-3.333	-11.96 to 5.293	No	NS	0.8711
C_1(MeOH 35%) vs. 1:1 FEt	0	-6.715 to 6.715	No	NS	>0.9999	0.3333	-8.293 to 8.960	No	NS	>0.9999
C_1(MeOH 35%) vs. C_2 (MeOH 70%)	-3.667	-10.38 to 3.049	No	NS	0.5894	-12.67	-21.29 to -	Yes	**	0.0015
1:1 BEt vs. 1:1 FEt	0.3333	-6.382 to 7.049	No	NS	>0.9999	3.667	-4.960 to 12.29	No	NS	0.8143
1:1 BEt vs. BEt	-1.333	-8.049 to 5.382	No	NS	0.9947	2.667	-5.960 to 11.29	No	NS	0.9508
1:1 BEt vs. FEt	-5.667	-12.38 to 1.049	No	NS	0.1387	-2.667	-11.29 to 5.960	No	NS	0.9508
1:1 FEt vs. BEt	-1.667	-8.382 to 5.049	No	NS	0.983	-1	-9.627 to 7.627	No	NS	0.9997
1:1 FEt vs. FEt	-6	-12.72 to	No	NS	0.1017	-6.333	-14.96 to 2.293	No	NS	0.259
C_2 (MeOH 70%) vs. BEt	2	-4.715 to 8.715	No	NS	0.9586	12	3.373 to 20.63	Yes	**	0.0027
C_2 (MeOH 70%) vs. FEt	-2.333	-9.049 to 4.382	No	NS	0.917	6.667	-1.960 to 15.29	No	NS	0.21
BEt vs. FEt	-4.333	-11.05 to 2.382	No	NS	0.3992	-5.333	-13.96 to 3.293	No	NS	0.4486

BE= bulb extract; FE= flower extract; t= treatment.

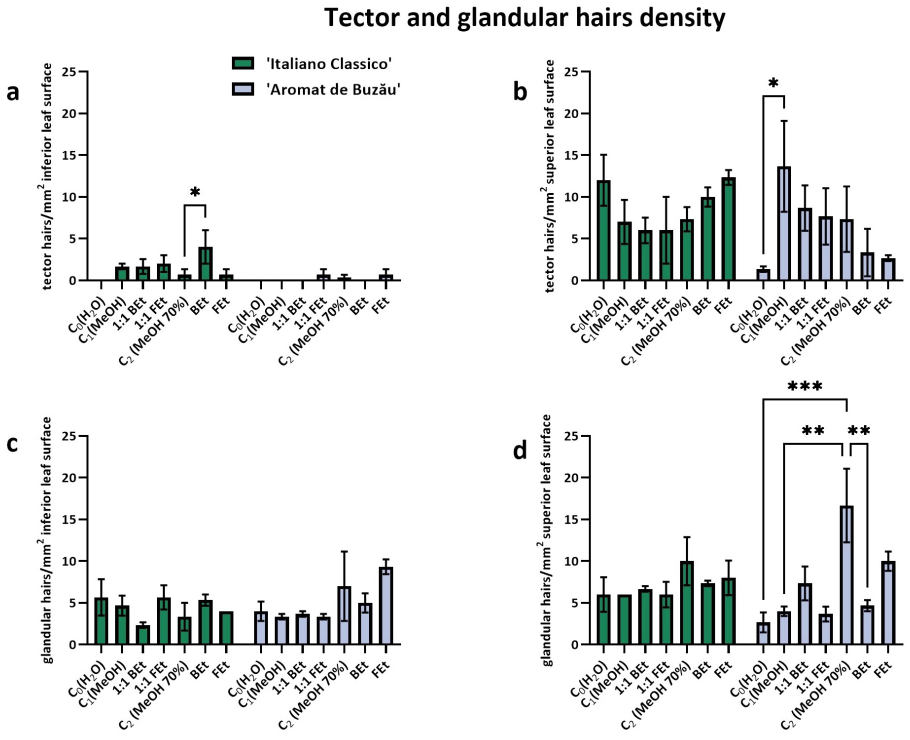

Fig. S1 - Tector and glandular hairs density on the abaxial (a, c) and adaxial (b, d) leaf surfaces of treated *O. basilicum* plantlets.

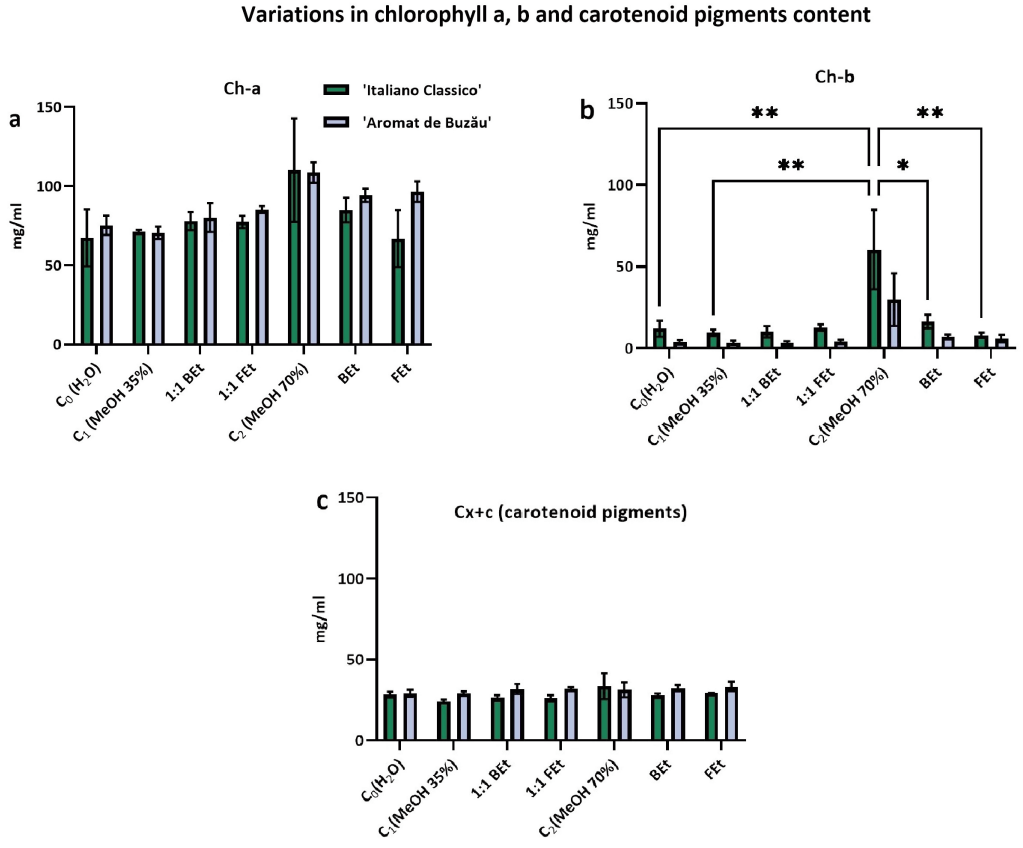

Fig. S2 - Variations in chlorophyll a, b and carotenoid pigments content in treated *O. basilicum* plantlets. BE= bulb extract; FE= flower extract; t= treatment; ** = p<0.01.

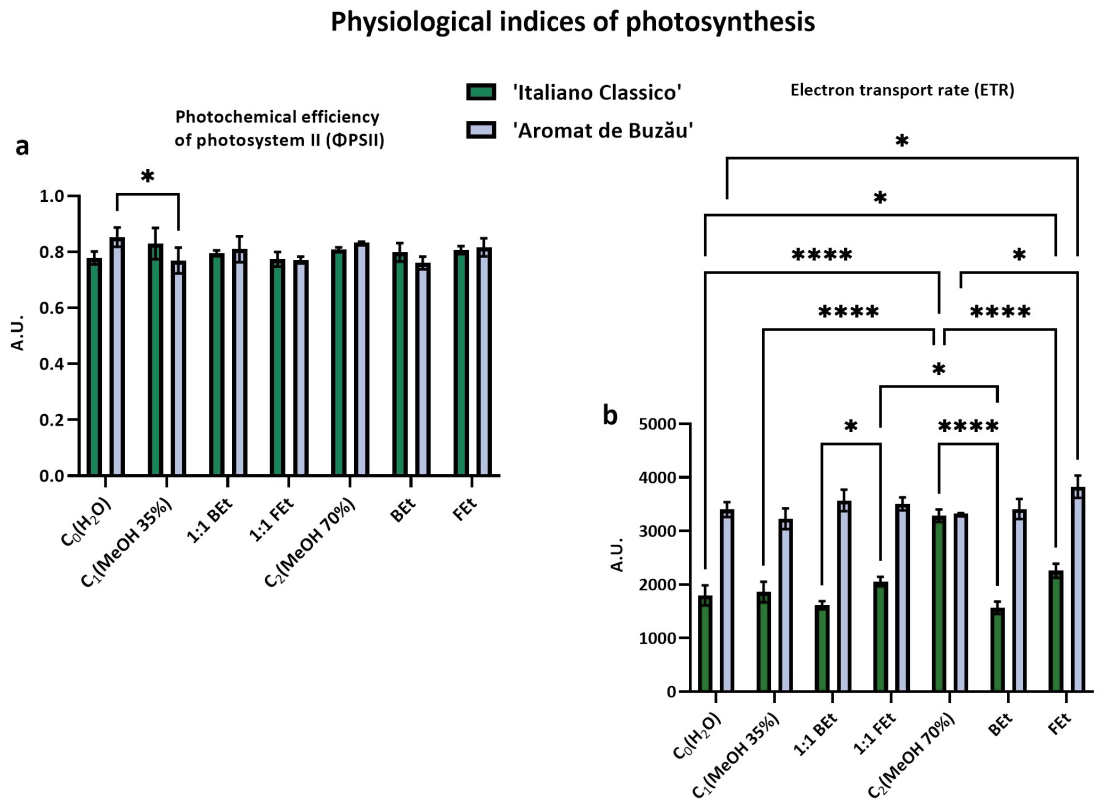

Fig. S3 - Physiological indices of photosynthesis determined through chlorophyll fluorescence assay in treated *O. basilicum* plantlets. BE= bulb extract; FE= flower extract; t= treatment; *= p<0.05; **** = p<0.0001.

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An analysis on the impacts of cryogenic freezing on raspberry quality

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Key words: Berries, liquid Nitrogen, *Rubus ideaus*, shelf life, storage.

Abstract: Counter-season supply of horticultural products is of increasing demand. Consumers are demanding annual supply of raspberries, which has historically been challenging due to their seasonal summer supply and characteristically high metabolism resulting in a short shelf life and limited period of availability. However, the development of freezing technologies for increasing the length of storage of raspberries offers an opportunity for continual supply of premium quality raspberries. We investigated berry quality after freezing whole fresh raspberries, comparing conventional freezing methods with a modern cryogenic freezing method over a period of six months. Significant increases in total soluble solids, titratable acidity, hue and chroma were found when raspberries were frozen compared to fresh raspberries. No overall difference in berry quality was observed between freezing methods for any parameter assessed. When assessed at time intervals, total soluble solids, pH, titratable acidity, chroma and hue were consistent between freezing methods for all durations of time frozen. These findings provide decision support for producers and distributors pursuing a novel counter season supply chain.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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1. Introduction

Raspberries (*Rubus ideaus* L.) are a perennial plant that produce a compound fruit composed of many drupelets (Wang *et al*., 2009). Raspberries grown for fresh production typically have a unique bright red colour with distinctive aroma and taste, as well as health benefits due to their antioxidant properties (Liu *et al.*, 2002). Raspberries are a non-climacteric fruit with a high metabolism, making them perishable with a very short shelf life (Tezotto-Uliana *et al*., 2014). The ability to maximise fruit quality whilst harvesting and during post-harvest is imperative to commercially viable raspberry production.

The development and adoption of innovative technologies has allowed conventional agricultural systems to pursue increased efficiency, yield, quality, food safety, and sustainability with reduced costs (Sunding and Zilberman, 2001). In the raspberry industry, freezing technologies for post-harvest storage offer producers the ability to extend the market window whilst preserving or increasing fruit quality standards. Additionally, freezing horticultural products allows access to diversified markets and

supply chains, creating increased economic feasibility and prosperity (Bora *et al.*, 2021; Safari *et al.,* 2021).

Demand by consumers for out-of-season supply of produce has driven research into the impacts of different storage methods on fruit and berry quality parameters *(*Boorse *et al.,* 1998; González *et al.*, 2002; Chassagne-Berces *et al*., 2010; Ceballos *et al*., 2012; Alhamdan *et al*., 2018). The perishable characteristics of raspberries caused by high rates of metabolism and limited seasonal production, makes counter season supply problematic for producers (Adobati *et al.*, 2015). Despite extensive literature investigating variances in quality parameters for horticultural products post conventional and cryogenic (liquid nitrogen) freezing, there is currently a gap in research comparing the impacts of the two freezing methods on the quality of whole fresh raspberries (Alhamdan *et al*., 2018; Otero *et al*., 2000). Some notable issues with freezing of berry fruit have been identified including ice crystal nucleation and subsequent cell membrane rupture and electrolyte leakage which compromises the eating quality of frozen fruit (Silva *et al*., 2008). The mouth feel of frozen fruit is related to cell turgor pressure which is driven by each cell having a separate functional semi permeable membrane that splits when ice nucleation occurs, resulting in soft textures in comparison to crisp fresh fruit (Grout *et al*., 1991). To reduce the damage to cell membranes by crystal nucleation during the freezing of berries, there have been several developments in freezing methodologies. Liquid nitrogen (~-80°C) applied to each berry individually reduces the thermal gradient and creates a snap freeze process that reduces the loss of quality through cell membrane damage caused by slower and higher temperature conventional freezing, resulting in a higher quality product (Ceballos *et al*., 2012). Studies have demonstrated that the loss of quality, particularly tissue damage and subsequent texture and colour from freezing, is impacted by the rate of freezing. Fruit frozen at lower temperatures (-40°C) have been shown to display better quality parameters than fruits frozen at higher temperatures (-14°C) (Chassagne-Berces *et al.*, 2010).

Whilst the demand for frozen horticultural products has seen considerable investment into studies on the impacts of freezing on vegetables and fruits, there is comparatively limited evidence on the specific effects of freezing on raspberry quality to support industry investment. González *et al.* (2002) found that berry variety, harvest timing and quality at harvest are critical factors influencing the impact freezing has on raspberry fruit quality. De Ancos *et al*. (2000) showed an increase in anthocyanin content during freezing; however, this conclusion was determined to be a result of anthocyanin extraction efficacy after ice nucleation, rather than a biological increase in anthocyanin content. Conversely, Kampuse *et al.* (2001) highlighted a decrease in anthocyanins in frozen raspberries, with varietal differences influencing the degree of anthocyanin depletion.

Cryogenic freezing technology has the potential to improve the quality of frozen berries. This study investigated if the application of freezing technologies can lead to improvements in raspberry fruit quality when frozen. Specifically, using a traditional commercially grown variety and a new propriety variety, we asked - Does freezing method (conventional and cryogenic) and freezing duration (2, 4, and 6 months) influence raspberry quality? The findings of this research are discussed in the context of industry pursuing continuous supply to a market that demands high quality and affordable produce throughout the year.

2. Materials and Methods

Experimental site

The experimental site was located at The Westerway Raspberry Farm (42° 47’ 26.34” S, 146° 47’ 26.34” E), an established 60-acre raspberry and mixed berry farm at Westerway, in southern Tasmania. The Westerway Raspberry Farm is situated on an alluvial soil deposit adjacent to the Tyenna River. The raspberry canes utilised for the experiment were oriented in a NE SW direction and were trained in a commercial vertical trellis system. Westerway receives an average annual rainfall of 764 mm (BOM, 2019). The traditional and popular ‘Willamette’ raspberry variety and a newly developed proprietary variety were investigated for the purpose of this experiment. The proprietary variety has been developed for the purpose of machine harvesting, with characteristics of diseases resistance, fruit firmness and low release force from plant.

Sample collection

Harvest coincided with the commercial raspberry season on 27 December 2018. Fruit was hand harvested from randomly selected rows within the block

with berries collected randomly along each row. Consistent with commercial practice, samples were collected into individually labelled commercial plastic 400 g punnets and stored in a commercial cool room (7°C) on site for five hours until transportation.

Treatments and storage conditions

For both raspberry varieties there were two different treatments (post-harvest storage technique and duration frozen) with five replicates for each treatment combination. Treatments were established as a fully factorial trial with two freezing techniques (conventional and cryogenic) and three lengths of storage (2, 4 and 6 months) tested with two varieties of raspberries.

Punnets containing fruit were immediately frozen on site in either a commercial conventional -8°C freezer (Gunter, Germany) or snap frozen in a liquid nitrogen (LN) tunnel at -80°C (Linde Cryoline MT 5-600 quick-freezing (IQF) tunnel, Munich, Germany) depending on treatment. For LN tunnel freezing, the berries were tipped out of the punnets and passed through the LN tunnel individually on the inbuilt conveyor belt and collected into labelled punnets immediately after freezing. All berries from both treatments were then stored in the commercial -8°C freezer for the duration of the storage period. During storage, all samples were stored in the commercial grade plastic punnets with lids and stacked into crates holding 50 punnets per crate, aligned with industry practice. Frozen samples remained in the freezer for three prescribed periods of up to six months.

Laboratory analysis

Samples were transported from the farm freezing facilities back to the laboratory at the Tasmanian Institute of Agriculture (University of Tasmania) for analysis in an ice chilled eski. The samples were then stored in a cool room (4°C) during the laboratory analyses, which were completed within 48 hr after being removed from the freezer.

The pH for every replicate was measured using a diluted sample in an auto-titrator (Metrohm 702 SM Titrino, Herisau, Switzerland). A subsample of 50 berries from each sample was juiced by squeezing berries through a microporous cloth (30-micron mesh filter cloth; Allied Filter Fabrics P/N M0032, Sydney, Australia). A sample of the juice (5 mL) was pipetted (BRANTECH 10 mL Transfer pipette S, San Francisco, America) into a 25 mL beaker and combined with 15 mL of distilled water. The solution was analysed for initial pH by electrode probes used in the auto-titration. The same solution used for pH was analysed for titratable acidity by the Metrohmauto-titrator (702 SM Titrino). The solution was mixed using a magnetic stirrer and combined with 1M sodium hydroxide to determine the titratable acidity (titration to pH = 8.2). Berry titratable acidity was expressed in g/L of citric acid. Total soluble solids (TSS) was determined using a portion of the juice (~3 mL) and a digital refractometer (Atago Pocket Refractometer PAL-1). The sample of juice was analysed immediately after juicing. Using a hand disposable plastic pipette, the sample was placed into the measuring chamber and analysed with the TSS figure (%) recorded manually. The chamber was cleaned between each sample using paper towels and distilled water to remove any residue.

The total anthocyanin content (mg cyanidin-3-glucoside equivalents/g berry fresh weight) was determined using the pH differential method (Lee *et al*., 2005). Subsamples (30 berries) were homogenised for 30 seconds at 7000 rpm using a Retsch Grindomix GM200 (Haan, Germany). Homogenised subsamples of 10 g were weighed into 50 mL centrifuge tubes and combined with 40 mL of acidified 70 % methanol [700 mL methanol, 300 mL distilled H2O, 0.1 mL concentrated HCl (0.01%v/v)]. The tubes were then placed into an ultrasonic bath (GRANT XUV Digital Ultrasonic Bath (Royston, UK) in darkness for 30 minutes at 10°C. The tubes were then dried and placed into a Hettich Benchtop Centrifuge (Universal 320 R Model Tuttlingen, Germany) and centrifuged for 10 minutes at 1520 g and 4°C. A 0.5 mL sample of the clear supernatant solution was then mixed with each of the buffers; buffer 1 solution being 0.025 M potassium chloride and buffer 2 being 0.4 M sodium acetate. The dilution used was a 1:10 dilution (0.5 mL supernatant to 4.5 mL of buffer). The solutions were then left to equilibrate for 30 minutes. Using a benchtop spectrophotometer (Thermo Scientific Genesys 10S UV-VIS, Waltham, America), the absorption of each solution was measured at 530 nm and 700 nm. This allowed the absorbance of the diluted sample to be calculated using the following equation (Lee *et al*., 2005):

$$\text{Absorbance} = (A530 - A700)\ pH1 - (A530 - A700)\ pH\ 4.5$$

where A530 is the spectral wavelength absorption measurement at 530 nm, and A700 is the spectral wavelength absorption at 700 nm.

Using the calculated absorbance value, the monomeric anthocyanin content was calculated using the equation (Lee *et al.*, 2005):

Monomeric Anthocyanin Pigment (mg/L) = (A x MW x DF x 1000)/ (e x 1)

where *A* = Absorbance, *MW* = 449.2 g/mol for cyanidin-3-glucoside, *DF* = dilution factor determined (1 in 10 dilution), 1000 = factor for conversion from g to mg, e = 26,900 molar extinction coefficient, in L X mol-1 X cm-1, for cyd-3-glu and 1 = path length in cm. Max used e = 30200.

The final value from the monomeric anthocyanin pigment (mg/L) was converted to the commonly expressed mg per 100 g fresh weight unit. This conversion was completed by knowing the total amount of anthocyanin in a litre and the initial fresh mass of raspberry sample used.

Anthocyanin (mg per 10 g Fresh weight) = (0.04 x anthocyanin Mg/L value) x 100 g/fresh weigh (g)

Where 0.04 = proportion of 40 mL of a litre, 100 g = final unit, Anthocyanin Mg/L Value = answer from monomeric equation and fresh weight = 10 g weighed initially

Using the homogenised samples, homogenate colour was measured using a colourimeter (Konica Minolta Chroma Meter CR-400, New York, USA) (Edgley *et al*., 2019). Spectrophotometer tubes were filled with homogenate from each sample and placed in the colourimeter. The values for l*, a* and b* were recorded manually and gave a three-dimensional colour space with each value interpreted as: L* a measure from opaque (0) to completely black (100), a positive a* indicates redness whilst negative a* indicates greenness and a positive b* indicates yellowness whilst negative b* indicates blueness on the hue-circle (Voss, 1992; Gonçalves *et al*., 2007). Chroma was obtained by the formula:
Chroma = $(a^{*2} + b^{*2})^2$ and Hue from the formula: hue= *arctg b*/a** (Gonçalves *et al*., 2007).

Statistical analysis

Analysis of variance and statistical significance of quality parameters (TSS, pH, titratable acidity, anthocyanins, hue and chroma) between treatment interactions and treatment main effects were analysed using the 2019 IBM SPSS Statistics package. Analysis of mean variance was determined using univariate linear models for each differing continuous variable. Post hoc tests were completed using Tukey's range test of statistical significance.

3. Results

There were no three-way interactions between variety, freezing method and length of duration frozen for all quality parameters assessed. However, there were two-way interactions for freezing method and variety, duration frozen and variety, and duration frozen and freezing method for a range of the quality parameters assessed.

Freezing method and variety

Raspberries of both proprietary and 'Willamette' varieties had significantly (P<0.05) increased TSS values after freezing, regardless of method, in comparison to their fresh TSS values (Fig. 1a). Conventionally frozen proprietary berries had significantly (P<0.05) lower pH values in comparison to fresh, however they were not significantly (P>0.05) different to cryogenically frozen proprietary berries (Fig. 1b). Cryogenically frozen 'Willamette' berries and frozen

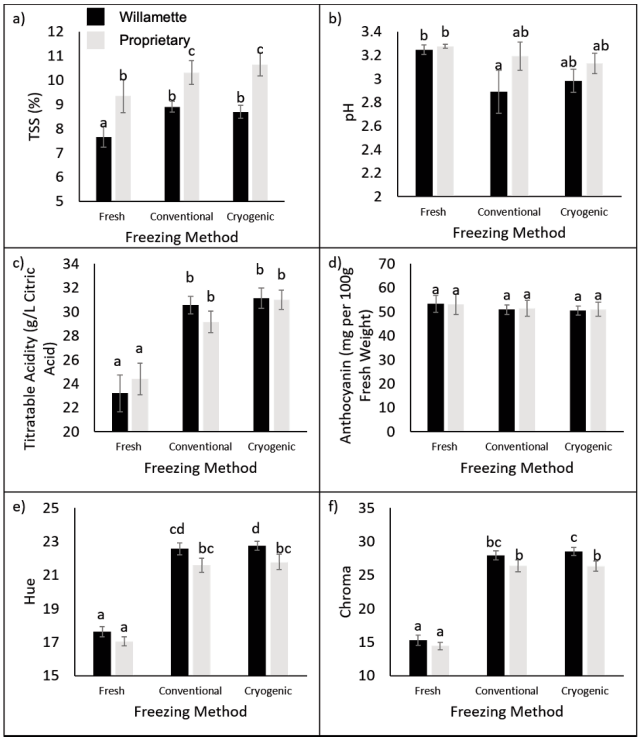

Fig. 1 - TSS (a), pH (b), Titratable acidity (c), Anthocyanin content (d), Hue (e) and Chroma (f) values for fresh and frozen propriety (grey bars) and 'Willamette' raspberries (black bars). Errors bars denote two times standard error. Letters above bars indicate significant differences from the mean at P<0.05.

proprietary berries had statistically similar pH values in comparison to their respective fresh berries (Fig. 1b.)

Freezing both 'Willamette' and proprietary raspberries significantly increased (P<0.05) titratable acidity (g/L citric acid), whilst the method of freezing made no significant (P>0.05) difference for either variety (Fig. 1c). Anthocyanin content of berries of both varieties were the same whether fresh or frozen, regardless of freezing method (Fig. 1d). Freezing both 'Willamette' and the proprietary variety significantly increased (P<0.05) both the hue and chroma values for berry colour, whilst the method of freezing did not have a significant impact on each variety individually (Fig. 1e and 1f). Proprietary raspberries exposed to cryogenic freezing however had significantly greater (P<0.05) hue and chroma values than 'Willamette' exposed to cryogenic and conventional freezing (Figs. 1e and 1f).

Duration frozen and variety

The TSS (%) for the proprietary raspberry variety was not significantly influenced (P>0.05) within the first two months of freezing, however TSS increased significantly (P<0.05) after four and six months frozen compared to the fresh samples (Fig. 2a). The 'Willamette' variety had significantly increased (P<0.05) TSS two and six months after freezing, however TSS for the four-month frozen sample was not significantly different (P>0.05) from the fresh samples (Fig. 2). Once frozen, there was no significant difference (P>0.05) in TSS for berries frozen for two, four or six months for either variety.

The 'Willamette' variety had no significant (P>0.05) change in pH for the duration frozen. pH of the proprietary variety berries continuously declined at each assessment date but was only significantly lower after being frozen for six months when compared to fresh berries (Fig. 2b).

TA (g/L citric acid) of both the 'Willamette' and proprietary variety significantly (P<0.05) increased once frozen but did not significantly change through the duration of freezing (two, four and six months) (Fig. 2c). The change in titratable acidity to TSS ratio was statistically similar across duration frozen.

The colour (hue units) of both 'Willamette' and the proprietary variety raspberries also significantly (P<0.05) increased when frozen (Fig. 2d). Once frozen, hue of the proprietary variety did not change for the duration of frozen storage, whilst hue for the 'Willamette' berries significantly (P<0.05) reduced at the six-month assessment compared to the second month assessments (Fig. 2d).

Duration frozen and freezing method

Berries that were initially cryogenically frozen had significantly (P<0.05) higher TSS values at four months compared to fresh raspberries, but no significant (P>0.05) difference at six months in comparison to fresh berries (Fig. 3a). For berries that were initially frozen in a conventional freezer, the two- and four-month frozen treatments were not significantly different from fresh raspberries in TSS, but at six months there was significantly (P<0.05) greater TSS

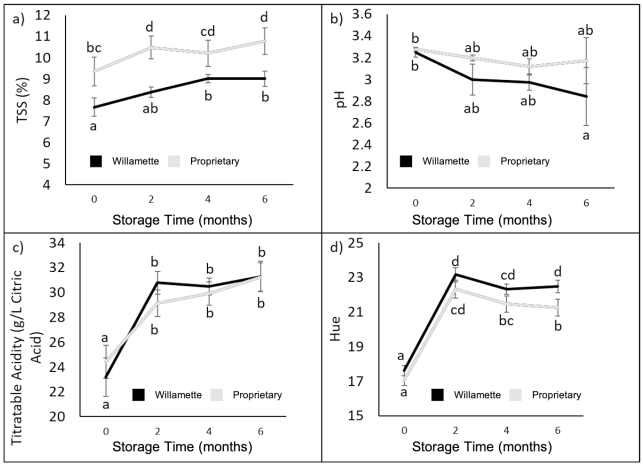

Fig. 2 - Brix (a), pH (b), Titratable acidity (c) and Hue (d) for both the proprietary (black bars) and 'Willamette' (grey bars) variety of raspberry after being frozen for up to six months. Errors bars denote two time standard error. Letters above bars indicate significance differences from the mean at P<0.05 of time frozen values.

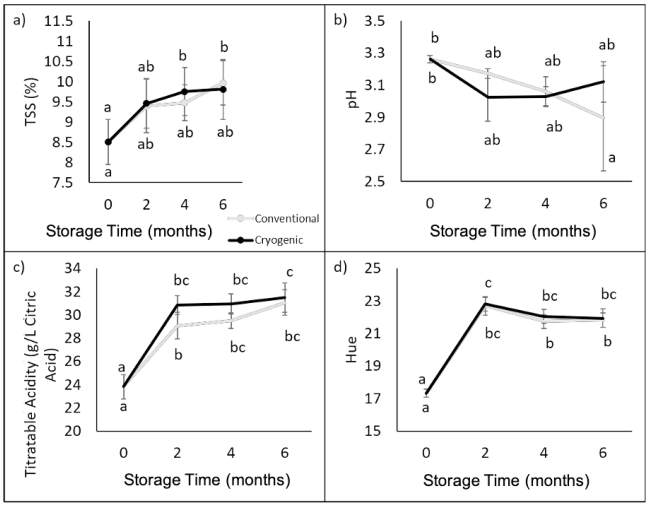

Fig. 3 - TSS (%) (a), pH (b), Titratable acidity (c) and Hue (d) for both the conventional (grey bars) and cryogenic (black bars) methods of freezing raspberries. Error bars denote two times standard error. Letters above bars indicate significance differences from the mean at P<0.05.

compared to fresh berries (Fig. 3a).

Cryogenically frozen berries had no significant ($P>0.05$) differences in pH value for the duration frozen in comparison to fresh berries (Fig. 3b). However, conventionally frozen berries at six months had significantly ($P<0.05$) lower pH compared to fresh raspberries.

There was a significant increase in TA once frozen, but at each assessment interval (two, four and six months), there was no significant difference in freezing method on the TA concentration (Fig. 3). For both conventional and cryogenic freezing, there was no significant change in TA between two, four and six months frozen (Fig. 3).

For hue, there was no significant difference between freezing method at any single duration measured for the duration of the experiment (Fig. 3). Both freezing methods did exhibit a significant increase in hue colour units initially after freezing, increasing by ~25 % between 0 and two months frozen (Fig. 3).

4. Discussion and Conclusions

Consumer demand for counter season supply of premium horticultural products is driving the need and innovation of storage techniques whilst maintaining premium quality produce (Kuchler and Arnade, 2015; Martindale and Schiebel, 2017). The results of this study found significant differences in quality parameters for whole raspberries after they were frozen compared to fresh berries. Measurements of total soluble solids, titratable acidity, hue and chroma all significantly increased when berries were frozen. This study found no difference however in berry quality once frozen between freezing methods for any parameter assessed. Total soluble solids, pH, titratable acidity and hue were consistent between freezing methods for all durations of time frozen. These results improve the understanding of the impacts of post-harvest storage on raspberry quality and provides critical information and decision support for producers to ensure optimal raspberry quality for consumers.

Raspberries are no exception in the unrelenting demand from consumers for premium quality counter season supply of horticultural products, however, the metabolic characteristics that result in raspberries being highly perishable makes the long-term storage of raspberries comparatively difficult (Gonçalves *et al*., 2018). For both the 'Willamette' and proprietary varieties, TSS increased once frozen regardless of freezing method. This finding of initial TSS increase after freezing is consistent with González *et al*. (2002) who showed a similar initial increase in TSS after freezing 'Heritage', 'Autumn Bliss', 'Zeva' and 'Rubi' raspberries. The treatments of two, four- and six-months freezing duration showed differences for TSS between varieties when fresh, with the 'Willamette' variety having greater than 20% higher TSS than the proprietary variety. Varietal difference as shown here is consistent with Shamaila *et al*. (1993) who found differences for fresh berries for the quality parameters pH, TSS, TA and anthocyanins between 'Chilcotin', 'Chilliwack', 'Meeker', 'Skeena' and 'Tulameen' varieties. This significant difference between varieties persisted for all freezing periods. However, once frozen, TSS values did not significantly change for both varieties for the duration of the trial, and the freezing method, conventional vs cryogenic, also had no impact on TSS. This finding contrasts with the González *et al*. (2002) study described earlier, which showed that TSS continued to significantly increase during duration frozen. The findings of consistency in TSS levels once frozen for the proprietary and 'Willamette' variety suggest the quality of these varieties once frozen is more stable compared to varieties such as 'Heritage', 'Zeva' and 'Rubi' used by González *et al*. (2002). This finding is likely a result of differing TSS profiles between varieties having differing stability when frozen, resulting in a variation in TSS level changes.

Titratable acidity contributes to the mouth feel a consumer experiences when stimulated by the multiple acids including citric, ascorbic and phosphoric acid found in raspberries (Haffner *et al.*, 2002; Marsh *et al*., 2004). Freezing is the most common form of long term storage for fresh produce, with the reduction in metabolism and respiration when frozen allowing raspberries to be stored for long periods of time (Chaves and Zaritzky, 2018). Cryogenic freezing facilitates the rapid transition into the frozen state which reduces membrane damage (and therefore maintains raspberry quality) from slow water crystallization and subsequent membrane damage that occurs during conventional freezing (Cao *et al*., 2018). Consistent with findings for changes in TSS, there was a significant increase in titratable acidity for frozen berries compared to fresh berries for both varieties, but there was no significant difference resulting from freezing method for either variety.

Titratable acidity significantly increased when initially frozen but did not change with length of duration frozen. These results are consistent with findings of increased total and conjugated acid in frozen raspberries by Mullen *et al*. (2002). However, de Ancos *et al*. (2000) who quantified ellagic acid concentrations, found a significant decrease (14-21%) in ellagic acid content when raspberries were frozen, highlighting that the increase in titratable acidity in this investigation may be due to increase in other acids found in raspberries.

Raspberry quality is derived from multiple parameters with each contributing to the consumer's sensory experience and as such, the ratio of TSS with TA is a major contributor to consumer experience. Despite possible variances of specific acids during freezing, it is the ratio of sugar to acid that influences the consumer's tasting experience (Shamaila *et al*., 1993). The increase in sugar when frozen is proportional to the increase in acidity and showed no significant difference between freezing methods and duration, and it is this consistency in ratio that is critical to consumer experiences. The consistency in the ratio between TSS and titratable acidity whilst frozen is also broadly consistent with research by González *et al.* (2002) who used different raspberry varieties and durations frozen. No physiological mechanism or chemical changes induced by freezing/thawing has been described for the increase in titratable acidity post-harvest for raspberries. The measurement of increased titratable acidity in this study may be a physiological response to storage or an increase in extraction efficiency post storage, and until further investigation quantifies the reason for the change, the implications cannot be fully understood. In strawberries, retention of quality parameters including acids, colour and anthocyanin content was associated with the thawing process, where rapid thawing was shown to be more favourable (Holzwarth *et al*., 2012).

The impact of freezing method and duration on the colour of raspberries is important for influencing consumer demand. A colour that is perceived to be preferable by the consumer increases both the acceptance and perception of other quality parameters such as taste and texture (Clydesdale, 1993). Both varieties had similar hue and chroma values when fresh, however once frozen, colour values increased significantly. When frozen conventionally, both varieties had similar coloured fruit; however, when frozen cryogenically, the proprietary variety had significantly higher hue and chroma unit values than 'Willamette' raspberries (Fig. 1). This was the only quality parameter analysed that showed similarities between varieties for fresh berries but when frozen showed a significant difference between varieties. This highlights a difference in the impacts of freezing methods between the varieties and the subsequent impact on colour. The contrasting influence of freezing between varieties is consistent with findings by other researchers. González *et al*. (2002), for example, showed that for the varieties 'Heritage' and 'Autumn Bliss', chroma and hue was not different when fresh, but after six and nine months of being frozen, hue and chroma values differed between varieties.

The significant change in hue and chroma between fresh and frozen berries was not accompanied by a change in anthocyanin levels. This is inconsistent with findings by Han *et al*. (2004), who attributed the darker colour of frozen raspberries to anthocyanin synthesis. However, for strawberries, Holcroft and Kader (1999) showed that pH affects the colour expression of anthocyanins, concluding that the red flavylium cation only remains stable in acidic conditions, and strawberries lose their red colour (become pale) when the pH increases. Therefore, if the anthocyanin stability and subsequent redness of raspberries is associated with the amount of acids present (acidity), the increase in titratable acidity observed in this study, may be associated with increasing the amount of stable red flavylium cations, causing the raspberries to be darker when frozen.

This investigation used commercial standards of raspberry quality as a benchmark to compare innovative freezing methods for post-harvest management of raspberry fruit. In this investigation, consistent quantitative results for the quality parameters, benchmarking the commercial standard, provides support for the application of new technology in producing a consistent high-quality product. However, the commercial implications of a non-consistent (significantly different) result are not known, as it may be of better or worse quality in the perception of the consumer. Therefore, results that highlight significant ($P<0.05$) differences present opportunities for further studies and evaluations of quality as well as potential for changing practices.

The results of this investigation broadly demonstrated consistent quality parameters between conventional freezing and the modern, innovative and comparatively expensive technique of cryogenic freezing. We suggest that based on these findings,

there is evidence-based support for the commercial retention of the cheaper conventional freezing over cryogenic freezing for post-harvest storage of raspberries. The study has provided critical decision support for post-harvest management of commercial raspberry production. This is particularly important for the uptake of horticultural technology that aims to access new markets and innovate logistics in supply chains. The findings of this study provide the framework and basis for the uptake and refinement of innovative technologies used in the raspberry industry, and allows freezing method choices to be evidence-based. The changes in titratable acidity during the freezing duration trial is unexplained here nor any published literature to date. Determining whether a physiological increase of titratable acidity occurs or if an increase in extraction efficiency of titratable acidity is the reason behind the results of this study would provide additional insights into the impacts of freezing on raspberries. The quantitative results of the current study also provide impetus for a sensory analysis to investigate consumer perceptions in parallel with the quantitative laboratory evidence provided in this study.

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Comparison of 18 Iranian caprifig cultivars based on some morphological and biochemical parameters

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Abstract: Caprifig is a valuable candidate for fig breeding programs as it typically grows naturally under non-optimal conditions. The present study was carried out to evaluate the biochemical/morphological characteristics of 18 caprifig cultivars indigenous to the Darab region/southern Iran with 4 replications in a completely randomized block design. From each cultivar, healthy uniform leaf samples and spring fruits were taken and analyzed. Our results showed that 'Naneghasem' had the highest leaf α-tocopherol and polyphenol concentration. The highest leaf ascorbic acid concentration was obtained from the Gol Khengi cultivar (17.03 µg g^{-1} fresh weight). Also, the contents of chlorophyll, carotenoids, and anthocyanins were significantly different among the studied cultivars. Various cultivars had different absorption potentials for essential elements as macro and micronutrients concentration in the leaves were statistically different in various caprifigs; 'Naneghasem' had the highest Ca (4.46 mg g^{-1} dry weight) and Fe (67.71 mg kg^{-1} dry weight) concentration and the highest leaf K concentration (22.46 mg g^{-1} dry weight) was observed in 'Mahali Layzengan'. In conclusion, 'Naneghasem' was evaluated as a cultivar which seems to be more morphologically- and biochemically-distant from other studied caprifig varieties and probably more adaptable/tolerant to environmental conditions.

1. Introduction

The fig, a deciduous tree of the Moraceae family and native to the southern Arabian Peninsula in the subtropical region, is an important horticultural crop that is grown for dry and fresh consumption. Dried figs are an excellent source of amino acids, vitamins, minerals, polyphenols, and crude fiber (5.8%, w/w) (Pourghayoumi *et al*., 2016). Satisfactory dry fig production is highly dependent on sufficient pollination. Caprifig is the only fig species that has both pistillate and staminate flowers. Growers collect the fruits of caprifigs, place them in cans, and hang the cans on Smyrna-type figs; *Blastophaga psenes* wasps, which live in the syconium of caprifigs, leave the profichi and enter the female flowers of the edible

figs and pollinate their pistillate flowers (Rahemi and Jafari, 2008).

In recent years, fig cultivation has developed rapidly due to the wide adaptability, early fruit and high yield, fewer pests and diseases, and simple management of this crop in various parts of the world (Zhang *et al*., 2020). This increases the importance of protecting and developing fig germplasm. Maintaining possible local varieties for commercial cultivation requires characterization of the available germplasm.

This type of thermophilic tree could be useful to adapt to the consequences of global warming, as fig trees can grow in dry regions where many other species may not survive (Sugiura *et al*., 2007). Fig trees have not been subjected to extensive breeding programs; consequently, many fig populations have rich diversity that cannot be fully exploited until correctly identified (Perez-Jiménez *et al*., 2012).

One of the first steps in breeding is the selection of promising species/cultivars. Previous studies show differences between various common fig cultivars (Aradhya *et al*., 2010; Perez-Jiménez *et al*., 2012; Zhang *et al*., 2020). These differences suggest varied levels of acclimation and/or tolerance potential to abiotic stress conditions. The plant's enzymatic and non-enzymatic antioxidant potential plays a vital role in this regard (Bonyanpour and Jamali, 2020). Maintenance of high antioxidant capacity to scavenge toxic reactive oxygen species (ROS) has been associated with better adaptation of plants to environmental conditions (Sharma *et al*., 2012). In addition, different cultivars have different abilities to uptake nutrients. The concentration of macro- and micronutrients in plant organs and tissues correlates with the plant's potential to tolerate prevailing environmental conditions (Jamali *et al*., 2016; Jamali and Bonyanpour, 2017; Tian *et al*., 2021).

Iran has one of the richest fig germplasm resources in the world; however, the majority of previous studies have focused on edible commercial cultivars, while studies on caprifig varieties are rare. Caprifig can be a valuable resource for breeding programs because it grows naturally under less than optimal conditions and is not domesticated. Strengthening research on caprifig germplasm's genetic/chemical diversity is of scientific importance for germplasm conservation, efficient breeding, and satisfactory production.

Typically, cultivar characterization studies involve determining a vast range of morphological, biochemical, molecular, etc., parameters to find promising varieties for future breeding projects. As part of a series of similar investigations on caprifig cultivars, the present study aimed to compare 18 cultivars indigenous to the Darab/Southern Iran region, focusing on phonological parameters and biochemical characteristics associated with non-optimal growing conditions.

2. Materials and Methods

Plant material

Uniform and healthy plants of eighteen mature autochthonous caprifig cultivars were selected. They were planted and grown in a completely randomized block design with 4 replications; each replication had 4 plants with a row spacing of 2.5 m and an inter-row spacing of 3.5 m in a rain-fed collection orchard in the Darab region, Fars province, southern Iran. Average annual climate parameters in the experimental region were: precipitation: 200 mm, relative humidity Max: 55%, Min: 18%, temperature Max: 42°C, Min: 4°C. The soil of the orchard was sampled and analyzed for soil texture, mineral content, organic matter, pH, and EC (Table 1). Cultivars included: 1-Mashgholamrezai (MGR), 2-Darabi (DRB), 3-Abbasali (ASA), 4-Khazraie (KZR), 5-Ieji (IJ), 6-Gol Khengi (GK), 7-Rastaghi (RS), 8-Naneghasem (NGM), 9-Mahali Layzengan (MLZ), 10-Cheshmbolboli (CMB), 11-Pouzehdonbali (PZD), 12-Danesibi (DS), 13-Pasras (PR), 14-Johari (JR), 15-Maseeh (MS), 16-Cho (CO), 17-Pasbehdari (PSD), and 18-Suzu (SZ). Leaf samples were taken from different orientations (north, south, west, and east) of the trees; 25 fully expanded mature leaves from each side of all trees (100 leaves per tree as bulk samples) were transported to the

Table 1 - Analysis of soil samples in the experimental region

Soil depth (cm)	Soil texture	Soil mineral content (mg kg^{-1})							Organic carbon (%)	EC (dS m^{-1})
		Nitrate	Ca	Mg	K	Fe	Zn	Mn		
0-50	Loamy clay	37	1110	170	220	7.00	1.7	5.50	0.70	0.72

laboratory. Leaves were of spring bloom, the middle third of the branch, at the height of 1-1.5 m, including the petiole. Leaves with abnormal symptoms, including mechanical lesions caused by diseases or pests as well as chlorosis, were avoided. Routine cultural practices were carried out during the experimental period.

Measurements

The following parameters were determined in caprifig cultivars for two consecutive years, with an average reported.

Leaf dry matter content

Three uniform leaves were washed with tap and distilled water. After drying with a clean towel, they were weighed with a digital scale to get fresh weight (FW) and then dried in an oven at 70°C for 72 hours and re-weighed to get dry weight (DW). The percentage of leaf dry matter was calculated according to the following formula:

$$\text{Leaf dry matters (\%)} = [\text{Leaf DW (g)}/\text{leaf FW (g)}] \times 100$$

Leaf relative water content (LRWC)

Ten leaf discs from each treatment were weighed (FW), then hydrated to saturation (constant weight) for 48 hours at 5°C in darkness (turgid weight, TW). Leaf discs were dried in an oven (DW). The relative water content was calculated according to the following formula (Jamali and Eshghi, 2015):

$$\text{LRWC (\%)} = (\text{FW-DW})/(\text{TW-DW}) \times 100$$

Leaf electrolyte leakage

Leaf electrolyte leakage (EL) was determined by recording leaf leachate's electrical conductivity (EC) in double-distilled water at 40 and 100°C. Leaf samples were cut into uniform-sized disks and placed in test tubes containing 10 ml of double-distilled water. The test tubes were kept at 40°C for 30 minutes and at 100°C for 15 minutes, and their respective electrical conductivities (EC1 and EC2) were measured using a conductivity-meter (METROHM Conductometer 644, Switzerland):

$$\text{Electrolyte leakage (\%)} = (\text{EC1}/\text{EC2}) \times 100$$

Leaf soluble carbohydrates

Leaf samples of 0.5 g (DW) were homogenized in 5 ml ethanol (95%) and centrifuged at 4500 × g for 15 min. The supernatant was removed from the sample, and the residue was resuspended in 5 ml ethanol (70%). The supernatant was centrifuged for final extraction. Both supernatants were combined. The anthrone sulfuric acid assay was used for determination (Irigoyen *et al*., 1992). An aliquot of 100 µl was added to 3 ml of the anthrone-sulfuric acid solution; the mixture was shaken, heated in a boiling water bath for 10 min, then cooled at 4°C. The absorbance at 625 nm was determined spectrophotometrically. Glucose (0-100 mg/l, Merk) was used as a standard (Jamali and Bonyanpour, 2017).

Leaf chlorophyll and carotenoids concentration

Leaf discs of 0.25 g were extracted in 2.5 ml of acetone (80%) and then centrifuged at 6,000 × *g* for 10 min. The supernatant was used to prepare a final volume of 50 ml of leaf extract. Extraction of the leaf tissue with the buffer was continued until decoloration. The absorbance of the extract was measured at 470, 645, and 663 nm spectrophotometrically. Acetone (80%) was used as a blank. Finally, the content of chlorophyll and carotenoids was calculated using the following equations (Lichtenthaler, 1987):

$$\text{Leaf total chlorophyll concentration (mg. g}^{-1}\text{ FW)} = [(7.15A663 + 18.71A645) \times v / 1000 \times W]$$

$$\text{Carotenoids (mg. g}^{-1}\text{ FW)}: 1000A470 - 1.82Chla - 85.02Chlb / 198$$

A = absorbance at ƛ (nm), W = sample weight.

Leaf anthocyanins concentration

Total anthocyanins in the leaves were determined by the pH differential method using a spectrophotometer and two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M), and sodium acetate buffer, pH 4.5 (0.4 M). 0.25 g leaf samples were extracted with 1 ml methanol: water: concentrated HCl solution (80:20:1 v/v/v). 0.4 ml of leaf extract was mixed with 3.6 ml of corresponding buffers and read against water as blank at 510 and 700 nm. Absorbance (A) was calculated as

$$A = (A515 - A700)\ pH\ 1.0 - (A510 - A700)\ pH\ 4.5$$

Then total leaf anthocyanins concentration was calculated using the following equation:

$$\text{Anthocyanin (µg. g}^{-1}\text{ FW)} = (A \times Mw \times DF \times 1000) / e$$

Where A is the absorbance of the diluted sample, DF is the dilution factor (10), Mw is the molecular weight of cyanidin-3-glucoside (449.2), and e = 26,900 L/mol.cm, the molar extinction coefficient of cyanidin-3- glucoside.

Leaf α-tocopherol concentration

The α-tocopherol was extracted using Chong *et al*. (2004) method. 200 mg lyophilized sample was homogenized in 1 ml acetone at 4°C with a pre-chilled mortar and pestle. 0.5 ml hexane was added, and the homogenate was first vortexed for 30 s, centrifuged at 1000 × *g* for 10 min. The upper hexane

layer was removed while the acetone layer containing α-tocopherol remained in the vial. A second aliquot of 0.5 ml hexane was added, and the extraction process was repeated three times. α-tocopherol was determined by the method of Kanno and Yamauchi (1997).

To 0.2 ml of pooled extract a 0.4-ml aliquot of 0.1% (w/v) 3-(2-pyridyl)-5,6- diphenyl-1,2,4-triazine was added. The volume was made up to 3 ml with absolute ethanol, 0.4 ml 0.1% (w/v) ferric chloride ($FeCl_3.6H_2O$) was added, and the content was gently mixed under dim light in a dark room to avoid photochemical reduction. After a 4-minute reaction at room temperature, 0.2 ml 0.2 M orthophosphoric acid was added, and the mixture was left for another 30 min. Absorbance was determined spectrophotometrically at 554 nm and reported as µg g^{-1} FW. The blank was prepared the same way; absolute ethanol was used instead of the sample. α-tocopherol (Sigma Chemical) was used as a standard.

Glutathione concentration

Two hundred mg of tissue was homogenized in 2 ml of ice-cold 5% TCA. The homogenate was centrifuged at 17,000 × g for 30 min at 4°C. A volume of 75 µl of the supernatant was added to a cuvette containing 300 µl of phosphate buffer (0.2 M, pH 8.0) and 750 µl of 0.6 mM DTNB (5,5´-dithiobis- 2-nitrobenzoic acid) in phosphate buffer. The absorbance at 412 nm was read, and glutathione concentration was derived against a standard curve prepared with known amounts of GSH in 5% TCA (Moron *et al*., 1979).

Leaf total polyphenols

This parameter was determined using the Folin-Ciocalteu reagent. One gram of freeze-dried leaf samples was placed in an Eppendorf tube, mixed with 1 ml of methanol (80%), ground at 4°C, then centrifuged at 10000 × *g* for 15 minutes. The extract was mixed with 0.5 ml of Folin-Ciocalteu reagent; diluted 1:1 with water, and then 1 ml of a 5% sodium carbonate solution was added. Absorbance was measured at 725 nm and expressed as mg per g FW after 30 minutes. Gallic acid was used as the standard phenolic compound (Bonyanpour and Jamali, 2020).

Lipid peroxidation

A sample of 0.2 g was homogenized in 2 ml 0.1% trichloroacetic acid (TCAA) solution and then centrifuged at 15,000 × *g* for 10 min at 4°C, the 0.5 ml of supernatant was added to 1.5 mL 0.5% TBA in 20% TCAA, followed by incubation of the mixture at 95 °C in a shaking water bath for 30 min. The tubes were placed in an ice-water bath to stop the reaction. The samples were re-centrifuged at 10,000 × *g* for 5 min, and the absorbance of the supernatant was determined spectrophotometrically at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The malondialdehyde (MDA) concentration was calculated using the extinction coefficient of 155mM^{-1} cm^{-1} (Cakmak and Horst, 1991).

Leaves proline concentration

Leaf samples were homogenized with 3% sulfosalicylic acid. The homogenate was centrifuged at 2500 × *g* for 20 min. The supernatant was treated with acid ninhydrin and acetic acid, boiled for 60 minutes, and then the absorbance of the solution was recorded at 520 nm. Leaves proline contents were expressed as µmol·g^{-1} FW. Proline amino acid was used as standard (Bates *et al*., 1973).

Leaf ascorbic acid concentration

This parameter was measured by the method of Omaye *et al*. (1979). To 1 g of freeze-dried leaf sample, 10% ice-cold TCA was added and centrifuged at 3500 × g for 20 min at room temperature. The supernatant (1 ml) was mixed with 0.2 ml of DTC reagent and incubated at 37°C for 3 h. Then 1.5 ml of ice-cold 65% sulfuric acid was added and mixed, and the solutions were allowed to stand at room temperature for another 30 min. The color developed was read at 520 nm spectrophotometrically and reported as µg g^{-1} FW.

Macro and micronutrients' concentration

Oven-dried leaf samples (0.5 g) were ground and ashed at 550°C in a porcelain crucible for seven h. The white ash was mixed in 2 M hot HCl, filtered, and finally made up to 50 mL with distilled water. Nitrogen (N) concentration was measured using the Kjeldahl digestion method. Potassium (K) concentration was determined using the flame emission method using a Sherwood Scientific Ltd model 360 flame photometer. An atomic absorption spectrophotometer (AA 6200, double beam atomic absorption spectrophotometer Shimadzu, Kyoto, Japan) was used to determine Ca, Mg, and micronutrient element, including Fe, Zn, and Mn concentrations. Phosphorus (P) concentration was determined colorimetrically (Kalra, 1998).

Leaf biometrics

Leaf area was determined using a leaf area meter

(Kaiser RS1) and expressed as mm^2; leaf and petiole length by using a digital caliper and expressed as mm.

Fruit fresh and dry weight

Spring fruits were harvested and weighed using a digital scale and reported as gram. Then they were oven-dried and weighed again.

Experimental design and statistical analysis

The experiment was carried out in a completely randomized block design with four replications. Data were analyzed by ANOVA test using SPSS (Ver. 9.1); means were compared using Duncan's multiple range test at 5% probability level. Cluster analysis was also performed according to Ward's minimum-variance method using SPSS (Ver. 9.1) to classify the cultivars.

3. Results

The leaf glutathione concentration in NGM (67.01 μg g^{-1} FW) was nearly 50% higher than MS (32.32 μg g^{-1} FW). CO (64 μg g^{-1} FW) was not statistically different when compared with NGM. The leaf MDA concentration in KZR (12.12 μg g^{-1} FW) was lower than MS and CO cultivars. Other caprifig trees were not statistically different (Table 2).

The leaf ascorbic acid concentration in the GK cultivar (17.03 μg g^{-1} FW), was 59% higher than DRB (7.14 μg g^{-1} FW). RS, CMB, PZD, JR, MS, and CO cultivars were not statistically different in comparison with GK. The leaf proline concentration in MS (6.77 μg g^{-1} FW) was not statistically different in comparison with GK, NGM, CMB, PR, JR, and MS cultivars. The leaf total polyphenols concentration in NGM (26.71 mg g^{-1} FW), was 56% higher when compared with the MGR cultivar. PZD, JR, and PSD were not statistically different compared to the NGM cultivar (Table 2).

The leaf dry weight in NGM was 26% higher when compared with MGR. CMB, PZD, MS, CO, PSD, and SZ cultivars were not statistically different in comparison with NGM. Leaf total sugars were not statistically different in all cultivars. LRWC in the NGM cultivar was 8.4% higher when compared with MS . IJ, GK, MLZ, DS, CO, and SZ were not statistically different in comparison with NGM. Leaf EL in MGR (22.63%) was significantly higher than DS (17.62%). This parameter was not statically different in other cultivars (Table 3).

Leaf nitrogen concentration was not statistically different in all cultivars. The leaf P concentration in DS (3.32 mg g^{-1} DW) was higher in comparison with PR, JR, and CO; other cultivars were not different.

Table 2 - The concentration of some non-enzymatic antioxidants and MDA in leaves of studied cultivars

Cultivar	Glutathione (μg g^{-1} fresh weight)	MDA (μg g^{-1} fresh weight)	α-Tocopherol (μg g^{-1} fresh weight)	Ascorbic acid (μg g^{-1} fresh weight)	Proline (μg g^{-1} fresh weight)	Polyphenol (mg g^{-1} fresh weight)
MGR	45.02 fg [z]	15.00 abc	92.92 d	7.40 c	2.83 d	17.06 e
DRB	44.30 fg	16.00 abc	110.41 ab	7.14 c	3.65 cd	17.17 e
ASA	53.3 de	14.07 bc	102.13 bc	8.88 c	3.33 d	18.19 e
KZR	57.20 cd	12.12 c	69.82 e	10.12 bc	4.41 bcd	18.88 e
IJ	50.10 ef	17.07 abc	97.04 cd	10.21 bc	3.45 cd	22.09 bcd
GK	61.11 bc	15.19 abc	72.20 e	17.03 a	5.55 abc	18.29 e
RS	53.44 d	15.41 abc	105.45 bc	12.00 abc	4.14 bcd	17.21 e
NGM	67.01 a	12.14 c	122.18 a	8.22 c	6.71 a	26.71 a
MLZ	48.15 ef	13.25 c	114.12 a	9.06 c	2.92 d	19.82 de
CMB	40.22 gh	17.15 abc	115.45 a	16.33 a	6.20 ab	18.85 e
PZD	52.36 de	18.02 abc	105.31 bc	12.14 abc	4.38 bcd	25.42 ab
DS	61.10 bc	14.14 bc	100.05 bc	7.63 c	2.78 d	23.41 bc
PR	45.16 fg	13.14 c	77.14 e	10.47 bc	5.61 abc	22.22 bcd
JR	37.35 hi	14.20 bc	98.32 cd	12.42 abc	5.99 ab	24.56 ab
MS	32.32 i	20.37 ab	77.11 e	15.15 ab	6.77 a	20.37 cde
CO	64.00 ab	21.07 a	100.07 cd	15.71 ab	4.70 abcd	20.17 cde
PSD	40.05 gh	15.3 abc	105.50 bc	9.16 c	3.77 cd	20.22 abc
SZ	41.07 gh	13.33 c	115.00 a	8.05 c	3.42 cd	22.15 bcd

[z] Means followed by the same letters within columns are not different at 5% probability using Duncan's test.

Table 3 - Leaf relative water content, electrolyte leakage, dry matter, and total sugars in studied caprifig cultivars

Cultivar	Leaf dry matter (%)	Leaf total sugars (mg g^{-1} dry weight)	Leaf relative water content (%)	Electrolyte leakage (%)
MGR	26.22 b [z]	27.33 a	77.92 d	22.63 a
DRB	28.30 b	26.05 a	81.21 abc	22.04 ab
ASA	27.33 b	27.17 a	78.89 bcd	21.87 ab
KZR	26.55 b	29.55 a	77.92 cd	19.12 ab
IJ	27.44 b	26.77 a	83.04 a	20.00 ab
GK	28.15 b	27.37 a	80.08 abcd	22.03 ab
RS	27.61 b	28.47 a	79.52 bcd	21.43 ab
NGM	33.05 a	29.14 a	83.60 a	17.96 ab
MLZ	28.15 b	30.13 a	81.11 abc	19.06 ab
CMB	30.31 ab	28.45 a	79.63 bcd	22.33 ab
PZD	29.55 ab	29.02 a	77.32 d	22.34 ab
DS	27.22 b	27.37 a	80.29 abcd	17.62 b
PR	29.71 ab	28.02 a	77.14 d	20.77 ab
JR	28.44 b	30.00 a	78.32 cd	19.72 ab
MS	29.81 ab	28.88 a	77.11 d	19.75 ab
CO	28.88 ab	30.11 a	81.00 abc	21.72 ab
PSD	29.02 ab	27.14 a	79.67 bcd	19.55 ab
SZ	28.87 ab	28.64 a	82.22 ab	19.15 ab

[z] Means followed by the same letters within columns are not different at 5% probability using Duncan's test.

The leaf K concentration in MLZ (22.46 mg g^{-1} DW) which was not statistically different compared with ASA, KZR, GK, RS, NGM, PZD, DS, PR, JR, MS, CO, PSD, and SZ. The leaf Ca concentration (4.46 mg g^{-1} DW) in NGM was 25% higher than PR. CMB had the highest leaf Mg concentration (2.48 mg g^{-1} DW); KZR had 42% lower leaf Mg concentration. Other cultivars were not statistically different (Table 4).

The leaf Fe concentration in NGM (67.71 mg kg^{-1} DW) was significantly higher than KZR, MLZ, PR, JR, MS, PSD, and SZ. The leaf Zn concentration in NGM (27.31 mg kg^{-1} DW) was not statistically different in comparison with DRB, MLZ, PSD, and SZ. Leaf Mn concentration was not statistically different in all cultivars (Table 5).

The leaf total chlorophyll concentration in NGM (1.43 mg g^{-1} FW) was not statistically different compared with DRB, ASA, KZR, MLZ, DS, MS, PSD, and SZ (Fig. 1). The leaf concentration of carotenoids in PSD was significantly higher than ASA, KZR, GK, CMB, PZD, RS, JR, and MS. Other cultivars were not statistically different in comparison with PSD (Fig. 2). The concentration of leaf anthocyanins in MGR was significantly higher than PSD. This parameter was not significantly different in other cultivars (Fig. 3).

Table 4 - Leaf macronutrients in studied caprifig cultivars

Cultivar	N (mg g^{-1} dry weight)	P (mg g^{-1} dry weight)	K (mg g^{-1} dry weight)	Ca (mg g^{-1} dry weight)	Mg (mg g^{-1} dry weight)
MGR	22.12 a [z]	2.60 ab	16.33 c	3.76 bc	2.00 ab
DRB	20.13 a	3.10 ab	17.16 bc	3.77 bc	2.22 ab
ASA	20.15 a	3.05 ab	19.65 abc	3.97 abc	2.02 ab
KZR	21.41 a	2.52 ab	18.27 abc	4.44 a	1.55 b
IJ	21.05 a	2.97 ab	17.05 bc	3.65 bc	1.95 ab
GK	21.77 a	2.55 ab	21.20 abc	3.80 bc	1.77 ab
RS	22.30 a	2.92 ab	18.85 abc	4.20 ab	1.67 ab
NGM	22.12 a	3.00 ab	20.38 abc	4.46 a	2.35 ab
MLZ	19.64 a	3.14 ab	22.46 a	3.95 abc	2.20 ab
CMB	20.51 a	2.46 ab	16.44 c	3.67 bc	2.48 a
PZD	22.35 a	2.81 ab	18.71 abc	3.72 bc	2.36 ab
DS	19.19 a	3.32 a	18.22 abc	3.90 abc	1.83 ab
PR	22.66 a	2.45 b	19.70 abc	3.55 c	2.11 ab
JR	20.44 a	2.37 b	19.00 abc	3.63 bc	2.07 ab
MS	20.11 a	2.88 ab	20.77 abc	4.18 ab	1.61 ab
CO	22.00 a	2.44 b	18.00 abc	3.76 bc	2.18 ab
PSD	21.02 a	3.11 ab	21.56 ab	4.02 ab	2.14 ab
SZ	21.48 a	3.23 ab	22.07 ab	3.89 abc	2.25 ab

[z] Means followed by the same letters within columns are not different at 5% probability using Duncan's test.

Table 5 - Leaf micronutrients in studied caprifig cultivars

Cultivar	Fe (mg kg^{-1} dry weight)	Zn (mg kg^{-1} dry weight)	Mn (mg kg^{-1} dry weight)
MGR	60.66 ab [z]	23.33 bcd	47.22 a
DRB	60.47 ab	26.50 ab	50.16 a
ASA	62.23 ab	22.74 cd	49.44 a
KZR	58.36 b	22.46 cd	50.33 a
IJ	63.33 ab	23.00 bcd	49.31 a
GK	64.44 ab	21.20 d	48.56 a
RS	60.96 ab	22.85 cd	47.91 a
NGM	67.71 a	27.31 a	48.05 a
MLZ	57.22 b	25.44 abc	50.20 a
CMB	61.22 ab	22.07 cd	50.27 a
PZD	63.42 ab	23.08 bcd	49.22 a
DS	60.75 ab	22.77 cd	48.71 a
PR	59.29 b	23.12 bcd	48.05 a
JR	57.33 b	21.90 cd	49.66 a
MS	59.40 b	22.35 cd	50.23 a
CO	60.05 ab	22.68 cd	51.44 a
PSD	58.63 b	24.88 abcd	50.63 a
SZ	59.34 b	25.14 abc	47.33 a

[z] Means followed by the same letters within columns are not different at 5% probability using Duncan's test.

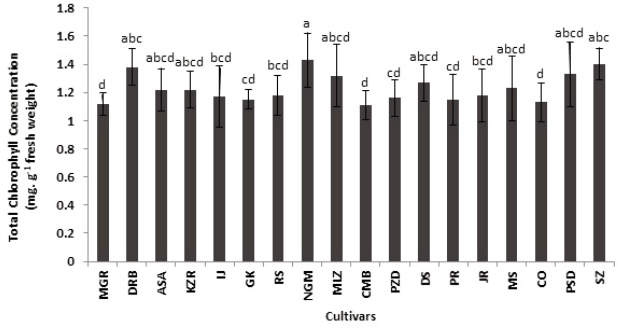

Fig. 1 - Total chlorophyll concentration in studied caprifig cultivars. Columns with the same letters are not statistically different at 5% probability using Duncan's test. Vertical bars indicate standard errors (n=4).

The highest fruit FW and DW were obtained from DS. NGM had the highest leaf length (154.66 mm). PR, MS, PZD, CMB, RS, and KZR were not statistically different. The leaf area (11581.2 mm^2) and petiole length (66.69 mm) in NGM were significantly higher than all other cultivars (Table 6).

The cluster analysis based on Ward's method divided the cultivars into three major clusters, including 8 cultivars in cluster 1, 9 cultivars in cluster 2, and 1 cultivar in cluster 3. The first cluster consisted of

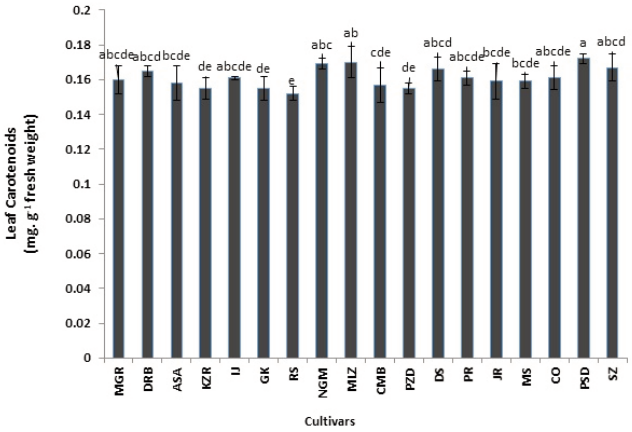

Fig. 2 - Concentration of leaf carotenoids in studied caprifig cultivars. Columns with the same letters are not statistically different at 5% probability using Duncan's test. Vertical bars indicate standard errors (n=4).

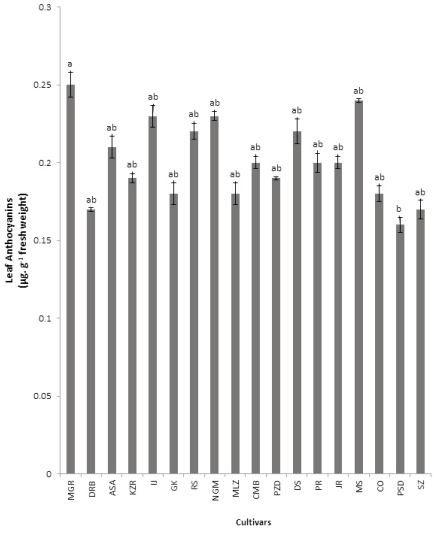

Fig. 3 - Concentration of leaf anthocyanins in studied caprifig cultivars. Columns with the same letters are not statistically different at 5% probability using Duncan's test. Vertical bars indicate standard errors (n=4).

two subclusters: subcluster 1 (MGR, IJ, ASA, and DS) and subcluster 2 (DRB, PSD, SZ and MLZ). Cluster 2 also had 2 subclusters: subcluster 1 (KZR, MS, and RS) and subcluster 2 (GK, CO, CMB, JR, PZD, and PR). Cluster 3 had only one cultivar: NGM (Fig. 4).

4. Discussion and Conclusions

Non-enzymatic antioxidants were statistically different among investigated cultivars in our study, which was consistent with previous studies. Jamali

Table 6 - Fruit fresh and dry weight and leaf and petiole length, and leaf area in studied caprifig cultivars

Cultivar	Fruit fresh weight (g)	Fruit dry weight (g)	Leaf length (mm)	Leaf area (mm^2)	Petiole length (mm
MGR	4.77 hi [z]	0.90 fg	78.97 g	1879.5 j	23.45 h
DRB	5.77 gh	1.09 ef	89.70 fg	3217.3 efg	29.36 fgh
ASA	6.48 efg	1.20 de	83.54 g	2232.6 hij	23.08 h
KZR	7.60 cde	1.21 de	121.63 bc	6352.9 b	37.46 cd
IJ	4.94 hi	0.95 fg	98.97 ef	2005.9 ij	28.60 fgh
GK	8.06 cd	1.48 bc	107.57 de	4433.1 d	def 10/33
RS	8.64 c	1.76 a	118.04 bcd	7090.9 b	50.72 b
NGM	6.63 efg	0.94 fg	154.66 a	11581.2 a	66.69 a
MLZ	4.01 i	0.72 g	79.28 g	2814.7 fgh	26.57 gh
CMB	7.12 def	1.09 ef	116.12 bcd	3698.2 def	38.30 cd
PZD	5.92 fgh	0.96 f	117.25 bcd	4373.1 d	35.95 de
DS	12.40 a	1.71 a	98.90 ef	2522.8 ghij	30.79 efg
PR	6.39 efg	1.03 ef	113.67 bcd	4554.1 d	37.77 cd
JR	6.37 efg	1.07 ef	99.53 ef	3980.3 de	32.84 defg
MS	10.88 b	1.58 ab	125.09 b	6518.2 b	42.68 c
CO	6.76 efg	1.02 ef	112.09 cd	5459.2 c	43.36 c
PSD	6.67 efg	1.36 cd	81.50 g	3334.1 efg	28.24 fgh
SZ	4.94 hi	0.9 fg	87.78 fg	2965.6 fgh	29.02 fgh

[z] Means followed by the same letters within columns are not different at 5% probability using Duncan's test.

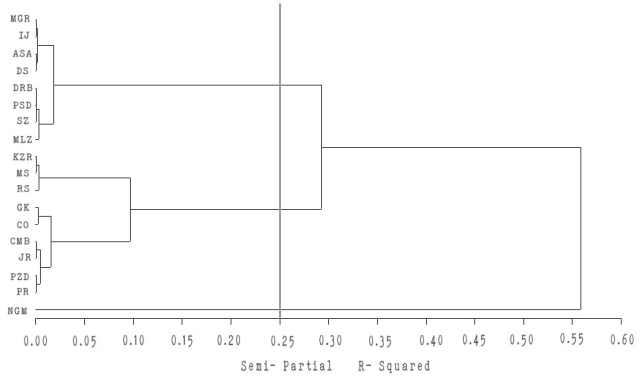

Fig. 4 - Cluster analysis dendrogram of 18 Iranian caprifig cultivars based on Ward's method.

and Bonyanpour (2017) determined leaf mineral composition and also some biochemical parameters, i.e., concentration of non-enzymatic antioxidants in leaves, in seven Iranian pomegranate cultivars for selecting probable more tolerant cultivars. They found that cultivars with a higher concentration of non-enzymatic antioxidants (e.g., glutathione, α-Tocopherol, proline, etc.) had better adaptability to the prevailing environmental conditions. As mentioned earlier, various species and cultivars have inherently different potential to tolerate non-optimal growth conditions. One of the possible reasons for this difference is their enzymatic/non-enzymatic antioxidant responses and/or their ability to absorb macro/micro nutrients. Therefore, determination of these characteristics can be used as biochemical markers for cultivar comparison/screening.

Gholami *et al*. (2012) compared four fig cultivars (Deyme Ahvaz, Sabz Estahban, Siah, and Shahanjir) under normal, drought, and recovery conditions. According to their results, some of the non-enzymatic and enzymatic antioxidants in leaves differed significantly under optimal conditions. Their findings show that the fig cultivars demonstrated a clear difference in their response to water stress and recovery. They evaluated Deyme Ahvaz as a more tolerant cultivar than Sabz Estahban. Deyme Ahvaz had higher leaf concentration of glutathione and anthocyanin in comparison to Sabz Estahban under non-stress conditions.

Samec *et al*. (2021) reviewed the role of phenolic compounds in inducing tolerance to non-optimal conditions in plants. They reported that the potential tolerance of a plant species to stress conditions can be associated with this diverse family of chemicals which is universally present in plants and includes

more than 8000 molecules. Phenolic compounds play an essential role in plant responses, especially in defense mechanisms. They have strong antioxidant properties and neutralize the detrimental effects of reactive oxygen species (Balasundram *et al*., 2006). The absolute concentrations of these chemicals in plant organs vary significantly depending on the determination method, cultivar, or tissue, but it can be concluded that higher phenol concentrations correlate with increased stress tolerance.

Arteaga *et al*. (2020) have proposed the use of proline as a biochemical marker for rapid and simple large-scale screening of different genotypes for drought and salt tolerance. Transgenic plants, especially those overexpressing genes for proline accumulation, show higher adaptation to abiotic stresses (El Moukhtari *et al*., 2020; Ghosh *et al*., 2022. Similarly, endogenous proline concentration has been linked to a relatively higher stress tolerance when comparing different cultivars by various authors in different species (Kapuya *et al*., 1985; Misra and Gupta, 2005; Goharrizi *et al*., 2020).

The lower MDA content in NGM, RS, or MLZ may be due to the higher content of antioxidants such as α-tocopherol in these two caprifigs. This is consistent with previous studies (Assaha *et al*., 2015; Amoah *et al*., 2019; Nawaz and Wang, 2020).

Except for leaf N and Mn concentration, other macro and micronutrients were significantly different in various caprifig cultivars. This was in agreement with previous studies on different fig cultivars (Anac *et al*., 1982; Aksoy *et al*., 1987; Askin *et al*., 1998; Hakerlerler *et al*., 1998; Bougiouklis *et al*., 2020).

The higher leaf DW or LRWC in NGM could be attributed to the higher leaf concentration of K, Ca, Fe, or Zn in this caprifig in comparison with other studied cultivars. This was in agreement with previous studies as various cultivars absorb macro/micronutrients differently. Hegwood (1972) reported a significant varietal effect on leaf mineral composition during full bloom and harvest in eleven snapbean cultivars. Jordão *et al.* (1999) studied 15 olive cultivars and found that the mean effect of cultivar on leaf concentrations of essential elements, including N, P, K, Ca, Mg, S, Mn, Zn, and B, was significant. There are also many other examples of the effects of cultivar or root system of different species on leaf mineral composition (Tsipouridis and Thomidis, 2005; North and Cook, 2006; Tomala *et al*., 2008; Kviklys *et al*., 2012).

This difference in endogenous macro/micronutrient composition can affect numerous aspects of plant growth and development. In other words, the presence of a particular macro- or micronutrient (e.g., Ca) above a critical concentration can alter the plant's response to non- optimal growing conditions (Pilbeam and Morley, 2007; Taiz and Zeiger, 2010). For instance, Fageria (2013) reported an increased root DW in 12 lowland rice genotypes after adding K fertilizer. They found that increase in root DW at high K levels was 246% compared to the low level of K.

Leaf chlorophyll and carotenoids concentration were significantly different in studied cultivars. Chlorophyll concentration is a sensitive biochemical marker indicating cellular metabolic state (Chutipaijit *et al*., 2011). Previous studies have shown that chlorophyll concentration and stability correlate with plants' high tolerance potential against abiotic stresses such as salinity or drought (Hasanuzzaman *et al*., 2013). Carotenoids protect the photosynthesis apparatus by scavenging free radicals keeping its integrity against photo-oxidative damages (Dall'Osto *et al*., 2007; Andrade-Souza *et al*., 2011). As an ABA precursor, higher carotenoid concentration means lower photo-oxidative damage and elevated potential for regulating plant growth under stress conditions (Götz *et al*., 2002; Han *et al*., 2008).

Caprifig cultivars in our study were clustered into three main groups indicating lower level of variability within clusters and higher variability levels between clusters. Morphological and biochemical markers have long been used for screening and characterizing different genotypes, as they are the first steps in describing/classifying any germplasm (Cantini *et al*., 1999). Previous studies on figs show the usefulness of these markers in documenting variability among genotypes (Salhi-Hannachi *et al*., 2006; Saddoud *et al*., 2008; Podgornik *et al*., 2010).

NGM had the highest leaf glutathione, α-Tocopherol, proline, polyphenols, Ca, and Zn concentrations. Leaf length, leaf area, and petiole length were the highest in this cultivar. Cluster analysis indicated that NGM was the only cultivar in cluster 3. In conclusion, NGM was evaluated as a genotype which seems to be more distant (morphologically and biochemically) from other studied caprifigs and probably more adaptable/tolerant. Our findings can be used as an overture for complementary studies for selecting promising genotypes for breeding and introducing new tolerant fig cultivars.

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Short note

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Grafting compatibility between Okra cultivars and root-knot nematode resistant Kenaf

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Key words: *Abelmoschus esculentus* L. Moench, *Hibiscus cannabinus* L., intergeneric grafting, *Meloidogyne* spp.

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All relevant data are within the paper and its Supporting Information files.

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Abstract: The use of intergeneric grafting has been reported as an alternative to manage root-knot nematodes in okra, but the compatibility for grafting has only been tested in a few okra (*Abelmoschus esculentus* L. Moench) cultivars. The kenaf (*Hibiscus cannabinus* L.) is resistant to root-knot nematode species and is a potential rootstock for okra. The objective was to study the compatibility of kenaf as rootstock with okra cultivars. It was used a completely randomized design, in factorial scheme 3x10, with five repetitions. The compatibility was assessed by measuring several vegetative characteristics. All cultivars are compatible for grafting with kenaf as rootstock. Grafting onto kenaf may be an option to control root-knot nematodes.

1. Introduction

The okra (*Abelmoschus esculentus* L. Moench) is mainly cultivated for its immature fruits but presents several industrial applications (Dantas *et al*., 2021). In Brazil, okra is widely cultivated, mainly by family farmers, as it is considered a low production cost crop that adapts to tropical and subtropical climates. Although it is rustic, a limiting factor for the crop has been the root-knot nematodes (*Meloidogyne* spp.) (Silva *et al*., 2019 a), which are favored by high temperature and humidity, which are also necessary for the development of the crop.

To control the root-knot nematode, it is necessary to integrate several management practices, from the choice of the planting area to pre-sowing, such as prevention, crop rotation, fallow, and the use of antagonistic plants (Collange *et al*., 2011; Nascimento *et al*., 2020). Other approaches, such as organic fertilization, biological control, and heat-based methods are also a possibility (Mahalik and Sahoo, 2019). However, the use of resistant cultivars is considered the most efficient method, but, to date, there are no reports of genetic resistance effectively incorporated in commercial cultivars.

Recent studies have been exploring promising alternatives, such as intergeneric grafting with species resistant to root-knot nematodes. Among the possible rootstocks, some *Hibiscus* spp. have been shown to be interesting for being resistant and compatible for grafting with okra (Marin *et al*., 2017; Silva *et al*., 2019 b; Andrade *et al*., 2020). In addition to genetic resistance, rootstocks recommended to manage some type of pathosystem must be compatible with the scion, ensuring normal vegetative and reproductive development. It is noteworthy that, by obtaining resistant and compatible rootstock, crop yield can be enhanced by minimizing negative effects generated by the nematodes on the plant.

Research has tended to focus on only two open-pollinated cultivars, namely 'Santa Cruz 47' and 'Colhe Bem', with the need to verify whether compatibility also occurs in other cultivars, as the compatibility may vary according to rootstock-scion combinations (Reig *et al*., 2018). Given the above, the objective of the present study was to assess the compatibility of the kenaf (*Hibiscus cannabinus* L.) with commercial okra cultivars.

2. Materials and Methods

The experiment was carried out at the Sector of Vegetables and Aromatic-Medicinal Plants, School of Agriculture and Veterinary Sciences, Unesp, Campus of Jaboticabal, SP (21°14'05'' S, 48°17'09" W, 614 m of altitude), from March to May 2019. The climate type is Aw, described as a tropical winter dry season, which occurs from April to September and rains are concentrated from October to March, with transition to Cwa. The average temperature and relative humidity during the period of the experiment is presented in figure 1.

A completely randomized design in a factorial scheme 3x10 with five replications was used. The first factor was seedling production modalities, being non-grafted, self-grafted, and grafted onto kenaf. The second factor consisted of ten okra cultivars, being 6 hybrids and 4 open-pollinated cultivars (Table 1).

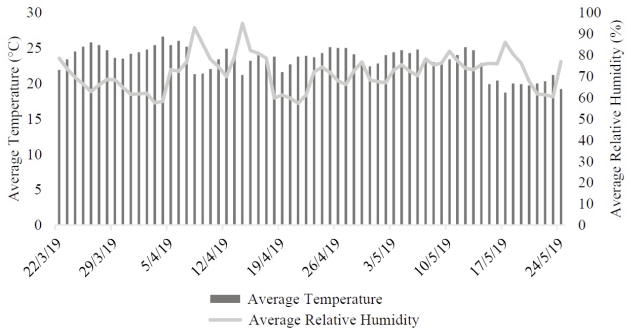

Fig. 1 - Changes in temperature and relative humidity during the experiment.

The okra seeds were immersed in acetone for 30 minutes to break dormancy, whilst kenaf seeds were immersed in water for five hours. Then, the genotypes were sown in 128-cell expanded polystyrene trays, filled with commercial substrate coconut-fiber based Bioplant® (Nova Ponte, MG, Brazil) to produce vegetable seedlings. The trays were placed in a greenhouse equipped with a sprinkler irrigation system.

Table 1 - Identification and origin of the okra and *Hibiscus* genotypes used in the experiment

Cultivar	Origin	Pollination
Brutus	Feltrin Sementes	Hybrid
Canindé	Isla Sementes Ltda.	Hybrid
Clemson	Isla Sementes Ltd.	Open
Colhe Bem	Sakata Seed Sudamérica	Open
Esmeralda	Agristar do Brasil Ltd.	Hybrid
Guará	Isla Sementes Ltd.	Hybrid
Santa Cruz 47	Feltrin Sementes	Open
V8	Agristar do Brasil Ltd.	Hybrid
Valença	Feltrin Sementes	Open
Xingó	Eagle Flores, Frutas e Hortaliças Ltd.	Open
Kenaf	Embrapa Hortaliças	Open

The seedlings were cleft-grafted as described by Silva *et al*. (2019 b) when the seedlings presented the cotyledonary leaves fully exposed. The scion was inserted in a cut of approximately 1 cm between the cotyledonary leaves of the rootstock. The scions were standardized to 3 cm in length. The scion was fixed so that it did not shade the cotyledonary leaves. After positioning the scion, a clip was placed to ensure the connection with the rootstock, until complete healing of the grafting site. The grafting was carried out on the seedling production trays.

After the grafting was carried out, the plants were placed in a humid floating chamber to secure high temperature and humidity, which is adequate for okra grafting healing. The grafted seedlings remained in the humid chamber for 11 days, and then transplanted to pots containing autoclaved clayey red latosol (oxisol) soil. The pots were placed in a greenhouse.

The percentage of grafting success was assessed

for the self-grafted and grafted onto kenaf treatments. The number of leaves (NL), the number of internodes (NI), and the number of internodes up to the first flower (NIF) were counted. The plant height (PH) and first flower height (FFH) were evaluated with the aid of a ruler graduated in centimeters. The diameter of the rootstock (DR) was measured with the aid of a digital caliper. The plants were cut close to the ground when the plants started flowering, which occurred 49 days after grafting, followed by weighing the fresh shoot mass (FM), and the dry shoot mass (DM) was weighed after drying in a forced air circulation oven, set at 60°C for 72 hours.

To meet the assumptions of ANOVA, the data was transformed using the Box-Cox method (Box and Cox, 1964), except for first flower height, number of internodes up to the first flower and diameter of the rootstock. Then, the data was subjected to two-way ANOVA and post-test. The means were compared using the Scott-Knott test at 5% significance, using the AgroEstat statistical software (Barbosa and Maldonado, 2015).

3. Results and Discussion

The percentage of grafting success was evaluated, however, it was not statistically analyzed, since the self-grafted and grafted onto kenaf treatments reached 100% of success for all cultivars, so there was no variance. Okra and kenaf are both malvaceous species but belonging to different genera, which theoretically hamper grafting healing process as physiological, morphological, and botanical discrepancies are expected to occur (Silva *et al*., 2019 b). However, despite the distinguishing characteristics, adequate development was verified for grafted okra seedlings of all cultivars. Thus, all cultivars are compatible with the kenaf rootstock as they present vegetative development similar to the self-grafted and non-grafted seedlings, except for number of leaves, plant height, and first flower height (Table 2). According to Belmonte-Ureña *et al.* (2020), there is compatibility when the plant formed by scion and rootstock has the capacity to develop as a single plant. These results confirm the compatibility with

Table 2 - Analysis of variance and test of comparison of means of vegetative variables of okra cultivars and seedling production modalities

	NL	NI	NIF (cm)	PH (cm)	FFH (mm)	DR (mm)	FM (g)	DM (g)
Cultivar (C)								
Brutus F1	8.13 a	8.40 a	6.13 b	42.47 c	28.53 b	14.40	49.70	9.61 a
Canindé F1	8.67 a	6.66 c	4.33 d	47.93 b	18.40 d	14.35	40.44	7.50 b
Clemson Americano 80	6.00 b	7.06 c	4.86 d	40.40 c	21.80 c	14.46	38.08	7.03 b
Colhe Bem	7.33 a	8.53 a	7.80 a	37.53 d	34.26 a	14.44	45.39	7.96 b
Esmeralda F1	6.40 b	7.60 b	5.40 c	42.27 c	25.33 c	14.44	41.21	8.25 a
Guará F1	6.33 b	6.53 c	4.33 d	72.80 a	28.13 b	14.30	37.23	9.79 a
Santa Cruz 47	6.33 b	8.33 a	7.80 a	35.00 d	33.47 a	14.43	40.63	6.65 b
V8 F1	5.87 b	7.80 b	6.20 b	37.97 d	27.87 b	14.35	41.87	6.82 b
Valença	6.73 b	7.26 c	4.86 d	41.47 c	22.60 c	14.41	45.05	8.75 a
Xingó F1	6.33 b	7.53 b	5.40 c	44.80 b	28.13 b	14.49	51.29	9.37 a
Test F	4.56 **	8.93 **	26.18 **	40.70 **	19.59 **	1.07 NS	0.95 NS	1.98 *
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.3960	0.4873	0.0471
Seedling production (S)								
Non-grafted	8.58 a	7.68	5.80	47.64 a	29.44 a	-	44.27	8744
Self-grafted	5.94 b	7.62	5.82	46.78 a	28.52 a	14.44	43.64	8145
Grafted onto kenaf	5.92 b	7.42	5.52	38.37 b	22.60 b	14.37	41.35	7625
Test F	37.77 **	1.34 NS	1.55 NS	35.62 **	36.27 **	3.81 NS	0.86 NS	1.74 NS
P-value	<0.0001	0.2669	0.2169	<0.0001	<0.0001	0.0543	0.4262	0.1808
Interaction C × S	2.40 **	1.57 NS	1.48 NS	2.53 **	1.58 NS	1.41 NS	1.18 NS	1.46 NS
P-value	0.0027	0.0799	0.1105	0.0015	0.0749	0.1975	0.2884	0.1159
CV (%)	9.47	6.19	16.84	11.20	16.23	1.25	17.21	33.97

NS, **, * not significant or significant at 1 or 5% probability. Data transformed according to the Box-Cox method.
Means followed by the sameletter do not differ by the Scott-Knott test, P<0.005.
NL = number of leaves, NI = number of internodes, NIF = number of internodes up to the first flower, PH = plant height, FFH = first flower height, DR = diameter of the rootstock, FM = fresh shoot mass, DM = dry shoot mass.

okra that, until then, had been demonstrated only for the open-pollinated cultivars 'Colhe Bem' and 'Santa Cruz 47' (Marin *et al*., 2017; Silva *et al*., 2019 b).

The interaction between cultivar and seedling production modality was significant only for the number of leaves and plant height (Table 2). Thus, the seedling production modality influenced the cultivars equally for most traits, even though they are genetically different. The grafting influenced the number of leaves of the cultivars Brutus, Clemson Americano 80, Esmeralda, Valença, Xingó, and V8, as the non-grafted showed higher average than the other seedling production modalities (self-grafting and grafting onto kenaf) (Table 3). It should be considered that grafted plants are subjected to additional stress that involves the grafting and healing process (Melnyk, 2017). After grafting, to heal the wound, there is callus formation followed by tissue differentiation to reestablish the vascular connections (Xie *et al*., 2019). Thus, it is expected that the non-grafted plants have a faster initial development, which does not necessarily indicate grafting incompatibility.

The cultivars differed as to the number of leaves (Fig. 2). For non-grafted plants, 'Brutus'and 'Valença' had the highest number of leaves. 'Canindé' presented the highest number of leaves self-grafted and grafted onto kenaf (Fig. 2). The grafting, either self-grafting or onto kenaf, reduced the number of leaves and plant height of five cultivars (Brutus, Esmeralda,

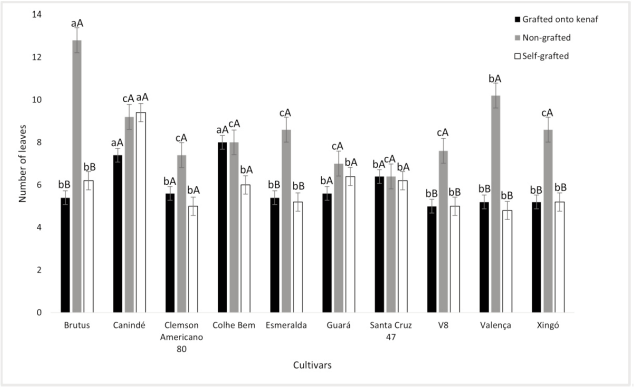

Fig. 2 - Interaction between cultivars and seedling production modality for the number of leaves. Means followed by the same uppercase (between seedling production modalities for each cultivar) and lowercase (between cultivars for each seedling production modality) letter do not differ by the Scott-Knott test (P<0.005).

V8, Valença, and Xingó), which may indicate slower initial development due to the stress caused by the grafting procedure.

Higher non-grafted cultivars tended to result in higher plants when grafted, either self-grafted or onto kenaf (Fig. 3). In this sense, 'Guará' presented the highest value of plant height in all seedling production modality. 'Brutus', 'Canindé', 'Clemson Americano 80', 'Guará', 'Valença', and 'Xingó' presented shorter plants when grafted onto kenaf (Fig. 3). The plant height should be considered concomitantly with the number and length of internodes (Sandeep *et al*., 2022), as fruit production is directly correlated with number of internodes. Higher plants

Table 3 - Post-analysis of the interaction between cultivars and grafting for the number of leaves of okra cultivars and seedling production

Cultivar	Number of leaves			Test F	P-value
	Non-grafted	Self-grafted	Grafted onto kenaf		
Brutus F1	12.80 aA	6.20 bB	5.40 bB	23.53 **	<0.0001
Canindé F1	9.20 b	9.40 a	7.40 a	2.29 NS	0.1059
Clemson Americano 80	7.40 bA	5.00 bB	5.60 bB	2.92 NS	0.0579
Colhe Bem	8.00 b	6.00 b	8.00 a	1.90 NS	0.1534
Esmeralda F1	8.60 bA	5.20 bB	5.40 bB	6.42 **	0.0022
Guará F1	7.00 b	6.40 b	5.60 b	1.00 NS	0.3708
Santa Cruz 47	6.40 b	6.20 b	6.40 a	0.03 NS	0.9702
V8 F1	7.60 bA	5.00 bB	5.00 bB	4.30 *	0.0158
Valença	10.20 aA	4.80 bB	5.20 bB	15.40 **	<0.0001
Xingó F1	8.60 bA	5.20 bB	5.20 bB	7.31 **	<0.0001
Test F	4.69**	3.34**	2.09*		
P-value	<0.0001	0.0011	0.0353		

The data presented is original, but for statistical analysis, the data were transformed into $(x+0.5)^{1/2}$.
Means followed by the same uppercase (row) and lowercase (column) letter do not differ by the Scott-Knott test (P<0.005).
NS = Not significant; ** Significant at 1% probability; * Significant at 5% probability.

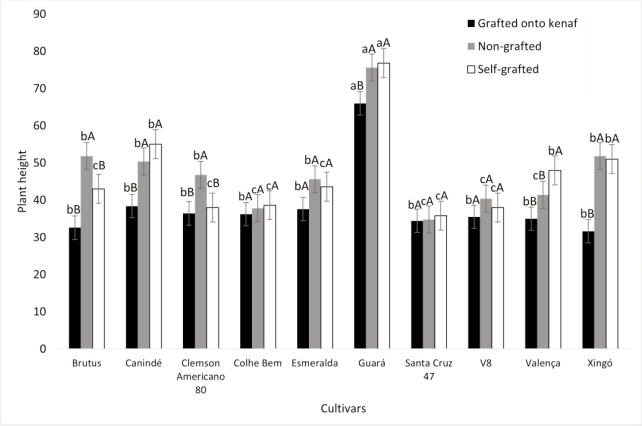

Fig. 3 - Interaction between cultivars and seedling production modality for plant height. Means followed by the same uppercase (between seedling production modalities for each cultivar) and lowercase (between cultivars for each seedling production modality) letter do not differ by the Scott-Knott test (P<0.005).

with long internodes are not ideal, as the plant will probably reach a stature that will hamper harvesting the fruits sooner.

The genotypic variability and the differential behavior as for the seedling production modalities can be confirmed from the analysis of variance and the mean test (Table 2). Significant differences between cultivars were detected for the variables number of internodes, number of internodes to first flower, first flower height, and scion diameter. Furthermore, the seedling production modalities differed as to first flower height and scion diameter.

The cultivars were grouped into two groups for the variables scion diameter and dry shoot mass, into three groups for the variable number of internodes, and into four groups for the variables number of internodes up to the first flower and first flower height. There were no significant differences for the variables diameter of the middle portion of the plant, rootstock diameter and fresh shoot mass.

The cultivars Colhe Bem, Brutus, and Santa Cruz 47 had higher number of internodes. The cultivars Colhe Bem and Santa Cruz 47 had higher NIF; The cultivars Colhe Bem and Santa Cruz 47 had the highest first flower height; the cultivars Xingó, Colhe Bem, Esmeralda, V8, Valença, Guará, and Brutus had higher scion diameter and the cultivars Guará, Brutus, Xingó, Valença, and Esmeralda had higher dry shoot mass.

As for the factor seedling production modality, besides number of leaves and plant height, differences were detected for the first flower height and scion diameter, with smaller and larger means for grafting onto kenaf, respectively. This indicates that most variables are not affected by grafting. No differences were detected for number of internodes to first flower, which suggests that the first flower insertion height differed due to internode length of plants grafted onto kenaf. The yield potential of okra depends considerably on the number of nodes per plant as the inflorescence consists of a single flower (Bhatt and Rao, 2009), so the smallest first flower height insertion can be advantageous as production starts lower.

The scion diameter was greater in plants grafted onto kenaf than self-grafted plants. This differs from the findings reported by Andrade *et al*. (2020), who verified largest diameters for self-grafted 'Santa Cruz 47' plants compared to roselle (*Hibiscus sabdariffa* L.) and other malvaceous rootstocks. The scion diameter depends greatly on the water and nutrients translocation, which can be influenced by the compatibility between rootstock and scion. Silva *et al.* (2019 b) previously reported that kenaf is a better rootstock for okra as it presents greater compatibility compared to other malvaceous genotypes. This result may be associated to the large root volume of kenaf plants (Alexopoulou *et al*., 2013), which improve water and nutrient absorption (Gaion *et al*., 2017).

Okra grafting is a potential tool to cope with root-knot nematodes, which are one of the main phytosanitary issues in okra cropping. Although most okra farmers employ little technology, we anticipate that grafted seedlings will be a feasible option as grafting will become cheaper when this process is automatized (Silva *et al*., 2019 b). Furthermore, grafting onto resistant rootstocks is a sustainable strategy that contributes to lessen pesticides use, which are potentially damaging to the environment and human's health (Thies, 2021).

The cultivars Brutus, Canindé, Clemson Americano 80, Colhe Bem, Esmeralda, Guará, Santa Cruz 47, V8, Valença, and Xingó are compatible with the kenaf rootstock, therefore the grafting can be used in okra as a strategy to control root-knot nematodes in all analyzed cultivars. Although it is an intergeneric grafting combination, the grafted seedlings showed no sign of incompatibility until flowering. However, further studies are necessary to confirm compatibility throughout the cycle. Furthermore, the agronomic performance should be evaluated under field conditions to validate the use of this technique in the okra crop.

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