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Reduction of leaf tip burns of *Ornithogalum dubium* by controlling the temperature during bulb storage and greenhouse forcing to produce quality plants

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Key words: Boron toxicity, controlled flowering, leaf morphology, optimum temperature, quality criteria, scape growth.

Abstract: Production of quality potted *Ornithogalum dubium* Houtt. plants were investigated under multiple conditions: pre-planting treatment at 10, 16, and 22°C for 40 days from Sept. 21 (stage A; ST-A) during bulb storage and then bulbs were potted. After potting, post-planting treatment at 15/12, 18/15, and 21/18°C (day/night) during stage B for 35 days from Nov. 2 (stage B; ST-B), and at 15/12 and 21/18°C during stage C for 30 days from Dec. 7 (stage C; ST-C) during greenhouse forcing was applied. Leaf tissue analyses for macro- and micronutrients were performed to investigate the cause of leaf tip burn symptom (LTB). Three criteria for quality of the plants at flowering were established: (1) LTB occurs on less than 1.5 leaves per plant. (2) the number of days to flower is less than 115 days, the length of the third leaf counted from the crown (the junction of the shoot and roots) is shorter than 11.5 cm, and the width is narrower than 2.5 cm; the scape length is shorter than 15 cm, and there are more than 45 flowers. (3) the leaf spread and morphology (leaf spread) and the pattern of the scape curvature (scape growth) have a score of less than 1.5. The following conditions are optimal to produce quality plants based on these three criteria: (1) Pre-planting bulbs treatment was applied at 10 or 16°C during ST-A, and forcing was performed at 15/12°C during ST-B and 21/18°C during ST-C. These conditions accelerated flowering, produced straight scape growth and upward (erect) growing leaves, and yielded acceptable leaf length and width. (2) The incidence of LTB was minimal at 10°C or possibly 16°C during ST-A, and at 15/12°C in ST-B and at 21/18°C in ST-C during greenhouse forcing. Leaf tip burn symptom was observed in both young and old leaves and was caused by a high boron (B) concentration (218-230 ppm) and possibly a high zinc (Zn) concentration (155-159 ppm) in *O. dubium*. A low calcium (Ca) concentration was not the cause of LTB. Although LTB cannot be avoided, it can be minimized by temperature manipulation during pre- and post-planting phase to produce high quality potted plants.

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1. Introduction

Ornithogalum dubium Houtt. is a geophyte native to South Africa with yellow or orange flowers with a dark greenish-brown center (Du Plessis *et al*., 1989; Littlejohn and Blomerus, 1997). Temperature treatments during the pre-planting phase before potting the bulbs and the greenhouse forcing phase after planting the bulbs should be optimized to produce quality *O. dubium* potted plants. "Leaf tip scorch" was believed to be caused either by genetics or by high salt levels in the growing medium after treatment with 5.5 grams of a slow-release fertilizer. However, no information on the salt levels in the potting medium or leaf tissue analyses was presented (De Hertogh and Gallitano, 1997).

The boron (B) concentration of healthy and necrosed leaves of the Oriental hybrid lily 'Star Gazer' did not significantly differ, although those of Ca, magnesium (Mg), manganese (Mn), and copper (Cu) were higher in necrosed than in healthy leaves (Chang, 2002). In the older leaves of *Curcuma* 'Chiangmai University Pride' ('CMU Pride'), a high B concentration (122 ppm) at the margin of leaves may have caused the marginal leaf burn (Roh *et al*., 2006). It is unclear whether LTB in *O. dubium* is similar to marginal leaf burn observed in the older leaves of *Curcuma* 'CMU Pride'.

Leaf scorch symptoms were also reported in *Lilium* (Berghoef, 1986; Chang *et al*., 2008). In *Lilium ×elegans*, 'Red Carpet' and 'Sterling Star,' the scorch symptoms were caused by a low calcium (Ca) concentration resulting from an inefficient translocation of radioactive calcium (^{45}Ca) to the tip of the leaves (Roh, unpublished data). The critical Ca concentration associated with upper leaf necrosis of *Lilium* 'Star Gazer' was 0.3 to 0.4%, and upper leaf necrosis was caused by low Ca concentration (Chang, 2002) although "it is difficult to isolate a single characteristic to explain the observed cultivar variation to upper leaf necrosis in Oriental hybrid lilies" (Chang *et al*., 2008).

Although forcing techniques in geophytes, such as tulip and lily, have been well documented (De Hertogh, 1974), forcing techniques for *O. thyrsoides* and *O. dubium* to produce quality potted plants are still lacking, especially regarding the incidence of LTB (Jansen van Vuuren and Holtzhausen, 1992; Littlejohn and Blomerus, 2000; Suh *et al*., 2000; Luria *et al*., 2002; Reinten *et al*., 2011; Lee and Miller, 2015). Flowering of *O. dubium* was accelerated when the plants were forced at 27/22°C (Luria *et al*., 2002) or 19/13°C (Suh *et al*., 2000) compared with 17/12°C or 13/10°C, respectively. Flowering was accelerated in several new cultivars forcing at 22/18°C during a short day between the visible bud stage to flowering (Lee and Miller, 2015). The highest (visual) quality *O. dubium* cultivars were produced when forcing occurred at constant 17 to 19°C (Lee and Miller, 2015), which is comparable to 22/18°C for forcing seed-raised bulbs (De Hertogh and Gallitano, 1997). However, the criteria used to assess the visual quality were not specified (Lee and Miller, 2015).

The objectives of this research were to produce quality potted *O. dubium* plants by reducing the incidence of LTB, accelerating flowering while ensuring the maximum number of flower buds, and desirable morphologies as influenced by temperature treatment during the bulb handling and greenhouse forcing stages to establish the optimum temperature regimes that reduce the incidence of LTB, and accelerate flowering with desirable morphologies. The number of leaves showing LTB, the time of flowering, and the leaf and floral morphology were used to develop criteria for quality *O. dubium* potted plants. The macro- and micro-elements of the leaf tips showing LTB/no LTB during growth and development were analyzed to determine the cause of LTB.

2. Materials and Methods

General cultural practices

Ornithogalum dubium bulbs (5-6 cm in circumference) purchased from Agrexco (Jamaica Plain, NY, USA) were used in the experiment conducted between 1999 and 2002. The bulbs were stored dry or potted, with one bulb per 6.3 or 10 cm pot, filled with ProMix (Pro-Mix BX mycorrhizae, Quakertown, PA, USA). Temperature treatments were performed in growth chambers as specified in each experiment with a 12 h day (29 $W \cdot m^{-2}$)/night (day: 08:00-18:00 hr; night: 18:00-08:00 hr). At planting, 0.8 g of a slow-release fertilizer (14 N - 6.2 P - 11.6 K, Osmocote, Scotts Co., Marysville, OH, USA) was applied to the surface of the growing medium. In addition, the plants received 200 ppm N from 15 N - 16 P - 18 K water-soluble fertilizer (JR Peter's Laboratory, Allentown, PA, USA) once a month during culture in the greenhouse.

The effect of the pre-plant bulb (ST-A) and post-plant forcing (ST-B and ST-C) temperatures on growth, flowering, and plant quality

Orntihogalum dubium bulbs (7-8 cm in circumference) from the previous experiment were harvested and stored dry at 20°C until potting on June 1 and then potted per 10 cm pot filled with ProMix and grown at 18/15°C. Leaf samples were collected for tissue analysis on July 12, 2000 from leaves without any LTB.

The tips and basal portions of the leaves were separated, and the width at mid-leaf was 2 cm. Samples were collected from the third and fourth leaves of each plant, counted from the tip of the bulbs, and were combined into a sample. The third leaf was collected from 15 plants, and the leaves were divided into three segments. The two-cm long distal end (tip) did not show any incidence of LTB, the proximal end (base) of each leaf, and mid portion between the tip and base was used for macro- and micro-element tissue analysis.

In another experiment for macro- and micro-element analysis, bulbs were harvested after forcing in 2000, and were potted in 2001. On Jan. 13, 2002, flowers began to develop and the flowers had fully developed color and were ready for anthesis. Leaves from the distal end (tip) with and without LTB (Fig. 1) and the proximal end (base) were collected from 15 plants during forcing on Jan. 14 and 26, Feb. 3 and 10, and Mar. 4. The leaf samples were dried at 70°C for 4 days and were ground into fine particles using a mortar and pestle. Two replicates of the dried leaves were sent to the JR Peters Laboratory (Allentown, PA, USA).

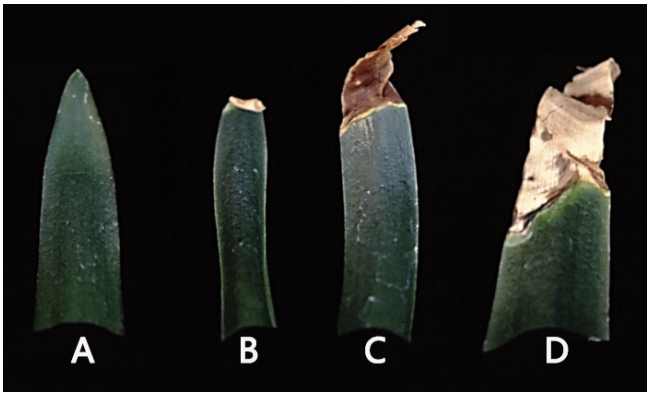

Fig. 1 - Various degrees of leaf tip burn (LTB) symptoms in *Ornithogalum dubium* leaves. Leaf without evident LTB symptom (A) and different degree of LTB symptoms (B, C, and D).

Bulbs purchased on Sept. 15, 1999 were used in this experiment. Before potting, *O. dubium* bulbs were stored at constant temperatures of 10, 16, and 22°C for 40 days from Sept. 21 (ST-A). Bulbs were planted 2 cm deep of the nose from the surface of the growing medium, and leaf emergence through the growing medium was recorded from potting when temperature treatment of ST-A started. Bulbs were potted (one bulb per 6.3 cm pot) filled with the ProMix medium, and the pots were placed in a greenhouse maintained at 15/12, 18/15, and 21/18°C for 35 days from Nov. 2 (ST-B). On Nov. 2, a slow-release fertilizer was applied to the surface of the growing medium, and plants received 200 ppm N water-soluble fertilizer once a month. The leaves emerged (about 0.5 cm or less above the nose of bulbs) before potting on Sept. 21. On Dec. 7, plants were divided into two groups and grown in a greenhouse maintained at 15/12 and 21/18°C for 30 days (ST-C) (Table 1). There were 16 plants per treatment. Starting from Jan. 6, all plants were grown in a greenhouse maintained at 19/16°C until flowering, and data were collected at anthesis.

Criteria establishment for quality evaluation

When 3-4 flowers reached anthesis, the date of flowering, the number of total leaves showing LTB (Fig. 1) excluding the incipient LTB (Fig. 2) and the scape length, number of flowers, leaf spread, scape growth (Fig. 2, 3) were recorded. The leaf spread was based on the angle of the leaf measured from the base to the tip using the following scores: 1: greater than 60° (pointing upward, erect); 2: between 30 and 60°; 3: old leaves prostrate and drooping downward at less than 15°. In addition, the scape growth was used as a quality criterion using the following scores: 1: no bending of the scape; 2: leaning sideways without bending at the base of scape; 3: scape at the base starting to grow at about 40° and continued sideways growth.

Data analysis

Data were analyzed using SAS® System Version 9 for Microsoft® Windows® (SAS Institute Inc., 2002). The number of total leaves, number of leaves with LTB, and the scores of the leaf spread and scape orientation were analyzed following a square root transformation $(x + 0.5)^{1/2}$. Means were compared using Duncan's Multiple Range test (DMRT) at the significant level indicated in the tables.

Table 1 - The number of total leaves and of leaves showing leaf tip burn by leaf position, and the total number of leaves showing leaf tip burn symptoms in *Ornithogalum dubium*

Individual plant (number)	10°C ST-A, 15/12°C ST-B, 21/18°C ST-C [y]			22°C ST-A, 18/15°C ST-B, 15/12°C ST-C [x]		
	No. of total leaves per plant	Tip burn symptom		No. of total leaves per plant	Tip burn symptom	
		Leaf position [z]	Total number of leaves		Leaf position number	Total number of leaves
1	5	2.5	2	5	1,3,4,5	4
2	4	1,2,3,4	4	6	2,3,4,5,6	5
3	4	-	0	6	3,4,5,6	4
4	5	5	1	6	5.6	2
5	4	-	0	6	1,3,4,5,6	5
6	5	3	1	5	3,4,5	3
7	6	4.5	0	5	1,2,3,4,5	5
8	6	4	1	6	3,4,5,6	4
9	5	-	0	6	2,3,4,5,6	5
10	6	-	0	5	1,2,3,4,5	5
11	5	4,5,6	3	5	2,3,4,5	4
12	5	-	0	8	1,2,3,4,5,6,7,8	8
13	6	-	0	4	1,3,4	3
14	5	-	0	6	3,4,5,6	4
15	5	1,2,3,4,5	5	5	3,4,5,6	4
16	6	5	1	6	2,3,4,5	4
Average	5.13		1.13 (22%) [w]	5.62		4.31 (76.7%)

[z] Leaf position was counted from the crown and upward, the first leaf (leaf number 1) is the most matured leaf.
[y] Bulbs received 10°C from Sept. 21 (ST-A), 15/12°C from Nov. 2 (ST-B) and 21/18°C from Dec. 7 (ST-C).
[x] Bulbs received 22°C from Sept. 21 (ST-A), 18/15°C from Nov. 2(ST-B) and 15/12°C from Dec. 7(ST-C).
[w] Percentage of leaves showing LTB symptoms.

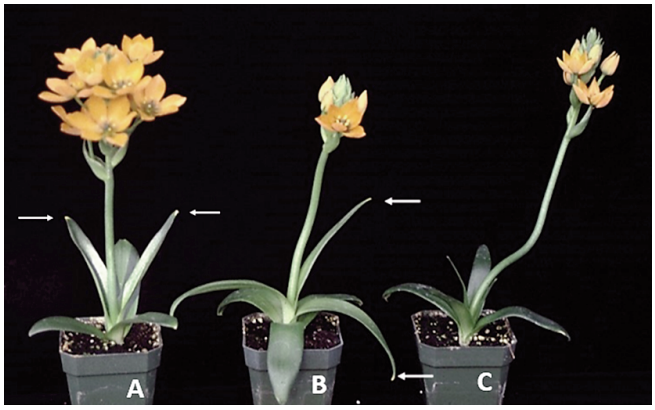

Fig. 2 - Patterns of scape growth of *O. dubium*. A) straight growth and no bending of the scape (score 1). B) leaning to sideway without bending at the base of scape (score 2). C) scape at the base started to grow at about 30-degree angle, and continue to grow leaning to sideway (score 3). Incipient leaf tip burn (LTB) symptoms are indicated with arrow sign. Plant A is considered the most ideal and high-quality plant.

3. Results

Leaf tip burn symptoms and analysis of macro- and micro-elements in different leaf parts

The number of total leaves ranging from 4.8 to 5.6 was not affected significantly by the temperature treatments (data not presented). When bulbs were stored at 10°C/ST-A, followed by forcing at 15/12°C/ST-B and 21/18°C/ST-C, the number of leaves showing LTB was 1.1 out of 5.1 leaves. When the bulbs were stored and forced at 22°C/ST-A, 18/15°C/ST-B, and 15/12°C/ST-C, 4.3 out of 5.6 leaves showed LTB (Table 1).

Fig. 3 - Leaf spread of *Ornithogalum dubium*. A) all leaves growing upward (erect) (score 1), B) Old leaves showing prostrate growth (spreading), while young leaves growing upward (score 2), and C) Old leaves drooping and curved inward toward the pot (prostrate) (score 3). Evident leaf tip burn (LTB) symptoms are indicated with arrow sign.

The macro- and micro-element analysis of the leaves without apparent LTB revealed that the B concentration at the tip (distal end) of the leaves was 119 ppm, which is within the suggested acceptable range (30-150 ppm) for general ornamentals (Table 2). The B concentrations in the middle and base (proximal end) of the leaves were 34 and 21 ppm, respectively, which was close to the lower end of the range.

When the samples were collected in the early growth stages when no or incipient symptoms were observed on Jan. 14, Jan. 26, and Mar. 4, the B concentrations were 109, 96, and 108 ppm, respectively, which fell within the suggested acceptable range (Table 3). Macro- and micro-element analysis of the leaves without apparent LTB revealed that the B concentration in the tip of the leaves when collected on Jan. 14 was lower than 109 ppm (Table 3). However, B concentrations of leaves showing incipient symptoms and severe LTP when the leaves were collected on Feb. 3 and Feb. 8 were 218 and 230 ppm, respectively, and these concentrations exceed the suggested acceptable range.

The Ca concentration was high in the leaf tips (0.76%), and the concentrations of the other macro- and micro-elements fell within the acceptable ranges for each element, even when LTB was observed. The exception was zinc (Zn), whose concentration was slightly above the upper level (155 and 159 ppm) of the suggested range (150 ppm) (Table 3). The nitrogen (N) concentration was above the lower level of the suggested range in all samples, regardless of leaf position, LTB symptoms, and leaf sampling time. The concentrations of Ca and Zn were lowest and highest, respectively, in the tip (distal end) and lowest in the middle and bottom (proximal end) of the leaves when LTB was not observed. At the tip of the leaves show-

Table 2 - Macro- and micro-elements of *Ornithogalum dubium* from the distal-, mid- and basal portion of third leaves from the base of the scape that do not show leaf burn symptom

Leaf position	Macro- and micro- elements										
	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	B (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	Mo (ppm)
Tip (distal end)	3.9 c [z]	0.97 a	3.1 a	0.76 c	0.29 c	119 c	87.2 a	40 a	18 a	56 c	3.1 a
Mid	3.5 b	1.11 a	3.8 a	0.69 b	0.27 b	34 b	76.2 a	34 a	17.1 a	46 b	2.3 a
Base (proximal end)	2.8 a	0.88 a	4.3 a	0.55 a	0.19 a	21 a	60.4 a	29 a	15.3 a	37 a	1.4 a
Level of significance [y]	***	NS	NS	***	***	***	NS	NS	NS	***	NS
Suggested acceptable range [x]	3.5-5.5	0.35-1.0	2.0-8.8	0.8-3.0	0.2-1.5	30-150	60-200	50-200	mag-25	30-150	0.5-5

[z] Comparisons of means by Duncan's multiple range test, $P<0.01$, *F*-test.
[y] ***, NS= significant at $P<0.001$ and not significant.
[x] JR Peter's Laboratory (Allentown, PA. USA).

Table 3 - Macro- and micro-elements of leaf analysis of *Ornithogalum dubium* from the tip and base of third leaves from the crown showing different degree of leaf burn symptom

Leaf position	Leaf tip burn	Sampling date	Macro- and micro- elements										
			N (%)	P (%)	K (%)	Ca (%)	Mg (%)	B (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	Mo (ppm)
Base	No	Jan. 14	5.30 c [z]	1.31 a	6.3 b	1.55 b	0.33 bc	25 a	67.5 a	40 e	10.9 d	62 a	1.4 d
Tip	No	Jan. 14	4.78 b	1.59 c	7.3 c	1.36 a	0.2 7 b	109 c	103.5 e	31b	13.1 e	114 bc	1.3 c
Base	No	Jan. 26	5.21 c	1.32 a	5.5 a	1.96 de	0.37 c	22 a	59.1 a	35 d	6.9 a	51 a	1.2 b
Tip	Incipient	Jan. 26	4.62 b	1.60 d	6.1 b	1.76 c	0.30 b	96 b	84.3 d	30 b	8.8 b	106 b	1.1 a
Tip	Evident	Feb. 3	3.92 a	1.44 b	6.0 a	1.94 de	0.30 b	**218 d**	91.6 bc	27 c	8.3 b	**155 e**	1.2 ab
Tip	Severe	Feb. 10	3.83 a	1.49 c	5.7 a	2.08 e	0.37 c	**230 d**	91. bc	25 bc	10.2 d	**159 e**	1.6 de
Tip	No	Mar. 4 (flowering)	3.90 a	1.31 a	5.4 a	1.50 b	0.19 a	108 c	93d	22 a	8.2 b	118 d	1.71 e
Level of significance [y]			***	***	***	***	***	***	***	***	***	***	**
Suggested and acceptable range [x]			3.5-5.5	0.350-1.0	2.0-8.8	0.8-3.0	0.2-1.5	30-150	60-200	50-200	mag-25	30-150	0.5-5

[z] Comparisons of means by Duncan's multiple range test, $P<0.01$, *F*-test.
[y] ***, ** = significant at $P< 0.001$, and $P<0.01$, *F*-test.
[x] JR Peter's Laboratory (Allentown, PA. USA).

ing evident and severe LPB Ca concentration was higher than in leaves showing incipient LTB symptom. The Mn concentration ranged from 25 to 40 ppm, which was lower than the lower level of the range (50 ppm). The concentrations of phosphorus (P), potassium (K), iron (Fe), Mn, Cu, and molybdenum (Mo) did not differ between the tips, middle, and bottom of the leaves.

The effect of the pre-planting and post-planting forcing temperatures on growth, flowering, and LTB expression

Leaf emergence was affected significantly by temperature treatments during ST-A and ST-B and the interaction of ST-A and ST-B (Table 4). Leaves emerged late when bulbs were stored at 10°C before potting during ST-A (10°C/ST-A) compared to when bulbs were stored at 16 and 22°C/ST-A, and the forcing temperatures were 15/12°C or 18/15°C/ST-B and 15/12°C or 18/15°C/ST-B (Table 4). The number of days to flower was affected significantly by temperature treatment during ST-A, ST-B, and ST-C and the interaction of ST-A and ST-B. The earliest flowering (87 days) occurred when bulbs were treated at 10°C/ST-A and 21/18°C/ST-B and ST-C. Bulbs stored at 22°C/ST-A took longer than 112 days to flower, regardless of the temperature during ST-B and ST-C. The average days to flowering was 116 days, whereas the days to flowering was <100-105 days at 21/18°C/ST-C.

The number of flowers (<36 flowers) was only affected by bulb storage temperature during ST-A

Table 4 - The effect of temperature treatment during three different growth and development periods on the growth and flowering of *Ornithogalum dubium*

Treatment stage (ST-)/ temperature (°C) [z]			Number of days [y] to		No. of flowers	Scape length (cm)	No. of leaves showing leaf tip burns	Score of angle u		Leaf	
A [y]	B [x]	C [w]	Leaf emergence	Flowering				Scape	Leaf	Length	Width ½ length
10	15-dic	15-dic	47.9 a	113 f	33 c	20.5 ij	2.00 b	1.0 a	1.0 a	8.4 a	2.2 ab
10	15-dic	21/18	47.9 a	98 c	33 d	20.7 hij	1.13 a	1.5 b	1.2 ab	8.5 a	2.1 a
10	18/15	15-dic	47.6 a	105 ef	36 d	22.7 j	3.31 fgh	1.1 ab	2.5 ab	9.6 abc	2.3 ab
10	18/15	21/18	47.5 ab	90 b	27 a	20.4 hij	2.50 bcde	1.1 ab	2.8 c	10.4 cdef	2.2 ab
10	21/18	15-dic	46.4 cd	100 c	25 a	22.6 ij	2.44 bcd	1.4 b	2.9 ab	9.5 abc	2.2 ab
10	21/18	21/18	46.4 cd	87 a	29 ab	19.4 h	0.50 a	1.5 b	2.5 b	9.9 bcd	2.2 ab
16	15-dic	15-dic	46.0 d	113 f	55 ef	15.3 defg	2.19 bc	2.3 c	1.8 c	9.1 ab	2.5 abc
16	15-dic	21/18	46.2 d	105 ef	44 d	15.8 fg	2.44 bcd	2.3 c	1.9 c	10.1 bcde	2.4 abc
16	18/15	15-dic	46.1 d	123 g	52 ef	15.4 defg	3.13 efg	2.9 e	2.7 de	11.5 fgh	2.4 abc
16	18/15	21/18	46.0 d	107 ef	51 e	20.3 hi	4.00 j	3.0 e	2.7 de	11.4 fg	2.5 abc
16	21/18	15-dic	46.0 d	116 fg	52 ef	12.4 ab	3.31 fgh	2.5 cd	2.8 e	11.3 efg	2.6 bcd
16	21/18	21/18	46.1 d	102 cd	48 e	13.6 bcdefg	2.94 defg	2.3 c	2.5 d	11.3 efg	2.4 abc
22	15-dic	15-dic	47.1 abc	129 h	52 ef	13.3 bcde	2.75 cdef	2.4 cd	2.8 e	12.1 h	2.8 cd
22	15-dic	21/18	47.0 bc	116 fg	58 fg	13.5 bcdef	3.50 ghi	2.6 de	2.9 e	12.2 hu	3.1 d
22	18/15	15-dic	46.8 bcd	125 g	59 fg	14.7 bcdefg	4.31 j	2.9 e	2.7 de	13.9 hi	2.8 cd
22	18/15	21/18	46.8 bcd	114 f	63 gh	12.8 bc	3.50 ghi	2.4 cd	2.8 e	12.8 hi	2.9 cd
22	21/18	15-dic	46.4 cd	123 g	61 gh	10.4 a	3.50 ghi	2.8 de	2.9 e	12.0 gh	2.6 bcd
22	21/18	21/18	46.4 cd	112 f	57 fg	13.2 bcd	3.88 hij	2.4 cd	2.7 de	13.6 i	2.6 bcd
Level of significance [t]											
ST-A			***	***	***	***	***	***	***	***	***
ST-B			***	***	NS	**	***	NS	***	***	**
ST-C			NS	***	NS	NS	NS	NS	**	NS	NS
ST-A × ST-B			***	***	NS	NS	***	NS	***	NS	***
ST-A × ST-C			NS	NS	NS	NS	**	NS	***	NS	NS
ST-B × ST-C			NS	NS	NS	NS	NS	NS	NS	NS	NS
ST-A × ST-B × ST-C			NS	NS	NS	NS	NS	NS	NS	NS	NS

[z] Comparisons of means by Duncan's multiple range test, $P<0.01$, F-test.
[y] ***, **, NS = significant at P< 0.001, P<0.01, and not significant.
[x] JR Peter's Laboratory (Allentown, PA. USA).

when treated at 10°C/ST-A, 18/15°C/ST-B, and 15/12°C/ST-C (Table 4). More than 44 flowers were produced when bulbs were treated at 16 and 22°C/ST-A, regardless of the temperature during ST-B and ST-C. The scape length was >20.4 cm when bulbs were stored at 10°C/ST-A, regardless of the temperature during ST-B and ST-C. When the temperature during ST-A was increased from 10 to 22°C, a significantly higher number of flowers was produced on short scapes when bulbs were stored at 22°C.

For plants that required an average of 115 days to flower, the scape length was 12.4 cm, and 52 flowers were produced in the treatment of 16°C/ST-A, 21/18°C/ST-B, and 15/12°C/ST-C (Table 4). However, a significantly higher number of leaves (3.31) showing LTB was produced compared to 0.50 leaves in treatment (10°C/ST-A, 21/18°C/ST-A, and 21/28°C/ST-C) and 1.13 leaves in treatment (10°C/ST-A, 15/12°C/ST-A, and 21/28°C/ST-C). As the bulb storage temperature was increased to 16 or 22°C, the number of leaves showing LTB increased to 2.19 leaves (16°C/ST-A, 15/12°C/ST-A, and 15/12°C/ST-C) to 4.31 leaves (22°C/ST-A, 18/15°C/ST-A, and 15/12°C/ST-C).

The scape growth was primarily influenced by the temperature during ST-A, resulting in scores of <1.5 when bulbs were stored at 10°C/ST-A, regardless of the temperature during ST-B and ST-C (Table 4). The scape growth when bulbs were stored at 16 and 22°C/ST-A had scores of >2.3, regardless of the temperature during ST-B and ST-C. The leaf spread showed a complex response; ST-A, ST-B, and ST-C had a significant effect, as did the interaction of ST-A and ST-B and ST-B × ST-C. However, the leaf spread had scores <1.9 when the plants were forced at 15/12°C/ST-C, followed by storing the bulbs at 10 or 16°C/ST-A. In contrast, the scores were >1.9 regardless of the temperature treatments during ST-A, ST-B, and ST-C. The length of the leaves was<10.4 cm when bulbs were stored at 10°C/ST-A, regardless of the forcing temperature during ST-B and ST-C, and was >12 cm when bulbs were stored at 22 °C/ST-A. Bulbs stored at 16°C/ST-A had leaf lengths between those of the 10°C and 22°C/ST-A treatments. The leaf width was <2.3 cm when bulbs were stored at 10°C/ST-A.

4. Discussion and Conclusions

Quality criteria of the finished potted plants

Criteria for the quality of finished potted *O. dubium* plants have not been established even in 2019. The quality can be evaluated in reducing the incidence leaf tip burn (LTB) at the tip of the leaves (Fig. 1) and by the rate of flowering without sacrificing the number of flower buds. Areas of LTB are indicated by the arrows in *O. dubium* seedlings in figures 2 and 3. However, the criteria used to assess the visual quality were not specified (Lee and Miller, 2015).

Therefore, the following three quantitative criteria to assess the plant quality were established in this study. Criterion I: LTB occurs on <1.5 leaves per plant. The lower the score of the scape growth and leaf spread, the higher the plant quality is. Criterion II: the number of days to flowering is <115 days, the length <11.5 cm, the width of the third leaf from the crown is <2.5 cm, the scape length is <15 cm with >45 flowers. Criterion III: the leaf spread and scape growth have a score <1.5.

Analysis of the macro- and micro-elements in the third and/or fourth leaves showing various symptoms and suggested concentration for quality plant production

The cause of LTB was investigated based on the macro- and micro-element concentrations in the tissue. Macro- and micro-elements analysis of *O. dubium* leaves without apparent LTB revealed that the B concentration in the leaf tips (distal end) was 119 ppm, which was within the suggested acceptable range for general ornamentals (30-150 ppm) (Table 2 and 3). The concentrations in the middle and bottom (proximal end) of the leaves were slightly higher or lower, respectively, than the lower level of the range of B concentration. When the samples were collected in the early growth stages when no or incipient LTB symptoms were observed on Jan. 14, Jan. 26, and Mar. 4, the B concentrations were 109, 96, and 108 ppm, respectively, which fell within the suggested acceptable range.

Leaf tip burn in *O. dubium* in young and old leaves is considered to be different from marginal leaf burn observed in old leaves of *Curcuma* 'CMU Price'. The latter is related to high concentrations of B (119 ppm), Fe (189 ppm), and Mn (273 ppm) and low N concentration (1.7%) (Roh *et al*., 2006). Boron may accumulate at the tip or margin of leaves (Jones, 1970) and may cause toxic effects (Oertli, 1962). Therefore, LTB in *Ornithogalum* is a unique physiological expression resulting from high B concentration at the leaf tips and developing regardless of the leaf age; these symptoms were observed in *O. thyr-*

soides and *O. arabicum* (Roh, personal observation). The incidence of LTB in *Watsonia laccata* (Jacquin) Ker Aawler is considered a similar disorder as in *O. dubium* and adversely affects potted plant production in *W. laccata* (Suh *et al*., 2011). Leaf tip scorch observed in *O. dubium* (De Hertogh and Gallitano, 1997), upper leaf necrosis observed in Oriental hybrid lilies (Chang *et al*., 2008), and burn appearing at certain developmental stages before the visible bud stages in *Lilium* 'Pirate' (Berghoef, 1986) may be different from LTB in *O. dubium*.

The LTB is, therefore, caused by a high B concentration (218-230 ppm) and possibly a high Zn concentration (155-159 ppm), which was higher in this study than the high level of the suggested range, even when grown in medium low in B and Zn. Boron concentration in the growing medium was <6 ppm (Tech Data PRO-MIX_HP_Mycorrhizae_4253; accessed on Nov. 1, 2020). The source of B accumulated at the tip of leaves showing LTB in *Ornithogalum* is considered resulting from B accumulation at the margin of old leaves after translocation from rhizome and leaves as discussed in *Curcuma* (Roh *et al*., 2006; Roh and Lawson, 2009). Since Zn has intermediate mobility and occurs in the middle leaves (Shelp *et al*., 1995; McCauley *et al*., 2009), it may not be associated with LTB observed in the young and old leaves of *O. dubium*.

The effect of the temperatures in the pre-planting bulb (ST-A) and post-planting forcing periods (ST-B and ST-C) on growth, flowering, and plant quality

Regardless of the temperature treatments during the pre-planting bulb (ST-A) and post-planting forcing (ST-B and ST-C) periods, the LTB symptoms were not entirely eliminated in *O. dubium*, which was in agreement with the results for *W. laccata* (Suh *et al*., 2011). In our experiment, the number of leaves showing LTB was 1.1 out of 5.1 leaves which was the fewest when received 10°C/ST-A, followed by forcing at 15/12°C/ST-B and 21/18°C/ST-C (Table 1). Therefore, the flowering responses and leaf and scape morphologies, as evaluated in criteria II and III, respectively, were further considered. The overall average number of days to flower was 116 days (Table 4), which was close to the 115 days established for criterion I. Therefore, it is suggested to store bulbs at lower than 22°C, which is lower than the 25°C suggested by De Hertogh and Gallitano (1997). Our data suggested that storing bulbs at 10°C ensures that plants will flower in <115 days.

Low pre-planting temperature treatments in the range of 9 to 27°C for 3 weeks were shown to accelerate flowering (Lee and Miller, 2015). However, this depends on the cultivar and bulb-handling methods since leaves did not emerge and plants failed to flower when bulbs were treated at 10°C. The initiation of the inflorescence can be inhibited by more than 6 weeks because breaking dormancy may require temperatures higher than 10°C (Roh and Joung, 2004). The optimum pre-planting temperature was reported as 22 to 28°C immediately after harvesting the bulbs to promote the early development of the first inflorescence and initiation of the second inflorescence (Roh and Joung, 2004). In *O. arabicum*, flower initiation and development were accelerated by storing initially at 30°C for 12 weeks, followed by 20°C for 4 weeks, and finally at 13°C for 8 weeks before planting (Shoub and Halevy, 1971; Shoub *et al*., 1971).

Forcing at 21/18°C/ST-B and ST-C after potting the bulbs caused the plants to flower in 100 to 105 days, and the response was faster when bulbs were treated at 16°C/ST-A. Therefore, the recommended temperatures for storing bulbs to ensure flowering in <115 days are 10 or 16°C/ST-A followed by forcing at 21/18°C/ST-B or preferably 21/18°C/ST-C (10°C/ST-A or at 16°C/ST-A, 21/18°C/ST-B or 21/18°C/ST-C). De Hertogh and Gallitano (1997) suggested to force bulbs in a greenhouse at a minimum of 22/18°C to flower in 96 days (this is similar to the period in ST-B and ST-C in this study). Temperatures below 15°C during an unspecified growth period did not increase the growth rate (Littlejohn and Blomerus, 2000), although a constant temperature of 17 to 19°C was most suitable for forcing, considering the forcing time, plant height, and visual quality of *Ornithogalum* cultivars. However, the criterion for visual quality was not specified (Lee and Miller, 2015).

The number of flowers was significantly influenced by bulb storage temperature during ST-A, and plants produced <36 flowers (10°C /ST-A, 18/15°C /ST-B, and 15/12°C/ST-C). However, more than 44 flowers were produced when bulbs were treated at 16 and 22°C during ST-A, regardless of the temperature during ST-B and ST-C. The number of flowers was reduced when forcing occurred at 32/27°C; however, the flowering rate was not mentioned (Luria *et al*., 2002). A significantly higher number of flowers (52 flowers) produced on the short scapes (12.4 cm)

when bulbs were stored at 22°C/ST-A and forced at 21/18°C/ST-B) and 15/12°C/ST-C. Depending on the bulb size, 20 flowers each were produced on two flower stalks, and the bulbs stored at 9°C produced floral stalks (31.3 cm) (De Hertogh and Gallitano, 1997).

Three criteria were established to determine the quality of *O. dubium* plants. Criterion I focus on the occurrence of LTB that are observed in less than 1.5 leaves per plant. Leaf tip burn cannot be avoided, but can be controlled by maintaining the forcing temperature at a minimum of 10°C or potentially 16°C during ST-A. This treatment does not delay flowering. Leaf tip burn was observed in *O. dubium* in both young and old leaves and was caused by a high B concentration (218-230 ppm) and possibly by a high Zn concentration (155-159 ppm). Calcium was not the primary cause for LTB. Criterion II focuses on growth and flowering, leaf and scape morphologies; flowering occurs less than 115 days, the length and width of the third leaf is shorter than 11.5 cm and narrower than 2.5 cm, respectively, the scape length is shorter than15 cm, and there are more than 45 flowers. Criterion III focuses on the leaf morphology and the scape growth have a score of less than 1.5. The recommended treatment to meet the three criteria and to produce high quality plants at flowering (plant A, Fig. 2) is to treat bulbs at 10 or 16°C before planting (ST-A), and at 15/12°C in ST-B and at 21/18°C during ST-C during forcing. However, it was not possible to produce finished plants without severe LTB symptoms. Although LTB can not be prevented in *O. dubium*, it can be minimized by temperature manipulation.

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Profiling of primary metabolites of *Averrhoa carambola*, *Spondias dulcis* and *Syzygium malaccense* fruits revealed underpinning markers during "on-tree" maturation and ripening stages

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Key words: Acids, Carambola, June plum, maturation, otaheite, ripening, sugars.

Abstract: The study aimed to profile and quantify sugars and organic acids metabolites in carambola, June plum and otaheite fruits during three different "on tree" stages: immature, green-mature and ripe stages. Metabolites were profiled and quantified by gas chromatography-mass spectrometry (GC-MS). Results showed that glucose, fructose, galactose, arabinose, and the sugar alcohol myo-inositol were detected in all fruits, while sucrose was detected in carambola and June plum only. Organic acids identified in all fruits were malic acid, citric acid, propanoic acid, and acetic acid. Comparatively, June plum showed the highest content of total sugars and carambola the lowest, while the highest total in organic acids content was found in otaheite and the lowest in carambola. On the other hand, most sugars increased during ripening of the three fruits, while organic acids decreased. Total sugars increased by 37%, 8% and 46% in ripe carambola, June plum and otaheite, respectively. Total organic acids decreased by 20% and 49% in ripe carambola and otaheite, while they slightly increased by 3% in ripe June plum. Furthermore, sugars/organic acids ratio in all fruits increased during maturation and ripening stages. Principal component analysis (PCA) showed two main groups of highly scoring metabolites, while the hierarchical cluster analysis (HCA) showed that the metabolites were grouped into three main clusters. Conclusively, results showed that glucose, fructose, malic acid and tartaric acids were the key marker metabolites of the maturation and ripening stages of the three fruits.

1. Introduction

Carambola (*Averrhoa carambola*) belongs to the family *Oxalidaceae*

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originated in Asia but has since developed a tolerance for tropical climates (Shui and Leong, 2006). Presently, carambola is cultivated extensively in India and China (Narain *et al*., 2001), but Malaysia is the largest exporter (Abdullah *et al*., 2007). Work has been done to improve carambola cultivars in the United States in the 1930s, and since then, the fruit became more popular, while some sub species of star fruit exist in the Caribbean, Central America and tropical West Africa (Neto *et al*., 2009). Unripe carambola is moderately sour, but when ripe it is very sweet and used for juice, fruit salads, chutney, stewed fruits, garnish drinks and dishes, or adding it to fruit smoothies. Physiologically, carambola does not exhibit climacteric ripening behaviour even though it continues to synthesise carotenoids and develop its yellow-orange colour (Warren, 2009).

June plum (*Spondias dulcis* Forst. Syn. *Spondias cytherea* Sonn.), a drupe belonging to the family Anacardiacea, is native to the Society Islands of the South Pacific ranging from Melanesia to Polynesia and was first introduced to Jamaica in the year 1782, and later in 1792 by Captain Bligh (Graham *et al*., 2004 a). Although the fruit is well known, there are no named cultivars, but both forms exist, one is with a thick mesocarp and a more pleasant taste, and another has long spines, a woody endocarp with a pungent and resinous taste (Daulmerie, 1994). Ripe June plum is eaten mostly raw but is also used in making refreshing drinks, jam, chutney, sauce or served with meat and seafood. When harvested green and mature, June plum ripens, and studies showed that respiratory pattern is typical of a climacteric fruit (Daulmerie, 1994; Graham *et al*., 2004 b).

Otaheite (*Syzygium malaccense*) is a berry and belongs to the family Myrtaceae. It is thought to be native to the Indo-Malay or Southeast Asian region (Whistler and Elevitch, 2006). Otaheite is, however distributed in many tropical countries throughout the world, particularly in Africa and South America (Oliveira *et al*., 2011). Other English common names for this fruit include: Malay apple, mountain apple, pomerac, and rose apple (Batista *et al*., 2017). Two colour forms exist: one which produces red flowers and fruits and another, less common variety, which produces white flowers and fruit (Whistler and Elevitch, 2006). Ripe otaheite fruit is not very sweet, and often eaten raw. However, in some tropical countries, it is stewed with sugar to make jam or wine and refreshing drinks. No study is recorded on whether otaheite is a climacteric or non-climacteric fruit except the work of Basanta (1998) who reported that otaheite is a non-climacteric fruit.

Ripening can be defined as the total changes of fruit tissue metabolism, leading to the production of an attractive fruit which can be consumed, aiding in the release and dispersal of the seed (Adams-Phillips *et al*., 2004). The ripening process is characterized by softening of fruit tissue and an increase in volatile compounds as well as pigments such as carotenoids and flavonoids which results in a more appealing fruit (Giovannoni, 2001). The concentration of sugars and organic acids in fruits varies depending on the fruit variety and the environmental conditions of the parent plant (Haruenkit, 2004). Overall, there is a general decrease in organic acids and an increase in sugar content as fruit development progresses, due to decarboxylation of organic acids and breakdown of stored carbohydrates to produce sugars (Batista-Silva *et al*., 2018). According to Etienne *et al*. (2013), using advanced technologies, i.e. proteomics, transcriptomics and metabolomics, studies have shown evidence of a shift from the accumulation of organic acids to sugar synthesis in the final stage of fruit development in several species of fruit. Thus, the respiratory pathways commonly involved in the reduction of fruit sugars are glycolysis, oxidative pentose phosphate (OPP) pathway, and the tricarboxylic acid (TCA) pathway (Tucker, 2012).

Because most fruits reach their best sensorial and commercial quality attributes when they ripen on the plants, the correct maturity for harvest of fruits impacts their postharvest shelf-life and quality attributes during storage (Thompson, 2003). Gene expression resulting from natural processes and triggering fruit ripening induces many metabolic processes leading to the formation of hundreds and even thousands of different metabolites (Pech *et al*., 2013).

Although extensive literature is readily available on the metabolic changes during the maturation, ripening and senescence of fresh crops, few researches reported on changes in metabolite profiles during postharvest ripening and senescence (Benkeblia, 2016). However, a limited work was carried out on the metabolites variation during the development and ripening of peach (Lombardo *et al*., 2011), strawberry (Zhang *et al*., 2011), pear (Oikawa *et al*., 2015), and pitaya (Wu *et al*., 2019), while scarce work was reported on some tropical fruit (Fabi

et al., 2010).

In the present work, in order to explore the variation of the metabolic profile of three tropical fruits commonly consumed in the tropics, we performed a profiling study of primary metabolites which are the main indicators of the maturation and the ripening of fruits. For this purpose, we selected three fruits namely carambola (sweet type), June plum and otaheite. One of the goals of this study was to assess how metabolically different the "on tree" maturation and ripening stages of these three fruits are and to find out if there is any particular metabolic profile which could be associated with these two stages of the fruits. On the other hand, by evaluating the metabolomic pattern at both maturation and ripening stages of the three fruits. Overall, this study is aiming to explore a part of the chemical potential of carambola (sweet type), June plum and Otaheite which will aid in the future to know the primary metabolites of these fruits that may correlate to different stages and determine which metabolites might be used as maturation and ripening markers.

2. Materials and Methods

Fruits collection

For the purpose of the present study, three physiological stages of the fruits were investigated: green-immature, mature, and ripe stages (Fig. 1). The colour and softness of the fruits were the two criteria used for discriminating the different maturation and ripening stages. A period varying from seven to ten days elapsed between each harvesting (sampling) stage. The commercial (optimal) harvesting stage of carambola is stage 3 (ripe), while for June plum and otaheite is stage 2 (mature). The fruits carambola, June plum, and otaheite of the three stages of each fruit were collected from three trees of same location. The fruit June plum was collected from a farm in St. Elizabeth. Otaheite fruits were collected from a local farm in Mona, Kingston, and carambola samples were collected from Orange River Research Station in St. Mary. The three stages differentiated and sampled based on their size and colour. For each stage, three samples were collected from three different trees, and each sample consisted of at least six fruits. Fruits collected were controlled for absent of any defect, wound or disease. Immediately after being collected, fruits were placed in plastic bags, and the bags were placed on ice in a cooler and transported to the laboratory within few hours. Then, fruits were washed with mild detergent and rinsed thoroughly, followed by seed removal, dicing or slicing and frozen for 48 hours at -20°C.

Freeze-drying

Prior to the extraction of the profiled sugars and organic acids, samples were freeze-dried in a Labconco freeze-drier (Labconco Corp., Kansas, MO, USA). After six days and complete drying, samples were sealed in plastic bags under vacuum using a MULTIVAC C100 vacuum packer (MULTIVAC, Wolfertschwenden, Germany) and stored under dryness in a desiccator until further use.

Extraction of sugars and organic acids metabolites

Sugars and organic metabolites were extracted by the method described by Broeckling *et al*. (2005) with some modifications. In an Eppendorf tube, 300 mg of freeze-dried samples were mixed with 0.75 mL HPLC grade water containing 26 µg/mL Ribitol was added as internal standard and the tubes vortexed. After equilibrating to room temperature. The tubes were incubated in a shaker for 10 min at 80˚C, fol-

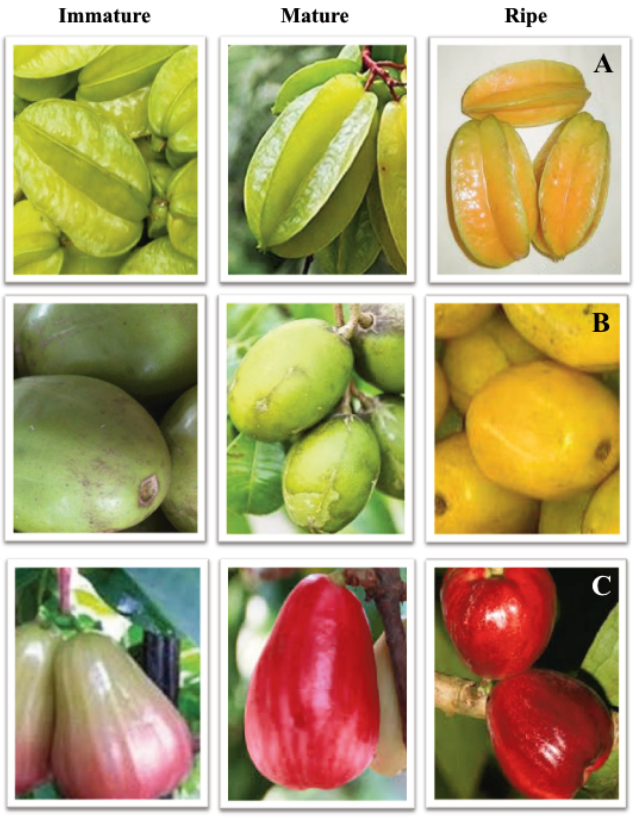

Fig. 1 - Carambola (A), June plum (B) and otaheite (C) at different development and ripening stages.

lowed by incubation at room temperature for c.a. 45 minutes. Afterwards, the tubes were cooled to 4˚C and centrifuged at 10 000 rpm for 15 minutes, the supernatant collected, and the pellet discarded. To the collected supernatants, 250 µL were mixed with 100 mL absolute EtOH and the samples dried under vacuum until dryness and stored at -20°C until GC-MS analysis.

Derivatization

Prior to GC-MS analysis, samples were derivatised as described by Broeckling *et al*. (2005) with some minor modifications. The dry residues were mixed with 80 µL of BSTFA+1% TMCS (Sigma Aldrich, St Louis, MI, USA) and 20 µL pyridine, vortexed and centrifuged for 10 seconds at 10 000 rpm. Afterwards, the mixtures were incubated for 20 minutes at 85˚C. After incubation and equilibrating to room temperature, 200 µL isooctane (2,2,4 trimethylpentane) (Sigma Aldrich, St Louis, MI, USA) were added to the mixture, vortexed and followed by a centrifugation at 10 000 rpm for 10 seconds. From the mixtures, 100 µL were transferred to a 300 µL glass insert for the GC-MS analysis.

GC-MS analysis of sugars and organic acids

The samples were analysed by GC-MS using an Agilent 7890B gas chromatograph coupled to an Agilent 5977A mass spectrometer scanning in the m/z range from 40 to 550. The column used was an HP 5MS (5% Phenyl Methyl Polysiloxane, 30 m × 250 µm × 0.25 µm) with helium as the gas carrier at a constant flow rate of 1.0 mL/min. The samples were injected at a 15:1 split ratio. Initially, the inlet line was held at 260˚C and the transfer line was held at 280˚C. Separation was achieved with an initial temperature program of 40˚C for 2 min, then ramped up at 4˚C per minute to 240˚C and held for 1 minute. The temperature was then increased to 10˚C per minute to 315˚C.

In order to produce the concentration curve, a mixture containing 250 µL of 26 µg/mL of ribitol was used. To the mixture, 20 µL of pyridine and 80 µL of BSTFA containing 1% TMCS was added, vortexed and injected into the GC-MS. Prior to the injection, 50 µL of the mixture were taken and 50 µL of isooctane were added. This was repeated by adding each time 50 µL of isooctane until 6 concentration curves were produced. From the curve a scatter plot with a trend line was generated. The concentrations of the different profiled sugars and organic acids metabolites were calculated from the generated trend line. The generated MS files were extracted, and the deconvolution and identification of the metabolites was carried out by using Agilent MSD Chem Station (Version F.01.01.2017) along with NIST library (Version 11 MS Mass Spectral Library) and AMDIS (Version 2.66) software.

Statistical analyses

For the analysis of each sample (three fruits for each ripening stage and for each species), six samples were analysed, and the data were averaged. The data were analysed and compared by running analysis of variance (ANOVA), Tukey's Honestly Significant Difference (HSD) Post Hoc test using SPSS software package (version 22.0, (IBM Corp., New York, USA). The significance level of all statistical hypotheses testing procedures was predetermined at $P < 0.05$ and 0.01. For the classification, clustering, and regression, PCA (principal component analysis) and HCA (hierarchical cluster analysis) were performed using SPSS software package (version 22.0, IBM Corp., New York, USA), while the clustered heatmap was generated using ClustVis free software (https://biit.cs.ut.ee/clustvis/).

3. Results

Profile and sugar contents of carambola, June plum and otaheite fruits

The profiling of sugars showed that seven saccharides and one sugars alcohol were detected in carambola, June plum and otaheite fruits (Table 1). Glucose, fructose, sucrose, galactose, arabinose, and myo-Inositol have been detected in carambola, June plum and otaheite, however, mannose and xylose were not detected in June plum while sucrose and mannose were not detected in otaheite. Overall, fructose, sucrose, galactose, in carambola and June plum, and glucose, fructose and galactose in otaheite increased during maturation and ripening stages, while the other sugars varied differently in the three fruits.

Interestingly the highest levels of glucose, fructose and sucrose were observed in June plum, highest levels of galactose and xylose in otaheite, and the highest levels of arabinose and myo-inositol in carambola. Results also showed that glucose and fructose were the most predominant monosaccharides in carambola and June plum, while in otaheite glucose, fructose and galactoses were predominant.

Table 1 - Profiled sugars and sugar alcohols and their contents (mg/g dry weight) in carambola, June plum and Otaheite during the three development and ripening stages

Metabolites	Immature	Mature	Ripe
Carambola			
Glucose	51.40 ± 3.27 a	68.20 ± 5.18 b	56.36 ±2 .99 ab
Fructose	30.69 ± 2.71 a	28.87 ±1.23 a	57.79 ± 7.98 b
Sucrose	1.97 ± 0.08 a	13.73 ± 2.05 b	3.59a ± 0.34 a
Galactose	9.50 ± 0.45 a	14.14 ± 0.52 b	11.11 ± 0.77 a
Mannose	n.d.	n.d.	1.23 ± 0.53
Arabinose	2.18 ± 0.42 a	1.86 ± 0.01 a	1.90 ± 0.03 a
Xylose	n.d	11.62 ± 1.85 a	6.28 ± 1 .11 b
Myo-Inositol	2.16 ± 0.11 a	3.30 ± 1.28 a	2.20 ± 0.08 a
Total	97.9 a	133.2 b	134.1 b
June plum			
Glucose	124.11 ± 7.62 a	112.38 ± 12.84 a	90.63 ± 18.41 b
Fructose	79.43 ± 3.26 a	72.68 ± 14.12 a	94.38 ± 18.01 b
Sucrose	7.46 ± 2.36 a	11.33 ± 1.76 a	35.03 ± 10.06 a
Galactose	13.01 ± 0.61 a	12.36 ± 1.77 a	23.67 ± 5.75 b
Mannose	n.d.	n.d.	n.d.
Arabinose	n.d.	1.28 ± 0.09 a	1.26 ± 0.56 a
Xylose	n.d.	n.d.	n.d.
Myo-Inositol	1.88 ± 0.08 a	1.99 ± 0.11 a	1.55 ± 0.61 a
Total	225.9 a	210.8 a	245.8 b
Otaheite			
Glucose	51.88 ± 3.12 a	103.40 ± 10.2 b	111.86 ± 6.59 b
Fructose	43.97 ± 2.75 a	62.40 ± 1.24 b	68.87 ± 9.78 b
Sucrose	n.d.	n.d.	n.d.
Galactose	34.33 ± 1.66 b	12.44 ± 2.45 a	10.79 ± 2.57 a
Mannose	n.d.	n.d.	n.d.
Arabinose	1.67 ± 0.77 a	n.d.	2.13 ± 0.61 a
Xylose	6.34 ± 2.58 a	n.d.	9.31 ± 1.44 a
Myo-Inositol	1.07 ± 0.81 a	n.d.	n.d.
Total	139.3 a	178.4 ab	202.9 b

Different letters of the same row indicate significant difference at P= 0.05.
n.d. = not detected.

On the other hand, a significant increase in total sugars was noted in carambola and otaheite, while in June plum sugars content increased slightly. The total increase of sugars averaged 38% and 45% in carambola and otaheite, respectively, but in June plum increases averaged 9%.

Statistically, total sugar contents in carambola and otaheite were significantly different and their contents in immature fruits were significantly different in comparison with mature and ripe carambola. Immature carambola and otaheite had 35% and 36%, and 28% and 46% less total sugars than the mature and ripe stages, respectively. However, statistical analysis showed no significant difference in sugar contents of June plum during the three maturation and ripening stages.

Profile and organic acids contents of carambola, June plum and otaheite fruits

With ten different acids identified in carambola, June plum and otaheite fruits, eight were detected in June plum, six in carambola and four in Otaheite (Table 2). Overall, malic acid, oxalic acid, propionic acid and acetic acids were the most abundant organic

acids. In carambola and otaheite, malic and tartaric acids were the most abundant, while in June plum malic and acetic acids were predominant. Malic, citric, propionic and acetic acids have been detected in carambola, June plum and Otaheite, while tartaric acid in carambola and otaheite only. On the other hand, ascorbic acid was detected in carambola only, and succinic, oxalic, threonic and gluconic acids were detected in June plum only. Results also showed that organic acids content decreased with development and ripening of carambola and otaheite, while a steady state was observed in June plum. Total organic acids decreased by 20% and 49% in carambola and otaheite, respectively, while in June plum variation of organic acids during the three stages averaged only 3%.

Table 2 - Profiled organic acids and their contents (mg/g dry weight) in carambola, June plum and otaheite during the three development and ripening stages

Metabolites	Immature	Mature	Ripe
Carambola			
Malic Acid	22.11 ± 4.87 b	13.68 ± 2.4 ab	6.73 ± 0.88 a
Citric Acid	2.30 ± 0.2 a	2.20 ± 0.07 a	1.77 ± 0.34 a
Propionic Acid	2.23 ± 0.01 a	2.15 ± 0.04 a	2.19 ± 0.12 a
Acetic Acid	2.01 ± 0.02 a	1.78 ± 0.39 a	1.84 ± 0.24 a
Tartaric Acid	16.67 ± 3.45 a	10.24 ± 2.44 a	10.65 ± 2.65 a
Ascorbic Acid	1.74 ± 0.34 a	1.80 ± 0.3 a	2.12 ± 0.02 a
Succinic Acid	n.d.	n.d.	n.d.
Oxalic Acid	n.d.	n.d.	n.d.
Threonic Acid	n.d.	n.d.	n.d.
Gluconic Acid	n.d.	n.d.	n.d.
Total	14.73 a	14.14 ab	11.71 b
June plum			
Malic Acid	7.34 ± 0.43 a	6.33 ± 0.67 a	6.45 ± 1.45 a
Citric Acid	2.12 ± 0.09 a	2.13 ± .009 a	2.13 ± 0.12 a
Propionic Acid	1.89 ± 0.05 a	3.47 ± 0.09 b	3.45 ± 0.41 b
Acetic Acid	3.24 ± 0.36 a	3.64 ± 0.25 a	3.62 ± 0.44 a
Tartaric Acid	n.d.	n.d.	n.d.
Ascorbic Acid	n.d.	n.d.	n.d.
Succinic Acid	1.82 ± 0.03 a	1.90 ± 0.1 a	1.88 ± 0.07 a
Oxalic Acid	4.52 ± 0.3 a	4.32 ± 0.18 a	4.03 ± 0.2 a
Threonic Acid	1.97 ± 0.05 a	2.26 ± 0.02 ab	2.34 ± 0.19 b
Gluconic Acid	1.84 ± 0.03 a	1.74 ± 0.24 a	1.73 ± 0.28 a
Total	24.74 a	25.79 a	25.63 a
Otaheite			
Malic Acid	22.11 ± 4.87 b	13.68 ± 2.4 ab	6.73 ± 0.88 a
Citric Acid	2.30 ± 0.2 a	2.20 ± 0.07 a	1.77 ± 0.34 a
Propionic Acid	2.23 ± 0.01 a	2.15 ± 0.04 a	2.19 ± 0.12 a
Acetic Acid	2.01 ± 0.02 a	1.78 ± 0.39 a	1.84 ± 0.24 a
Tartaric Acid	16.67 ± 3.45 a	10.24 ± 2.44 a	10.65 ± 2.65 a
Ascorbic Acid	n.d.	n.d.	n.d.
Succinic Acid	n.d.	n.d.	n.d.
Oxalic Acid	n.d.	n.d.	n.d.
Threonic Acid	n.d.	n.d.	n.d.
Gluconic Acid	n.d.	n.d.	n.d.
Total	45.31 b	30.05 ab	23.19 a

Different letters of the same row indicate significant difference at P= 0.05.
n.d. = not detected.

On the other hand, the ratio of sugars/organic acids plays an important role that can characterise the ripe stage of fruits. During the different stages, the ratio of sugars/organic acids maintained a significant rising trend especially in carambola and otaheite. In carambola, the ratio was 6.47, 9.42 and 11.45 in immature, mature and ripe, respectively. In June plum, the ration was 9.13, 8.17 and 9.59, in immature, mature and ripe, respectively. In otaheite, the ratio was 3.07, 5.93 and 8.75, in immature, mature and ripe, respectively.

Statistical analysis showed that total organic acid contents of carambola was not significantly different between either immature and mature, or mature and ripe stages. Malic acid content varied significantly during the development and ripening of carambola and otaheite, but not significantly in June plum. Malic acid was also the main organic acid accumulating in carambola and otaheite and its level was significantly different among the three stages of the maturation and ripening of the two fruit. The statistical analysis also showed that citric acid and acetic acid contents did not show significant difference among the three developmental stages of carambola. Comparatively, total sugars and total organic acids during the three stages showed different correlations. In carambola and June plum, weak correlation (R^2 =0.18 and R^2 = 0.33, respectively) was observed between sugars and organic acids contents, however, a moderate correlation (R^2 = 0.56) was observed between sugars and organic acids contents of otaheite during the three stages.

Factoring and clustering of the profiled metabolites

The principal component analysis of the data sets revealed two individual clusters that seem to be governed by the developmental and the ripening stages of the fruits (Fig. 2). The analysis showed that in PC 1, the metabolites in carambola with the highest loading scores were glucose (0.99), galactose (0.99), xylose (0.97), sucrose (0.96), myoinositol (0.93) and mannose (0.92), while in PC 2 ascorbic acid (0.99), fructose (0.98), citrate (0.95) and acetic acid (0.98) had the highest loading scores. In June plum, the highest loading sores metabolites in PC 1 and PC 2 were oxalic acid (0.90), acetic acid (0.85), sucrose (0.84) and gluconic acid (0.80), and malic acid (0.89), fructose (0.89), arabinose (0.82, galactose (0.82) and sucrose (0.77), respectively. In otaheite, the metabolites with the highest scores were galactose (0.98), myoinositol (0.98), malic acid (0.96), tartaric acid (0.96), acetic acid (0.89), citric acid (0.79) and

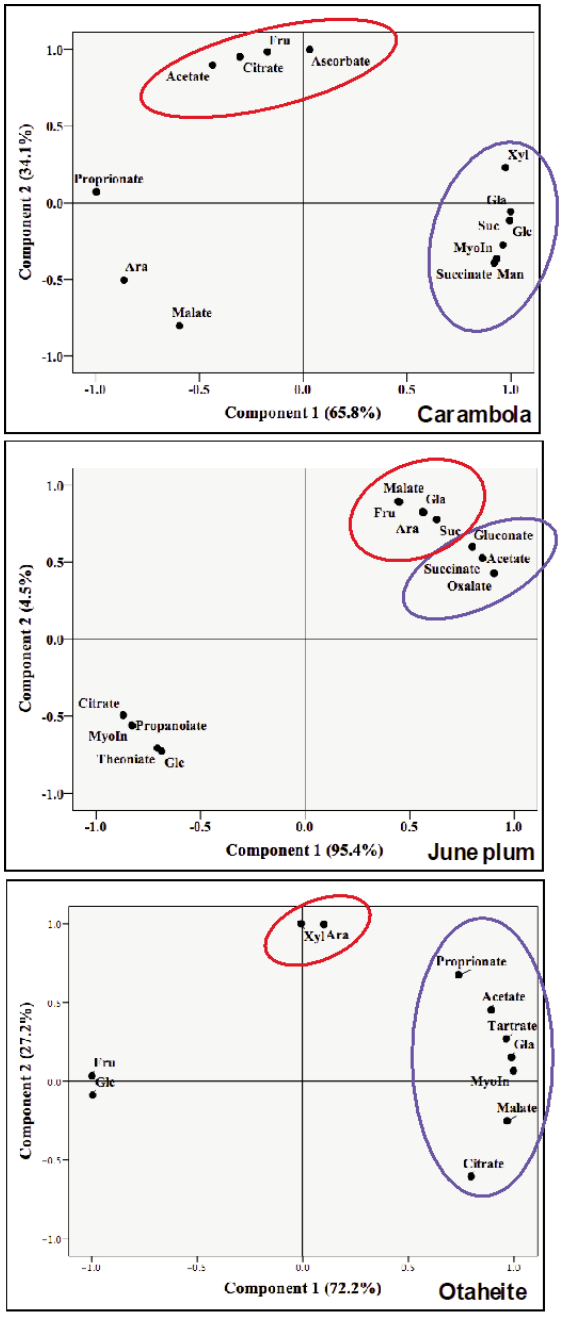

Fig. 2 - Principal component analysis (PCA) of the profiled and identified metabolites in carambola, June plum and otaheite fruits through different development and ripening stages. For PCA analysis, data sets were normalised for better comparison of the variable levels of the different metabolites.

propanoic acid (0.74), and arabinose (0.99) and xylose (0.99), respectively. Overall, principal components analysis (PCA) of samples based on the development and ripening stages revealed a difference between grouped metabolites. As suggested by the PCA in the figure 2, profiled metabolites were then divided into two classes, and loading values of sugars

and organic acids of fruits samples were found mostly in quadrant PC2+, illustrating the discriminated metabolites reflecting the development and ripening of fruits.

Hierarchical cluster analysis (HCA) was applied to a data set of the profiled and detected metabolites during the three stages of the three fruits. The dendrograms (Fig. 3) show that the profiled metabolites were quite homogeneous and tend to be distributed into three groups. According to the dendrograms of the HCA, at the distance of three, the metabolites can be grouped as shown in Table 3. Interestingly, three metabolites have been classified within the same groups of the three fruits. Myoinositol, citric acid and arabinose were classified in group 1, group 2 and group 3, respectively. The clusters of the different metabolites in the three fruits showed that the metabolites were quite clearly hierarchically separated, and these results were also clearly depicted by the PCA (Fig. 2) and the HCA (Fig. 3) which show the distribution of the metabolites into three main clusters. Furthermore, the heatmap (Fig. 4) also shows that June plum concentrates the highest levels of ten metabolites, while carambola and otaheite concentrate the highest levels of four and six other metabolites, respectively.

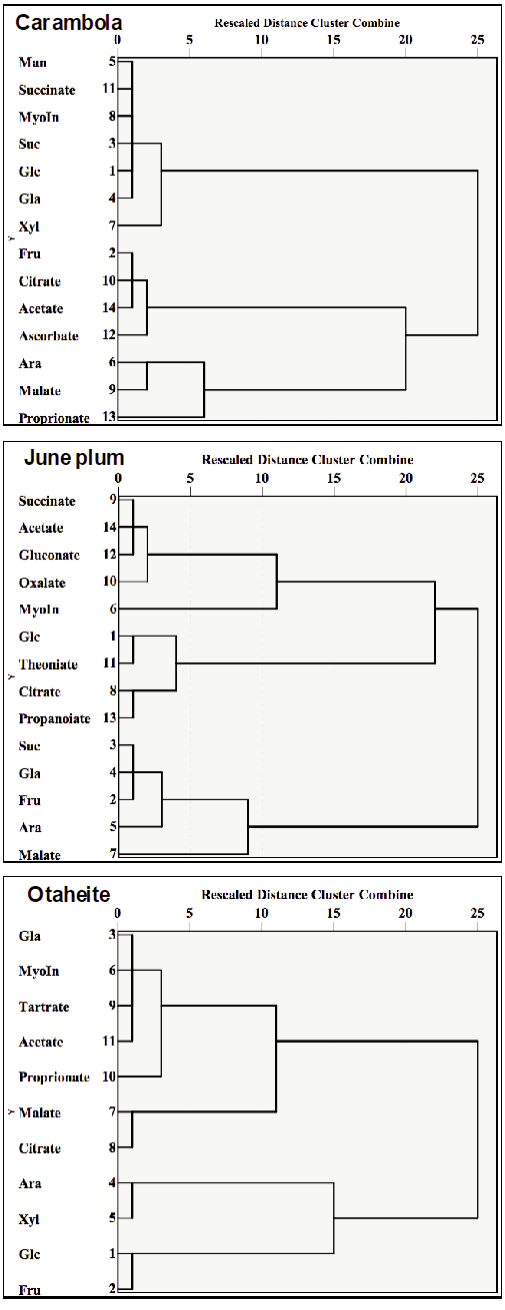

Fig. 3 - Dendrogram showing the Hierarchical Cluster Analysis (HCA) of profiled and identified metabolites in Carambola, June plum and Otaheite fruits through different development and ripening stages. To run HCA, data sets were normalised to reduce of the original data sets and hence allowing better clustering.

Table 3 - The hierarchical clusters distribution of the profiled metabolites of carambola, June plum and otaheite during the three stages (underlined metabolites are classified within the same groups of the three fruits)

	Group 1	Group 2	Group 3
Carambola	Mannose	Fructose	Arabinose
	Succinate	Citric acid	Malic acid
	Myo-inositol	Acetic acid	Propionic acid
	Glucose	Ascorbic acid	
	Galactose		
	Xylose		
June plum	Succinic acid	Glucose	Succinic acid
	Acetic acid	Threonic	Galactose
	Gluconic acid	Citric acid	Fructose
	Oxalic acid	Propionic	Arabinose
	Myo-inositol		Malic acid
Otaheite	Galactose	Malic acid	Arabinose
	Myo-inositol	Citric acid	Xylose
	Tartaric acid		Glucose
	Acetic acid		Fructose
	Propionic acid		

Indeed, primary metabolites profiling led to the identification 8, 6 and 5 sugars, and 6, 8 and 5 organic acids in carambola, June plum and otaheite, respectively. On the other hand, our results showed that the key marker metabolites of the maturation and ripening of the three fruits are glucose and fructose in carambola and otaheite, while in June plum glucose, fructose, galactose and sucrose were the key marker metabolites of the three different stages. Similarly, malic and tartaric acids were the key organic acids metabolites of the maturation and ripening

of carambola and otaheite fruits, while malic acid was the key marker metabolite of June plum maturation and ripening.

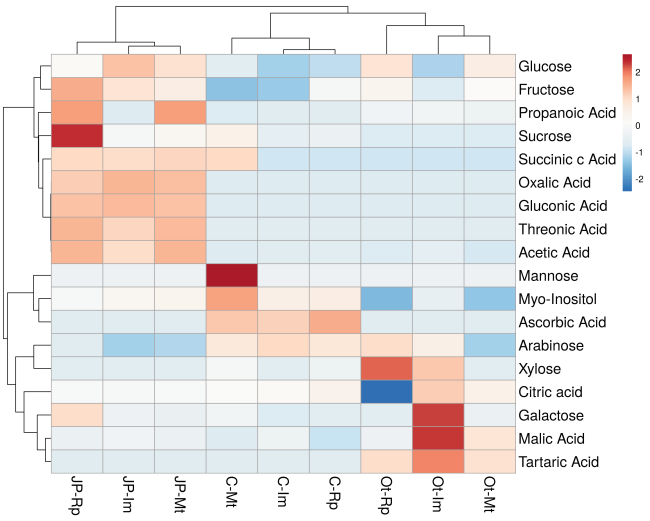

Fig. 4 - Heatmap of the profiled and identified metabolites in Carambola, June plum and Otaheite fruits through different development and ripening stages. The color scale represents the relative concentration of each metabolites.

4. Discussion and Conclusions

Although extensive literature is readily available, the variation of sugars and organic acids of many fruits including carambola and June plum at ripe stage, less and scattered work was done on the variation of the metabolites including sugars and organic acids in carambola, June plum and otaheite fruits during the development and ripening stages. On the other hand, most of the work carried out on carambola targeted the postharvest physiology and biochemistry of the fruit during storage.

In carambola, Campbell and Koch (1989) found that total soluble sugars concentration, mainly glucose and fructose, increased during ripening and varied between 22 and 27 mg/g fresh weight depending on the varieties, while Narain *et al*. (2001) investigated the variation of the chemical composition of carambola at three different ripening stages and found that total sugars increased from 2.91 to 5.60 g/100 g fresh weight. Later, Patil *et al*. (2010) reported the composition of the fruit at three stages of maturity (young, half-ripe and ripe), and they noted a tremendous increase of total sugars, oxalic acid and ascorbic acid by 100%, 89% and 65%, respectively. Similar increase by 33% and 90% of total sugars and ascorbic acid respectively were also reported by Ali and Jaafar (2012). Glucose, fructose and sucrose were reported to be the most predominant sugars in carambola (Mohd Zainudin *et al*., 2014; Benkeblia and López, 2015), however, Benkeblia and López (2015) reported an increase of glucose and fructose, but a slight decrease of sucrose in the ripe fruit compared to the green one, while Mohd Zainudin *et al*. (2014) noted an increase of the three sugars.

There is almost no work reporting on the composition of June plum fruit during maturation and ripening except from the one of Benkeblia and López (2015). The authors investigated the variation of glucose, fructose and sucrose in green and ripe June plum and found that in ripe fruit glucose and fructose increased in ripe fruit, while sucrose decreased significantly. Other scattered studies reported on the variation of sugars and organic acids in June plum fruit but at a specific stage. In a study carried out on immature green June plum, Franquin *et al*. (2005) investigated the composition at this early stage and found the concentrations of glucose, fructose, sucrose, citric acid, malic acid, oxalic acid and ascorbic acid were 1.5 (± 0.2), 1.2 (± 0.2), 3.1 (± 0.3), 0.9 (± 0.1), 0.2 (± 0.02,) 0.03 (± 0.01), and 52.0 (± 4.9) g/100 g fresh weight, respectively. In his study, Nahar *et al*. (1990) reported that 0.3% of fresh weight of the pulp is composed by free sugars, where glucose, fructose and sucrose were the most predominant (Nahar *et al*., 1990; Mahmood *et al*., 2012).

Similarly to June plum, few studies investigated the composition of otaheite during the maturation and ripening, however, few studies reported on ripe otaheite. Lu and Lin (2011) investigated the sugars in ripe otaheite and found that fructose yielded the highest content compared to glucose and sucrose which were detected at this ripe stage.

The untargeted profiling of primary metabolites during the maturation and ripening of fruits is a good approach to provide better insight into their metabolome changes during these stages. Different studies on metabolite analyses of fruits have focused on temperate and stone fruits such as tomato, peach, strawberry, and grape among many others, but scarce studies focused on tropical fruits. However, these studies revealed similar dynamic variations in the levels of sugars and organic acids, as well as many other primary and secondary metabolites during fruits maturation and ripening (Oikawa *et al*.,

2015). Our results showed patterns of variation in sugars and organic acids levels in carambola, June plum and otaheite during maturation and ripening, and provided fundamental metabolomic data that is useful for understanding fruits physiology. Our reported results are in agreement with those reported by Ramadan *et al*. (2020) who used metabolomics approach carambola fruit of different origins and at two different ripening stages. Mamat *et al*. (2019) and Parijadi *et al*. (2018) analysed the distribution of primary metabolites in mangosteen (*Garcinia mangostana* Linn.) fruit during ripening, and their results showed that fructose was the kye marker metabolite of mangosteen ripening. Similar results were reported by Ogawa *et al*. (2018) who noted that the increasing sucrose level might be a key marker metabolite of pineapple ripening.

Beside metabolomics, Pandit *et al*. (2010) used transcriptomics markers to understand the maturation and ripening programmes in mango (*Mangifera indica* L.) fruit. Among eighteen genes related to the fruit physiology and biochemistry, genes related to primary metabolism showed higher expression in comparison to that of the genes related to flavour production.

However, regardless of the origin and environmental zones, the maturation and ripening of fruits are complex and highly coordinated processes. Globally, the increase in sugar and decline in organic acids are one of the main changes associated with these processes (Giovannoni, 2001; Klee and Giovannoni, 2011; Osorio *et al*., 2013; Batista-Silva *et al*., 2018). During maturation and ripening of fruits, organic acids contents are inversely related to sugar contents. The rising trend of sugars is due to photosynthates import or starch degradation, while organic acids that accumulated in young fruits strongly decrease by being converted to other organic acids (Carrari *et al*., 2006; Beauvoit *et al*., 2018). Although environmentally different from tropical fruits, there have been a number of different studies reporting similar metabolic changes that occur in temperate fruits during maturation and ripening stages (Fait *et al*., 2008; Osorio *et al*., 2011, 2012). Sugars accumulation and organics acids decrease trend were reported in blueberries (*Vaccinium arctostaphylos* and *Vaccinium myrtillus*) (Ayaz *et al*., 2001), apple (*Malus domestica*) (Liu *et al*., 2016; Yang *et al*., 2021), apricot (*Prunus armeniaca* L.), plumcot (*Prunus armeniaca* x *Prunus salicina* L.), plum (*Prunus salicina* Lindl.), and peach (*Prunus persica* L.) (Bae *et al*., 2014), Damson plum (*Prunus domestica*) (García-Mariño *et al*., 2008), different citrus cultivars (Bermejo and Cano, 2012), grapes (Kurt *et al*., 2017; Liang *et al*., 2011; Muñoz-Robredo *et al*., 2011), loquat (*Eriobotrya japonica* Lindl.) (Amorós *et al*., 2003), mango (*Mangifera indica* L.) (Mokhtar *et al*., 2014), melon (*Cucumis melo* L.) (Wang *et al*., 1996), pomegranate (*Punica granatum* L.) (Nuncio-Jáuregui *et al*., 2014) and wolfberry (*Lycium barbarum* L.) (Zhao *et al*., 2015) among other reported fruits. Furthermore and in agreement with our finding, glucose, fructose, and sucrose were found to be the most predominant among mono and disaccharides, while malic, citric and tartaric acids were predominant organic acids (Wang *et al*., 1996; Liang *et al*., 2011; Mahmood *et al*., 2012; Kurt *et al*., 2017; Yang *et al*., 2021).

Indeed, the relative levels of sugars and organic acids in fruits are of great importance for harvesting time and are one of the determinants of the organoleptic quality attributes of fruits particularly sweetness (Itai and Tanahashi, 2008). Furthermore, the postharvest quality attributes of fruits, their shelf-life and even processed products are strongly associated to their sugars and organic acids levels (Matsumoto and Ikoma, 2012; Aprea *et al*., 2017). In order to preserve freshness and reduce economic losses, it is of great importance to understand the metabolic changes occurring during maturation and ripening which might contribute to accelerate fruits senescence and perishability after harvesting. In this sense, metabolomic profiling of key metabolites responsible for quality attributes such as sugars and organic acids can be a powerful tool for further understanding the biochemical basis of pre- and postharvest physiology and have the potential to play a critical role in the identification of the pathways affected by fruit maturation and ripening (Allwood *et al*., 2021; Pott *et al*., 2020; Tian *et al*., 2021).

The data presented here indicates that the profiled metabolites varied significantly during the maturation and ripening of the fruits. Glucose, fructose, galactose, sucrose and myoinositol were found predominantly in all the fruits and during the three stages, except sucrose in otaheite. Comparatively, June plum showed the highest content of total sugars and carambola the lowest, while the highest total in organic acids content was noted in otaheite and the lowest in carambola. On the other hand, most sugars increased during ripening of the three fruits, while organic acids decreased. Interestingly, the multi-vari-

able analysis showed than all the metabolites were clustered into three main clusters, and myoinositol, citric acid and arabinose shared group one, group two and group three, respectively. From the different profiled sugars and organic acids, our results are suggesting that glucose and fructose are the marker metabolites of the maturation and ripening of carambola and otaheite, while the ripening marker metabolites in June plum are glucose, fructose, galactose and sucrose. Because this study represents the first report on the profiling of sugars and organic acids in carambola, June plum and otaheite, it might be interesting to profile the secondary metabolites mainly phenolics and volatiles, and their variation during the maturation and ripening of these fruits.

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Postharvest performance interpretation and storage temperature optimization in some newly introduced melon hybrids

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Abstract: Temperature is a key factor in melon cold storage. Thus, optimizing storage temperature is an important goal in postharvest research. In this experiment, postharvest attributes of four inbred lines and five derivative hybrids were investigated under three storage temperatures (1, 4, and 12°C). Melon fruit were evaluated for their main characteristics directly after harvest and postharvest changes were monitored through cold storage period. Cluster analysis results showed that most of the hybrids clustered with their maternal parents illustrating the significant role of cytoplasmic inheritance for the studied traits. Similarly, principal component analysis clustered the studied types into three clusters according to their average postharvest behaviour. The best postharvest performance belonged to inodorus and cantalupensis netted melon with their intercrossing breeds. While the dudaim inbred line and its hybrid scored the highest postharvest changes. Response surface analysis showed that 1.8°C was the optimum storage temperature for inodorus and cantalupensis clusters, while 5.1°C was the best storage temperature of dudaim cluster. The results of the current study are similar to previous reports for optimum storage temperature in similar melon types.

1. Introduction

Melons (*Cucumis melo*) are among the most important fruit crop worldwide with a yearly production of more than 27 million tons (FAO, 2019). This fruit is of an exclusive importance in Mediterranean and Eastern Asia regions (Garcia-Mas *et al*., 2012). Melons are usually perishable with short shelf life and low storability (Fukuta *et al*., 2006; Briones *et al*., 2012). Thus, these fruits do not maintain their marketability for longer than 10 days in room temperature conditions. Therefore, introducing new melon types with enhanced storability, longer shelf life, and better shipping potentials is among the main aims of melon breeders. Additionally, investigating postharvest behaviour and optimizing storage

variables of this fruit is of high importance.

Persian melons (*Cucumis melo* var. *inodorus*) are reported to be a valuable material in melon breeding for postharvest purposes. These types of melons have firmer mesocarps compared to cantalupensis types and thus, endure shipping and handling better (Welbaum, 2015). For instance, including the line 'Cm-UTKH' in breeding programs by crossing with other cantalupensis melons reportedly introduced new types with promising postharvest attributes (Alabboud *et al*., 2020; Shajari *et al*., 2021. Therefore, investigating these newly introduced intergroup hybrids for their postharvest potentials under different storage temperature might be of high importance.

Weight losses, firmness losses, soluble solids fluctuations and colour changes are the main postharvest attributes in stored melons. Furthermore, temperature is the main factor affecting these attributes throughout cold storage (De Arruda *et al*., 2003; Yang *et al*., 2003; Žnidarĉiĉ *et al*., 2013; Hatami *et al.*, 2019). Therefore, minimizing postharvest changes through optimized storage temperature is the ultimate goal in cold storage research. However, monitoring these attributes in large populations can be a laborious quest especially when investigating optimum storage temperature. Thus, a more appropriate experimental method should be used in order to minimize experiment size and maintain an acceptable accuracy. For this purpose, principal component analysis (PCA) is considered a useful tool to explain diversity within a studied group of samples with minimum loss of information. Many researches implemented PCA for better understanding of melon fruit characteristics (Obando *et al*., 2008; Maietti *et al*., 2012; Farcuh *et al*., 2020).On the other hand, response surface method (RSM) is a direct application of regression theory to optimize an experimental input (Barton, 2013) with fewer number of experiments (Wani *et al*., 2012) which fits the goal of postharvest studies in melon.

Therefore, the main goals of this study were to investigate postharvest attributes of some newly introduced melon hybrids under different storage temperatures, and to find the optimum storage temperature for these hybrids through the application of response surface regression optimization.

2. Materials and Methods

Plant material

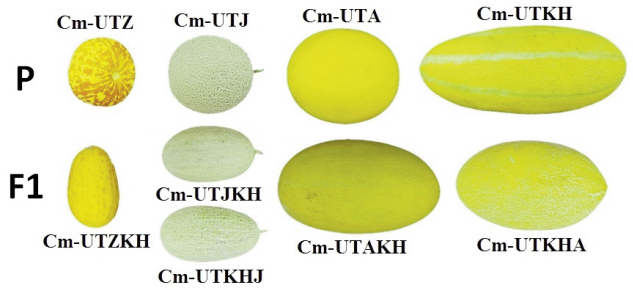
The experiment was carried out for the seasons of 2019 and 2020 at the Research Station of the Department of Horticultural Sciences (University of Tehran) in Karaj, Iran. Four melon inbred lines (Table 1) with five of the intercrossing hybrids were used in this experiment (Fig. 1). The seed of inbred lines and hybrids were planted in seedling trays in greenhouse and transformed to the field three weeks after germination. The seedlings were planted in rows with 1.5 m distance between rows and 0.8 m between plants on each row. Ripening was determined by the development of ½ to ¾ of abscission layer in types that tend to develop an abscission layer and after 42 days post-anthesis in types that lacks the ability to develop an abscission layer. The fruit were harvested at ripening and transferred to the cold storage facility at the Department of Horticultural Sciences. The harvested fruit were stored under three different temperature (1, 5, and 12°C) and a relative humidity of 90-95% for a period of one month.

Fruit attributes monitoring

The main fruit characteristics were monitored

Table 1 - The morphological characterizations of the inbred lines used for the crosses

Line name	Group and origin	Climacteric behaviour	Fruit shape	Rind	Mesocarp	Volatiles	Origin
Cm-UTKH	Inodorus,	Non-climacteric	Oval elongated	Yellow-Shallow netted	Greenish white	Odourless	Khorasan (South-eastern part of Iran)
Cm-UTA	Cantaloupensis x Inodorus derived inbred line	Non-climacteric	Oval-Round	Dark yellow - Without net	White	Odorous (Medium)	Abadan (Southern part of Iran)
Cm-UTJ	Cantaloupensis	Non-climacteric	Ova -Round	Green - Netted	Green	Odourless	Alborz (North-western part of Iran)
Cm-UTZ	Dudaim	Climacteric	Round flatted	Dark yellow with orange areas - Without net	White	Odorous (High)	Kerman (South-eastern part of Iran)



Breed	Maternal parent	Paternal parent
Cm-UTKHA	Cm-UTKH	Cm-UTA
Cm-UTAKH	Cm-UTA	Cm-UTKH
Cm-UTKHJ	Cm-UTKH	Cm-UTJ
Cm-UTJKH	Cm-UTKH	Cm-UTJ
Cm-UTZKH	Cm-UTZ	Cm-UTKH

Fig. 1 - The studied melon inbred lines (P) and their hybrids (F1). The first part in each hybrid name represents the maternal parent while the second represent the male parent.

directly after harvest and throughout storage period in 10 days intervals (i.e. days 0, 10, 20, and 30). Fruit weight was measured using an electronic scale. TSS% in the juice of extracted from a longitudinal section of the fruit was measured using a handheld refractometer. Mesocarp firmness was measured using a handheld penetrometer equipped with an 8-mm tip. The firmness was expressed as an average of three reads along the longitudinal surface of the fruit after the removal of the rind. Rind colour was expressed in CIE-lab scale as lightness (L*), chroma (C*) and hue angle (h°) using a chromameter (Konica Minolta, Japan).

The changes in fruit attributes throughout storage were presented as percentages and were calculated using the following formulas:

$$\text{Weight loss (\%)} = (FW0 - FWA)/FW0 \quad (1)$$

$$\text{Firmness loss (\%)} = (MF0 - MFA)/ MF0 \quad (2)$$

Where FW0 and FWA are fruit weight (g) at harvest and at analysis time, respectively; while MF0 and MFA are mesocarp firmness (Kg cm^{-2}) at harvest and at analysis time, respectively.

Colour changes were calculated using colour difference formulas according to Sharma *et al*. (2005) eq. 3 and using the same system described in Mohi-Alden *et al*. (2021).

$$\Delta C = [(L^* - L0^*)2 + (a^* - a0^*)2 + (b^* - b0^*)2]1/2 \quad (3)$$

where ΔC is colour changes during cold storage L0*, a0* and b0* are the values of colour parameters at harvest, and L*, a* and b* are the values of colour parameters after cold storage.

Data analysis

The two-factor experiment (evaluation date and storage temperature) was carried out in a split plot design. Fruit attributes mean at harvest were compared using Fisher's LSD test ($P<0.05$) with Minitab 19 software. Furthermore, fruit attributes were clustered using Ward's method on NCSS 12. The averages postharvest measurement for the studied lines and breeds were analysed using principal component analysis (PCA). The best Response surface method (RSM) was used to calculate the optimized storage temperature for each PCA cluster.

3. Results

Fruit characteristics and heterosis analysis

The main fruit characteristics of parents and F1 progeny were investigated (Table 2). The result showed that the parent 'Cm-UTKH' recorded the highest fruit length and fruit shape values and, the parent 'Cm-UTA' scored the highest results in terms of fruit diameter, mesocarp thickness, and cavity diameter. The highest firmness results were observed in 'Cm-UTJ' parent with 11.67 Kg cm^{-2} followed by its hybrid 'Cm-UTJKH' (10.98 Kg cm^{-2}), with no significant differences between the parent and the hybrid. The highest fruit weight, and TSS% were observed in the hybrids 'Cm-UTKHA' and 'Cm-UTJKH' with 2033.91 g and 15.3%, respectively. As for colour attributes, the highest L* and hue values were recorded in the parent 'Cm-UTA', while the highest chroma values were observed in the hybrid 'Cm-UTJKH' and the reciprocal 'Cm-UTKHJ'.

Moderately low heterosis results were recorded for fruit diameter, cavity diameter, firmness, TSS, and colour attributes. However, F1 progeny expedited higher heterosis values levels for fruit length (33.17%), fruit shape (22.71%), mesocarp thickness (17.48%), and weight (29.35%).

Cluster analysis of fruit attributes

Ward linkage cluster analysis of fruit attributes at day 0 can be seen in figure 2. The studied genotypes were clustered in three clusters the first cluster contained the hybrid 'Cm-UTZKH' with its maternal parent. The second cluster contained 'Cm-UTJKH' with

Table 2 - Fruit characteristics for the studied melon types directly after harvest (Day 0 observation). FL (Fruit length), FD (Fruit diameter, FS (Fruit shape), MT (Mesocarp thickness), CD (Cavity diameter), W (weight), F (Firmness), TSS (Total soluble solids), L* (Lightness), H (Hue), C (Chroma)

Type	FL (cm)	FD (cm)	FS	MT (cm)	CD (cm)	W (g)	F (Kg cm^{-2})	TSS (%)	L*	H	C
Cm-UTKH	27.77 a	11.33 cd	2.50 a	2.65 cd	6.09 b	1717.42 b	4.77 e	11.70 b	71.15 d	69.72 d	62.35 c
Cm-UTA	13.81 e	14.82 a	0.93 f	3.77 a	7.14 a	1520.51 c	9.84 b	6.33 e	78.24 a	78.77 a	60.37 d
Cm-UTJ	12.88 f	11.94 c	1.09 e	3.01 b	5.88 bc	1026.36 d	11.67 a	11.65 b	72.81 cd	22.06 e	65.21 b
Cm-UTD	6.21 g	7.57 e	0.80 f	1.05 e	5.44 c	201.84 f	5.63 de	6.67 e	68.72 e	70.99 cd	44.45 f
Cm-UTAKH	21.65 c	13.44 b	1.61 c	3.66 a	6.30 b	1738.41 b	7.46 c	8.70 d	74.23 c	75.76 b	60.13 d
Cm-UTKHA	22.29 c	13.15 b	1.69 c	3.54 a	5.98 bc	2033.91 a	6.51 cd	10.60 c	70.92 d	71.69 c	60.12 d
Cm-UTJKH	16.71 d	10.64 d	1.59 c	2.92 bc	4.82 d	917.40 de	10.98 ab	15.30 a	76.19 b	17.17 f	75.38 a
Cm-UTKHJ	25.98 b	13.79 b	1.95 b	2.78 bcd	6.97 a	1771.08 b	5.20 e	10.33 c	73.24 c	12.71 g	74.49 a
Cm-UTZKH	14.36 e	11.12 cd	1.32 d	2.49 d	6.16 b	760.31 e	7.38 c	6.80 e	72.74 cd	74.36 b	55.36 e
h%	33.17	8.87	22.71	17.48	-1.49	29.35	-5.91	13.85	1.01	-16.64	12.05

Types with similar letters in each column have no significant differences for the monitored trait in that column (P<0.05). h% represents heterosis in each trait compared to med-parents.

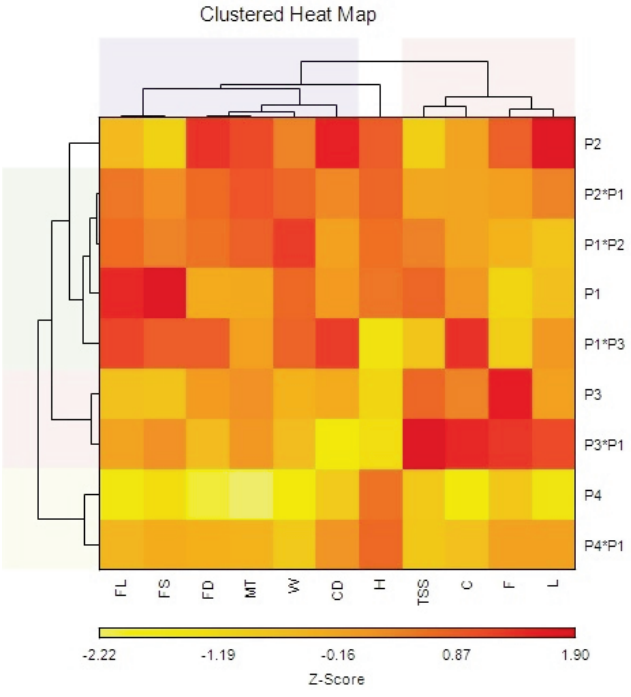


Fig. 2 - Ward's linkage cluster analysis for the studied melon types and their fruit characteristics. P1, P2, P3, and P4 represent the studied lines Cm-UTKH, Cm-UTA, Cm-UTJ, and Cm-UTZ, respectively while two letters genotypes represent F1 direct and reciprocal breeds. FL (Fruit length), FD (Fruit diameter, FS (Fruit shape), MT (Mesocarp thickness), CD (Cavity diameter), W (weight), F (Firmness), TSS (Total soluble solids), L* (Lightness), H (Hue), C (Chroma). The colors of the heat map represent the normalized values of raw data to Z score. Thus, red colors or yellow colors represent values higher or lower than the average value for each studied characteristic, respectively.

its maternal parent. While the final cluster constituted of the 'Cm-UTKHA', 'Cm-UTKHJ', and their maternal parent in addition to the hybrid 'Cm-UTAKH'. On the other hand, 'Cm-UTA' was not clustered with any of the studied types.

As for the studied fruit traits, two major clusters were noticed. The first contained TSS, chroma, firmness, and lightness (L). The second cluster contained the rest of the studied attributes (fruit length, fruit shape, fruit diameter, mesocarp thickness, weight, and cavity diameter), while hue was not clustered with any other attribute.

Principal component analysis results

The average postharvest performance data under three different storage temperature was subjected to principal component analysis (PCA) (Fig. 3). Scree distribution of factors showed that the first two factors were accounted for 68.7% of the total variance with 49.3% for the first factor (PC1) and 19.4% for the second (PC2). Additionally, the accumulated effect of the first three factors was accounted for 79.1% of the total variance among the studied types (Table 3). The most significant positive contributors to the PC1 were chroma, TSS, firmness, and colour changing index, while hue and firmness loss were the most significant negative contributors to this factor. On the other hand, PC2 was substantially positively influenced by fruit weight.

As for genotype clustering according to biplot of postharvest performance, three major clusters were noticed. The green cluster located on the positive

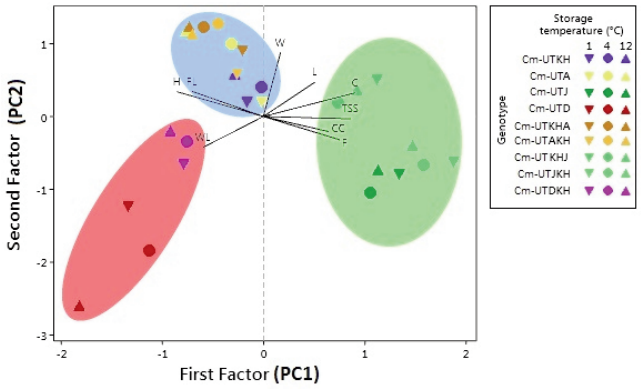


Fig. 3 - Principal component analysis for the studied melon. Loading factors represent the average performance of the studied types calculated as the mean for each loading variable of each type during storage period (the average of 0, 10, 20, and 30 days in cold storage). W (Weight), WL (Weight loss), F (Firmness), FL (Firmness loss). TSS (Total soluble solids), L (Lightness), H (Hue), C (Chroma), CC (Color changes).

quadrates of PC1 and contained 'Cm-UTJ' parent with its hybrid 'Cm-UTJKH' and reciprocal 'Cm-UTKHJ'. The blue cluster located on the positive PC2 and negative PC2 quadrate and was constituted of the parents 'Cm-UTKH' and 'Cm-UTA' with their hybrids 'Cm-UTKHA' and 'Cm-UTAKH'. The last cluster was located on the quadrate determined by negative PC1 and PC2 and contained the parent 'Cm-UTZ' and its hybrid 'Cm-UTZKH' (Fig. 3).

Table 3 - Principal component analysis unrotated factor loadings and communalities

Variable	Factor1	Factor2	Factor3
Weight	0.17	0.901	0.141
Weight loss	-0.594	-0.433	-0.418
Firmness	0.754	-0.328	-0.464
Firmness loss	-0.705	0.354	-0.139
TSS	0.868	-0.034	0.131
Lightmess	0.51	0.484	-0.661
Hue	-0.852	0.347	-0.108
Chroma	0.911	0.334	-0.02
Colour changes	0.651	-0.215	0.2
% Var	49.3	19.4	10.4
Accumulated	49.3	68.7	79.1

Response surface analysis throughout storage period

Contour plotting was used to demonstrate fruit postharvest performance during cold storage period can be seen in figure 4 and figure 5. The most pronounced weight loss was observed in the parent 'Cm-UTZ' under 12°C and after 20 days in in cold storage with more than 30% weight loss. On the other hand, the highest firmness losses were recorded under 12°C and after 30 days in cold storage with the line 'Cm-UTA' and its hybrids 'Cm-UTAKH' and 'Cm-UTKHA' with more than 80% firmness loss in the parent and more than 75% firmness loss in both hybrids.

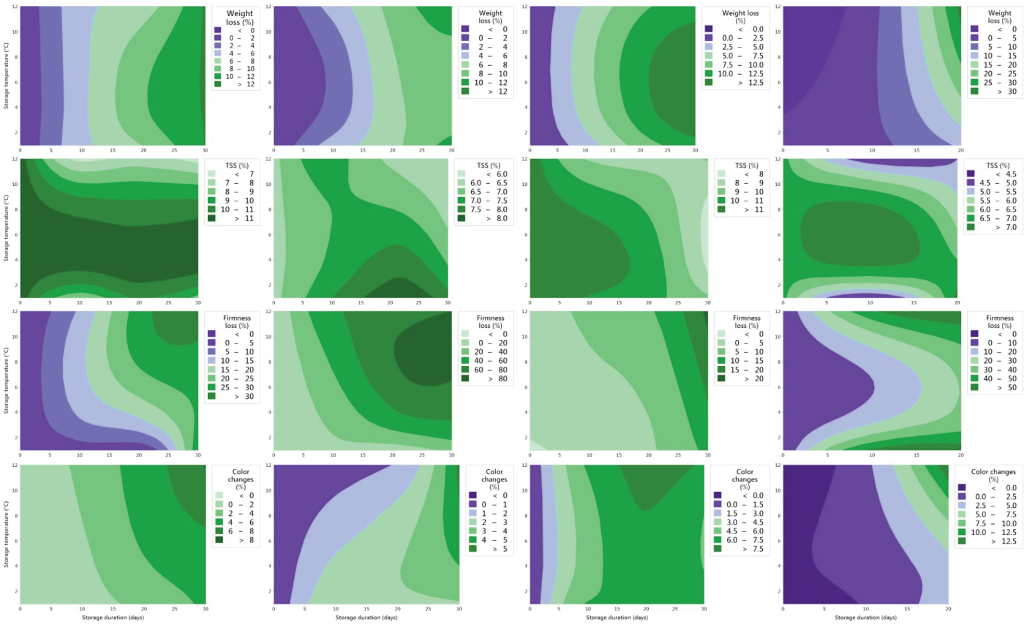


Fig. 4 - Contour plots of postharvest changes during cold storage period for the studied inbred lines. Each column of contour plots represents an inbred line (From left to right 'Cm-UTKH', 'Cm-UTA', 'Cm-UTJ', and 'Cm-UTZ', respectively). Each row of contour plots represents a postharvest attribute (From top to bottom Weight loss, TSS, Firmness loss, and Color changes, respectively). A significant difference between contour levels is present when the difference between contour levels are larger than 1.32, 1.67, 5.97, and 1.02 for Weight loss, TSS, Firmness loss, and Color changes, contours respectively ($P<0.05$).

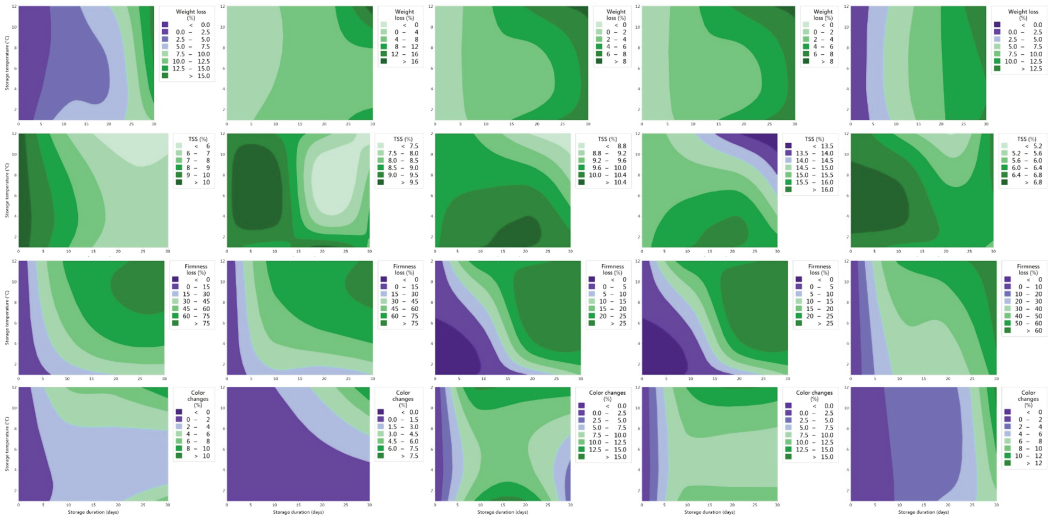


Fig. 5 - Contour plots of postharvest changes during cold storage period for the studied hybrids. Each column of contour plots represents a hybrid (From left to right 'Cm-UTKHA', 'Cm-UTAKH', 'Cm-UTKHJ', 'Cm-UTJKH', and 'Cm-UTZKH', respectively). Each row of contour plots represents a postharvest attribute (From top to bottom Weight loss, TSS, Firmness loss, and Color changes, respectively). A significant difference between contour levels is present when the difference between contour levels are larger than 1.32, 1.67, 5.97, and 1.02 for Weight loss, TSS, Firmness loss, and Color changes, contours respectively ($P<0.05$).

There were no significant colour changes during the first 10 days of storage under any of storage temperature in any of the studied lines except for 'Cm-UTZ'. However, all the studied hybrids showed significant colour changes after 10 days in cold storage and under all temperature except for 'Cm-UTKHA' which showed significant colour changes only after 20 days in storage and under higher storage temperature (>5°C). colour changes were steep and more pronounced in the hybrids 'Cm-UTKHJ' and 'Cm-UTJKH'.

There was no defined trend in TSS changes throughout storage period in the studied genotypes. A general decreasing trend was noticed in the lines 'Cm-UTJ' and 'Cm-UTZ' and similar decreasing overall trends were observed in all the studied hybrids. On the other hand, minimal TSS fluctuations were observed in the line 'Cm-UTA'. Furthermore, the line 'Cm-UTKH' experienced significant decrease in TSS during the first 10 days of storage under 12°C and then remained constant for the rest of storage duration. However, the line 'Cm-UTKH' kept an almost constant TSS under 4°C throughout storage period.

Response surface model was used in order to calculate the optimized storage temperature of the clusters obtained via PCA analysis. The monitoring date and storage temperature were set to be the continuous factors of the model, while weight loss (WL), firmness loss (FL), colour changes (CC), and TSS were set as responses. Stepwise method was used to omit unnecessary sources for better fit of each model (Table 4). Then, the optimized storage temperature for each cluster was calculated by setting optimization goals of WL, FL, and CC to a minimum and TSS to a maximum. The results showed that the optimized temperatures were 1.8 for the green and blue clusters, and 5.1 for the red cluster.

4. Discussion and Conclusions

Fruit characteristics

Cluster analysis showed that fruit shape was more correlated with fruit length than diameter even though these three attributes were located in the same cluster (Fig. 2). This relation was also observable in (Table 2) since the line 'Cm-UTKH' recorded the highest FL and FS values, while the line 'Cm-UTZ' recoded the lowest. In fact, both these traits had also a high heterosis values with 33.17% and 22.71% for FL and FS, respectively, which is similar to Monforte *et al*. (2005) reports of significant correlation between fruit shape and length. The same research suggested that the heterosis in fruit shape is a function of fruit elongation.

On the other hand, the segregation fruit diameter, mesocarp thickness, fruit weight, and cavity diameter might indicate that the more rounded the melon is, the thicker the mesocarp and thus, the heavier fruit will be. The relatively high heterosis in mesocarp thickness and fruit weight (17.48% and 29.35%,

Table 4 - Storage temperature optimization results for the clusters obtained by principal component analysis using response surface method

Model	Response surface regression	Sources			Optimization goal	Optimized storage temperature
		Linear	Square	interaction		
Green cluster	WL	D	-	-	Minimize	1.8
	FL	D, T	T x T	D x T	Minimize	1.8
	CC	T	T x T	-	Minimize	1.8
	TSS	T	-	-	Maximize	1.8
Blue cluster	WL	D, T	D x D, T x T	D x T	Minimize	1.8
	FL	D, T	T x T	-	Minimize	1.8
	CC	D, T	T x T	D x T	Minimize	1.8
	TSS	D, T	T x T	-	Maximize	1.8
Red cluster	WL	D, T	D x D	-	Minimize	5.1
	FL	D, T	T x T	-	Minimize	5.1
	CC	D, T	-	D x T	Minimize	5.1
	TSS	T	T x T	-	Maximize	5.1

WL= Weight loss, FL= Firmness loss), CC= Colour changes, TSS =Total soluble solids, D= Test date, T= Storage temperature.
Cluster colours (green, blue, and red) refer to the principal component analysis (PCA) clustering shown in figure 3.

respectively) and the low heterosis in cavity diameter might in favour of the previous debate. The current results showed that the hybrids 'Cm-UTKHA', 'Cm-UTAKH', and 'Cm-UTKHJ' scored positive weight heterosis values compared to best parents, while 'Cm-UTJKH' and 'Cm-UTZKH' that scored negative weight heterosis compared to mid parent results. This result is similar to Monforte *et al*. (2005) and Mohammadi *et al*. (2014) reports of positive to negative heterosis for melon fruit weight, while the general positive heterosis for fruit weight is similar to this reported by Feyzian *et al*. (2009). Furthermore, the results refer to the importance of the line 'Cm-UTKH' in breeding programs to increase average fruit weight.

It was noticed that each hybrid clustered with its maternal parent, except for the hybrid 'Cm-UTAKH' which was however the closest to its maternal parent 'Cm-UTA' although they were not clustered together (Fig. 2). This observation refers to the high maternal impact of the used lines and the cytoplasmic inheritance for the studied traits. The cytoplasmic inheritance and the high similarities between hybrids and maternal parents was previously reported for fruit length, fruit diameter, fruit shape, average fruit weight, mesocarp thickness, cavity diameter, TSS, and firmness (Y. Hassan Al-Hamdany, 2013; Shajari *et al*., 2021) while other study reported a non-significant reciprocal effect for fruit weight (Feyzian *et al*., 2009).

Postharvest performance

In the current work, PCA clustered the studied genotypes according to their average postharvest performance into three clusters with each hybrid clustered with its maternal parent. The high similarities in postharvest behaviour between hybrids and their maternal parent can be also attributed to the significant reciprocal effects for the studied traits, which were previously reported in similar population (Alabboud *et al*., 2020).

By comparing loading variables to samples distribution, it can be concluded that the 'Cm-UTJ' line with its hybrid 'Cm-UTJKH' and reciprocal 'Cm-UTKHJ' had lower weight loss and firmness loss during cold storage compared to 'Cm-UTZ' and its hybrid 'Cm-UTZKH'. Thus, the former samples had a better postharvest performance compared later (Fig. 3) which can be also noticed in (Fig. 4) and (Fig. 5). colour changes in the line 'Cm-UTJ' and its hybrids are attributed to chroma increase which indicates more saturated colour during cold storage.

According to PCA biplot (Fig. 3), the samples with higher TSS content were characterized by lower weight and firmness losses during cold storage; therefore, the types with higher TSS content performed better throughout cold storage. Previously a negative correlation was reported between sucrose content in blueberry and Polygalacturonase activity (Wang *et al*., 2020). Additionally, postharvest shelf

life of roquette leaves was extended in relation to higher sucrose content (Clarkson *et al*., 2005). Furthermore, it was reported that sucrose can act as a protective signal in fresh cut melon against wounding signal (Wu *et al*., 2020). Considering the fact that sucrose is the major component in melons' TSS content (Burger *et al*., 2000), it can be assumed that the current observed correlation between TSS content and enhanced postharvest performance is related to a higher sucrose level. However, this relation should be investigated thoroughly.

Storage temperature optimization

The decrease in fruit weight and firmness in addition to colour changes are among the usual observations throughout cold storage of fruit and vegetables. Weight loss is mainly attributed to water evaporation during storage period (lal Basediya *et al*., 2013) which is supposed to increase by increasing storage temperature as can be seen in all the studied types (Fig. 4 and 5). The differences in weight loss between different types during storage phase should correlated with fruit characteristics. For instance, the line 'Cm-UTJs' and its related hybrids are characterized with thicker rind and suberized periderm tissues which can efficiently block water evaporation (Nishizawa *et al*., 2017) especially when considering the non-climacteric behaviour of the aforementioned types compared to other climacteric types such as 'Cm-UTZ'. Firmness loss is the result of cell wall deterioration due to various enzymes activity throughout storage (Qi *et al*., 2011; Wu *et al.*, 2020). The current results showed lower and slower firmness loss under lower storage temperature, which is usually observed in stored melon (Hatami *et al*., 2019) due to the slower overall metabolism and the lower enzymatic activity.

The currently observed fluctuation in TSS during cold storage phase was observed in full melon fruit (Hatami *et al.*, 2019) and in fresh cut slices (Chong *et al*., 2015). In fact, most of the observations where TSS fluctuated were under lower storage temperature (1°C) (Fig. 4 and 5). Therefore, the most convincing explanation is that fruit metabolism under lower temperature was lower than that of higher storage temperature; therefore, water evaporation from fruit resulted in higher concentrations of soluble solids.

Response surface method (RSM) is a combination of experimental design, analysis of regression and stochastic response optimization (Barton, 2013). The main advantage of RSM analysis compared to other optimization methods is that fewer number of experiments are needed to monitor the interaction of the independent variables on the response (Wani *et al.*, 2012). PCA clustering results were used to produce response surface models. The postharvest related data of each cluster members were the inputs while the target was to find the storage temperature which minimizes weight losses, firmness losses and color changes while maintaining the highest TSS possible. RSM analysis showed that the optimized storage temperature for both green (netted cantalupensis melon) and blue clusters (inodorus) members was 1.8°C. This result is similar to previous recommended storage temperature for various inodorus group cultivars (Yang *et al*., 2003) where a storage temperature of 3°C was recommended, and netted cantalupensis group cultivars (De Arruda *et al*., 2003; Žnidarĉiĉ *et al*., 2013) where a storage temperature of 2-3°C resulted in the best storage outcomes. On the other hand, the optimum storage temperature for the red cluster (dudaim) was 5.1°C which is similar to the previous observations in dudaim group including the line 'Cm-UTZ' where 5°C resulted in better colour preservation and less weight, firmness, and TSS losses during cold storage period (Hatami *et al*., 2019). Therefore, RSM can be considered a feasible method of storage temperature optimization especially in newly established populations and hybrids.

The current study showed that the hybrid melon types of inodorus x cantalupensis and dudaim x inodorus crosses had more similarities with their maternal parent. These similarities were obvious not only on fruit characteristics directly after harvest, but also in the later postharvest performance in cold storage. This result illustrated the importance of cytoplasmic inheritance in melon. The best postharvest performance was that of crosses between inodorus x netted cantaloupe, while the worst performance was related to 'Cm-UTZ' line of the group dudaim and its hybrid with the line 'Cm-UTKH'. The current reported work flow of using PCA followed by RSM showed promising results in optimizing storage temperature which was similar to previous literature. Therefore, the use of this workflow is highly recommended in optimizing postharvest inputs especially when testing for newly introduced hybrids where the experimental work volume might be overwhelming.

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Decreasing postharvest chilling injury of guava fruit by using melatonin treatment

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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: Guava fruit is a tropical fruit thus sensitive to the chilling injury. In this study the effects of melatonin (known to protect membrane integrity and to help to face abiotic and biotic stress) is evaluated for reduction of chilling injury during postharvest. Guava fruits were dipped into 10, 100 and 1000 µmol L^{-1} melatonin solutions, then kept at cold storage (10±1°C and 90% relative humidity) for 21 days. Several parameters including chilling injury, malondialdehyde content, electrolyte leakage and increased total phenolic compounds and antioxidant activity, phospholipase D and lipoxygenase activity were measured after treatment. Measurements were made every 7 days during the storage. Results showed that melatonin decreased chilling injury, malondialdehyde content, electrolyte leakage and increased total phenolic compounds and antioxidant activity compared to the control. Also, results indicated that chilling injury of guava fruit by using melatonin decreased through increasing integrity of membrane and reducing phospholipase D and lipoxygenase activity. Thus, melatonin can be a useful treatment for decreasing postharvest chilling disorder of guava fruit.

1. Introduction

Guava (*Psidium guajava* L.) is one of the most important fruits of tropical and sub-tropical regions in the world. The fruits are delicious, rich in vitamin C and minerals (Deepthi *et al.,* 2016). There is a great demand of guava fruits in both domestic and international markets for fresh and processing purposes.

Cold storage is one of postharvest technologies for maintaining quality of horticultural crops until human consumption. However, guava is sensitive to chilling disorder of cold storage (temperature of below 12°C). Signs of chilling injury include irregular ripening and surface pitting on the fruit which decreases quality of fruit (Etemadipoor *et al.,* 2020). Resistance to the chilling temperature related to several factors. One of the most important factors is maintaining membrane integrity

(Wongsheree *et al.,* 2009). Membrane integrity can be measured using leakage, malondialdehyde content, lipoxygenase and phospholipase D (Aghdam *et al.,* 2014). Several methods used to decrease chilling injury symptoms of fruit rely on the use of hot water and UV-C (Pongprasert *et al.,* 2011).

Melatonin plays in fruit ripening and senescence and membrane integrity and protection against abiotic and biotic stresses (Rastegar *et al.,* 2020). Melatonin treatment maintained quality of in pear (Liu *et al*., 2019), peach (Gao *et al.,* 2016), and grape (Xu *et al.,* 2018) and tomato (Aghdam *et al.,* 2019) fruits during cold storage.

However, melatonin effects on reducing chilling injury of guava fruit have not been evaluated during cold storage. Therefore, the purpose of this study was to investigate melatonin effects on chilling injury reduction.

2. Materials and Methods

The guava fruits (green stage maturity) were bought from a commercial orchard in Hormozgan province, Iran and the uniform sized fruits were transferred to the laboratory. Twelve fruits per four replications were dipped into 10 (T2), 100 (T3) and 1000 (T4) µmol L^{-1} melatonin solutions for 10 min. Distilled water was used as the control (T1). Fruit were kept at cold storage (10±1°C and 90% relative humidity) for 21 days. Several parameters were measured soon after treatment, and then the measurements were made every 7 days during the storage. Finally, the parameters were checked again one day after exposing to the ambient temperature (25±1°C). The measurements included chilling injury index assessment, percentage of ion leakage, malondialdehyde content, weight loss, titratable acidity, soluble solids concentration (SSC), ascorbic acid, total phenolic content (TPC), antioxidant activity, phospholipase D and lipoxygenase activity.

Chilling injury (CI) index assessment, Percentage of ion leakage (EL) and Malondialdehyde (MDA) content

CI index was assessed subjectively with a scale from 1 to 5, where 5= >50% surface pitting area, 4= 31-50% surface pitting area, 3= 16-30% surface pitting area, 2= 1-15% surface pitting area, 1= 0% no chilling symptoms.

EL was measured by using method described by Madani *et al.* (2016) and results expressed as percentage.

The MDA content was determined based on the method described by Wang *et al.* (2015). The content of MDA was expressed as nmol g^{-1} FW.

Weight loss, Soluble solids concentration (SSC), Titratable acidity (TA), Ascorbic acid, Total phenolic content (TPC) and Total Antioxidant activity

Weight loss was measured based on initial and final experiment at 7-day intervals during storage using a digital balance, and results were expressed as percentage.

Soluble solids concentration (SSC) and TA of pulp tissues were measured by using the method of Ali *et al.* (2011) and the results were expressed as %SSC and %TA, respectively. Ascorbic acid was measured using dye method described by Ranggana (1986) and results were expressed as mg 100 g^{-1} fresh weight (FW).

TPC were assayed using Folin-Ciocalteu procedure (Singleton and Rossi, 1965). Results were expressed as mg of gallic acid equivalents (GAE) per gram of fresh weight (mg GAE g^{-1} FW). The DPPH assay was measured according to the method described by Mirshekari *et al.* (2019). Results were expressed as percentage.

Phospholipase D and Lipoxygenase activity

Phospholipase D and lipoxygenase assay was determined based on the method described by Aghdam and Mohammadkhani (2014). One unit of Phospholipase D was defined as the amount of enzyme that catalyzed the formation of 1 nmol D-nitrophenol h^{-1}. One unit of lipoxygenase was defined as the amount of enzyme which causes an increase in absorption of 0.01 min^{-1} at 234 nm and 25°C when linoleic acid was used as the substrate. Protein content was estimated according to Bradford (1976). Enzymes activities were expressed as units per milligram of protein.

Statistical analysis

Experiments were carried out using completely randomized design. Four replications per treatment used for this study. Data were analyzed using (SAS) version 8.2 (SAS Institute Inc., Cary, NC, USA). Variation Sources were storage days and treatments and means were compared with Duncan's Multiple Range Test (DMRT) at significance level of 0.05.

3. Results and Discussion

Chilling injury (CI), Electrolyte leakage (EL), and Malondialdehyde (MDA)

In the present study, melatonin treatment reduced chilling injury index of guava fruit after 7 days of chilling storage when compared with the control (Table1). Moreover, CI increased with storage time (Table 1). At the end of storage day control (T1) had the severe chilling injury index (4.8) with highest pitting signs, while T4 had the lowest chilling injury index of 3.2. Usually, CI happens at the cell membrane, and maintaining its integrity reduces CI (Mirdehghan *et al.,* 2007; Mirshekari *et al.,* 2020).

Accordingly, Electrolyte leakage has been used as an indicator of membrane damage. In this study, EL increased during storage for control and treated melatonin fruits (Table 1). However, at the end of storage day EL was lower in T3 and T4 compared to the T2 and T1 (Table 1). These results showed the role of melatonin in maintenance of membrane integrity. Comparable results have been stated for sapota fruit by Mirshekari *et al.* (2020). Researches have shown that melatonin treatments affect electron flow acceleration in mitochondria to maintain membrane integrity (Tan *et al.,* 2013).

As shown in Table 1, MDA of control fruit increased during storage. Also, lower MDA content were observed in all melatonin treated fruits compared to the (T1) after 21 days of chilling storage (Table 1). One of the first events in the CI is membrane lipid peroxidation. MDA is the final product of lipid peroxidation (Imahori *et al.,* 2008). Lower temperatures are the main inducers of oxidative damage which produce higher ROS and change the ratio of unsaturated fatty acids to saturated forms (Antunes and Sfakiotakis, 2008). Melatonin treatments lowered MDA accumulation of sapota fruit (Mirshekari *et al.,* 2020).

Weight loss, SSC, TA, Ascorbic acid, TPC and Total Antioxidant activity

Weight loss was at the highest rate (14.3 %) in the control fruits (T1) after 21 days. Treated fruit (T4) showed lower weight loss (6.7%) compared to the T1 and T2 at the end of storage day (Table 2). Weight loss is an index for assessing quality of fruits during storage (Yaman and Bayonidirli, 2002). Skin strength properties of fruit by using melatonin treatment might lower weight loss. Our results are comparable with Rastegar *et al.* (2020) who indicated that the weight loss was decreased by using melatonin treatment in mango.

SSC and TA concentration are main factors for fruit quality judgment. The initial SSC value of this study was 5.2% in control fruits (T1) and increased

Table 1 - Melatonin treatments (0, 10, 100 and 1000 µmol L^{-1}) effects on chilling injury index (CI), electrolyte leakage (EL) and malondialdehyde (MDA) content in guava fruit stored at 10°C for up to 21 days

Treatment (µmol L^{-1})	Storage (day)			
	0	7	14	21
CI				
0 (T1)	0 a D [z]	2.3 a C	3.5 a B	4.8 a A
10 (T2)	0 a D	2.1 a C	3.2 ab B	4.1 b A
100 (T3)	0 a D	1.1 b C	2.5 bc B	3.8 b A
1000 (T4)	0 a D	1.3 b C	2.1 c B	3.2 c A
EL (%)				
0 (T1)	6.5 a D	18.0 a C	32.3 a B	49 a A
10 (T2)	6.5 a D	16.5 a C	30.2 a B	47 a A
100 (T3)	6.5 a D	12.5 b C	22.2 b B	31.7 b A
1000 (T4)	6.4 a D	10.5 b C	20.5 b B	32.3 b A
MDA (nmol g^{-1} FW)				
0 (T1)	5.5 a D	8.3 a C	12.3 a B	15.6 a A
10 (T2)	5.3 a D	7.2 b C	11.6 a B	13.1 b A
100 (T3)	5.0 a B	5.6 c B	7.6 b A	8.5 c A
1000 (T4)	5.6 a B	5.9 c B	8.2 b A	9.1 c A

[z] Small and capital letters show significant differences by DMRT at P= 0.05 between treatments in columns, and storage time for each parameter, respectively.

Table 2 - Melatonin treatments (0, 10, 100 and 1000 µmol L^{-1}) effects on weight loss, soluble solids content (SSC), titratable acidity (TA), ascorbic acid, total phenolic content (TPC) and total antioxidant activity (TAA) in guava fruit stored at 10°C for up to 2

Treatment (µmol L^{-1})	Storage (day)			
	0	7	14	21
Weight loss (%)				
0 (T1)	0 a D [z]	4.5 a C	9.5 a B	14.3 a A
10 (T2)	0 a D	3.2 b C	8.3 b B	13.4 a A
100 (T3)	0 a D	2.6 c C	5.2 c B	8.6 b A
1000 (T4)	0 a D	2.1 c C	4.2 d B	6.7 c A
SSC (%)				
0 (T1)	5.2 a D	8 a C	12.1 a B	13.4 a A
10 (T2)	5..1 a C	7.3 ab B	11.7 a A	12.6 a A
100 (T3)	5.2 a D	6.6 bc C	8.2 b B	9.2 b A
1000 (T4)	5.2 a C	5.8 c BC	6.9 c AB	7.5 c A
TA (%)				
0 (T1)	0.9 a A	0.6 a B	0.4 b C	0.3 bc C
10 (T2)	0.9 a A	0.7 a B	0.4 b C	0.2 c C
100 (T3)	0.9 a A	0.8 a A	0.7 a A	0.5 ab B
1000 (T4)	0.9 a A	0.7 a AB	0.6 a BC	0.5 a C
Ascorbic acid (mg 100 g^{-1} FW)				
0 (T1)	135.5 a B	145.6 b A	121.5 b C	118.3 c C
10 (T2)	136.4 a B	149.2 ab A	126.5 b C	122.4 bc C
100 (T3)	136.2 a B	151.2 a A	132.7 a C	127.2 ab D
1000 (T4)	134.5 a B	150.3 ab A	133.8 a B	129.1 a C
TPC (mg GAE g^{-1} FW)				
0 (T1)	175 a D	210.6 c A	189.7 b B	181.5 d C
10 (T2)	172 a C	201.2 d A	191 b B	188.5 c B
100 (T3)	173.2 a D	233 a A	213.2 a B	200.7 b C
100 (T4)	174 a D	224.2 b A	218.7 a B	214.2 a C
TAA (%)				
0 (T1)	52.3 a C	62..1 b B	70 c A	65.2 b AB
10 (T2)	52 a C	64.2 b B	72.7 c A	67 b B
100 (T3)	53.2 a C	72.4 a B	78.5 a A	74.2 a B
1000 (T4)	52.7 a B	75 a A	77.2 ab A	75.5 a A

[z] Small and capital letters show significant differences by DMRT at P= 0.05 between treatments in columns, and storage time for each parameter, respectively.

during storage (Table 2). However, at the end of storage, T3 and T4 melatonin treatments decreased SSC content compared to untreated (T1) and T2 treatments. Lower SSC content in this research is in agreement with results of Liu *et al.* (2018) who showed that SSC decreased with melatonin treatments during storage. Lower amounts of SSC might be due to the slower respiration rate and a weaker metabolic activity due to the reduced rate of carbohydrate hydrolysis.

Table 2 shows TA content of treated and control fruits. TA content was the highest at harvest day (0.9%). However, TA content decreased during storage. At the end of storage T3 and T4 showed higher TA content compared to the T1 and T2 (Table 2). The higher amounts of TA in melatonin treated fruit can be related to the reduction of respiration rate during storage (Han *et al.,* 2004).

Ascorbic acid decreased during storage. After 21 days of storage, ascorbic acid was higher in T3 and T4 compared to the T1 (Table 2). One of the most significant signs of the nutrient value of fruits is ascorbic acid. The ascorbic acid reduction during storage can be related ascorbic acid oxidase (Choudhary *et al.,* 2016). Melatonin treatment increases oxidative stress resistance by increasing ascorbic acid (Gao *et al.,* 2016). Our results are comparable with Gao *et al.* (2016) who stated that melatonin treatment main-

tained ascorbic acid content of peach.

TPC and antioxidant activity decreased during chilling storage. However, melatonin treated fruit had higher TPC and antioxidant capacity compared with control (Table 2). It has been shown that melatonin treatment increased TPC by regulating gene expression in phenyl propanoid pathway (Zhang *et al.,* 2016). Moreover, Liu *et al.* (2018) stated that melatonin treatment increased TPC and DPPH scavenging capacity of strawberry. This indicated that melatonin showed positive effect on antioxidant activity of guava fruit.

Phospholipase D and Lipoxygenase activity

Phospholipase D and lipoxygenase activity increased during chilling storage. However, melatonin treatment decreased their activities during storage (Table 3). Similarly, melatonin treatment decreased CI signs and inhibited Phospholipase D and lipoxygenase activity of sapota fruit (Mirshekari *et al.,* 2020). Studies indicated that CI was achieved by the activities of membranous lipolytic enzymes like Phospholipase D and lipoxygenase which catalyse peroxidation of polyunsaturated fatty acids and are believed to be major contributors to CI in plant tissue (Aghdam and Mohammadkhani, 2014).

4. Conclusions

Results of this study showed that melatonin treatments during cold storage of guava fruit decreased chilling injury, soluble solids concentration, malondialdehyde content, electrolyte leakage, phospholipase D and lipoxygenase activity and increased titratable acidity, ascorbic acid, total phenolic compounds and antioxidant activity compared to the control. Accordingly, we found that melatonin application reduced CI of guava fruit with enhancing membrane integrity and decreasing phospholipase D and lipoxygenase activity.

Concerning the different treatments, we found out that while 10 µmol L^{-1} produced results which were not significantly different from control treatment, 100 µmol L^{-1} and 1000 µmol L^{-1} were significantly different for CI, weight loss, soluble solids concentration, total phenolic compounds and phospholipase D activity at the end of storage. In conclusion, melatonin application at 1000 µmol L^{-1} can be recommended to be used to decrease CI in guava fruit under cold storage.

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Table 3 - Melatonin treatments (0, 10, 100 and 1000 µmol L^{-1}) effects on phospholipase D (PLD) and lipoxygenase (LOX) in guava fruit during storage at 10°C for up to 21 days

Treatment (µmol L^{-1})	Storage (day)			
	0	7	14	21
PLD (U mg^{-1} protein)				
0 (T1)	24 a D [z]	31.5 b C	46.7 a B	51 a A
10 (T2)	22.5 a D	26.7 b C	43.5 a B	51.7 a A
100 (T3)	24.2 a C	24.5 b C	31.2 b B	44 b A
1000 (T4)	22.7 a C	25.5 b BC	28.2 b B	37 c A
LOX (U mg^{-1} protein)				
0 (T1)	1.45 a D	2.95 a C	5.12 a B	8.9 a A
10 (T2)	1.65 a D	2.85 a C	4.47 a B	8.52 a A
100 (T3)	1.72 a C	2.1 b C	2.95 b B	8.25 b A
1000 (T4)	1.47 a C	1.85 b C	3.27 b B	5.65 b A

[z] Small and capital letters show significant differences by DMRT at P= 0.05 between treatments in columns, and storage time for each parameter, respectively.

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Phytoprotective film for resistance induction, growth, and yield of organic strawberries

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Abstract: **The objective of this work was to evaluate a phytoprotective film of chitosan-pyroligneous extract in promoting growth, productivity, induction of systemic resistance in strawberry cultivars managed in an organic production system. Treatments consisted of rates (0, 25, 50, and 100 mL L^{-1}) of Chi-Pyro-Film and a reference resistance inducer (dipotassium hydrogen phosphate - K_2HPO_4), evaluated in three strawberry cultivars ('Albion', 'San Andreas' and 'Portola'). Growth, yield, anthracnose incidence, and enzymatic activity were evaluated. The experimental design was a randomized block design with four replications. Chi-Pyro-Film increases the growth, yield, and anthracnose resistance of strawberry plants. The best concentration of Chi-Pyro-Film varies between 50 and 60 mL L^{-1}, according to strawberry cultivar.**

1. Introduction

Strawberry (*Fragaria x ananassa* Duch.) is the most planted red fruit in Brazil. The high demand is due to its sensory characteristics such as color, texture, aroma, and taste (Ventura-Aguilar *et al*., 2018).

However, according to a report by the Brazilian Health Surveillance Agency (ANVISA) of 2016, 26% of strawberry samples collected between 2013 and 2015 presented nonconformities with pesticide residues. Pesticides detected not authorized for the crop, captan stood out, detected in 20.4% of the samples analyzed, among others such as dithiocarbamates, pyrimethanil, carbendazim, tebuconazole, iprodione, and azoxystrobin (ANVISA, 2016). Most of these fungicides aim to control or prevent anthracnose (*Colletotrichum* spp.), a disease that is considered main in strawberry fields in Brazil (Kososki *et al.*, 2001; Capobiango *et al*., 2016).

Currently, there is a search for technologies, which make agriculture sustainable and "smart", with practices and ways to minimize the excessive use of chemicals (Grewal *et al*., 2018). In this context, the use of resistance inducing products activates the plant's natural defenses, enabling disease control in the organic production system.

The resistance is associated with various defense responses, such as protein and phytoalexin synthesis, cell wall changes, and increased activity of various enzymes defense-related (Durrant and Dong, 2004). Responses that are associated with changes in the activity of various enzymes such as peroxidase (POX, EC 1.11.1), polyphenol oxidase (PPO, EC 1.14.18.1), and phenylalanine ammonia-lyase (PAL, EC 4.1.3.5) (Prasannath *et al*., 2014; Prasannath, 2017).

POXs have been implicated in many defense processes, such as hypersensitive response, lignification, phenolic and glycoprotein cross-linking, suberization, and phytoalexin production (Thakker *et al*., 2012; Prasannath, 2017). PPOs are a group of enzymes that catalyze the oxidation of hydroxyphenols to their quinone derivatives, which have antimicrobial properties (Prasannath, 2017). PAL (E.C.4.1.3.5) is the major enzyme in the phenylpropanoid pathway and acts in the synthesis of defense-related secondary compounds such as phenols and lignins (Hemm *et al*., 2004; Vanitha *et al*., 2009).

Among the compounds that have activating properties of defense mechanisms in plants are the chitosan and pyroligneous extract (Di Piero and Garda, 2008; Grewal *et al*., 2018; Souza *et al*., 2018).

Chitosan is a polycationic β-1,4 polymer bound to D-glucosamine chemically derived from crustaceans and soluble in organic acids and known to be a natural elicitor and triggers various physiological and biochemical responses in plants that act in the growth, production, and protection against disease (Chandra *et al*., 2015; Katiyar *et al*., 2015; Pichyangkura and Chadchawan, 2015). Chitosan has several characteristics that make this polymer advantageous for many applications: (1) has a defined chemical structure; (2) may be chemically and enzymatically modified; (3) is physically and biologically functional; (4) is biodegradable and biocompatible with many organs, tissues and cells; (5) can be processed into various products including flakes, powders, membranes, fibers and films (Badawy, 2012; van den Broek *et al*., 2015; Porto *et al*., 2019).

The pyroligneous extract is a liquor with strong smoke flavors, is a crude and acid condensate produced from the distillation of the smoke generated in the carbonization of wood. It consists of a complex mixture of compounds derived from the chemical decomposition of wood components through the condensation of vapors and gases generated during pyrolysis in a low oxygen concentration (Campos, 2018; Pimenta *et al*., 2018).

The pyroligneous extract is composed of water (80-90%) and more than 200 species of organic compounds (10-20%) (Theapparat *et al.*, 2018). The presence of phenolic compounds in the pyroligneous extract confers growth-promoting and antifungal properties, as found in the literature on *Helminthosporium sativum*, *Cochliobolus sativus*, Waltz, *Colletotrichum orbiculare*, *Alternaria mali* (Jung, 2007; Baimark and Niamsa, 2009; Wei *et al.*, 2010 a; Grewal *et al*., 2018).

In this context, the cationic character of chitosan in acidic conditions offers the possibility to establish electrostatic interactions with other negatively charged compounds, for example with the pyroligneous extract, considered a raw material obtained from renewable sources, and is a good solvent for chitosan (Campos *et al*., 2012; van den Broek *et al*., 2015; Porto *et al*., 2019).

The phytoprotective film of chitosan-pyroligneous extract (Chi-Pyro-Film) consists of the chitosan diluted in the pyroligneous extract, and its characteristics are the formation of a film with photoprotection capability against radiation (UV-B and UV-C), fungi toxic action, and inducing systemic resistance in plants (Campos *et al*., 2012).

The objective of the present work was to evaluate the effect of different concentrations of phytoprotective film formulated with chitosan and pyroligneous extract (Chi-Pyro-Film) and dibasic potassium phosphate (K_2HPO_4) on growth promotion, resistance induction to anthracnose, yield, and defense enzyme activity in strawberry cultivars in the organic production system.

2. Materials and Methods

The study was carried under field conditions, at Embrapa Temperate Climate Experimental Station, in Pelotas city, Rio Grande do Sul state, Brazil (31°40'49 "S 52°26'18" O, at 60 m altitude) (Fig. 1 a). The climate of the region, according to the Köppen classification is Cfa type, temperate and humid, with hot summers.

Raw materials

The pyroligneous extract was obtained through an extraction procedure of *Eucalyptus grandis* proposed by Campos (2018) and the distillation process was performed according to that described by Porto *et al.*

(2019). Chitosan was supplied by Nutrifarm™, with a 97% degree of deacetylation, determined by proton magnetic resonance (Porto, 2011).

Plant material

The seedlings of neutral-day strawberry cultivars, 'Albion', 'San Andreas' and 'Portola', were planted in May and were grown under a low tunnel system with mulching and drip irrigation. The spacing between lines and between plants was 0.30 m, with three lines per bed. The area had a history of many years with severe anthracnose incidence in the strawberry plants (Fig. 1b, 1c). Liming and fertilization were performed according to the recommendation for strawberry organic production. Climatic data from the experiment period are shown in figure 1d.

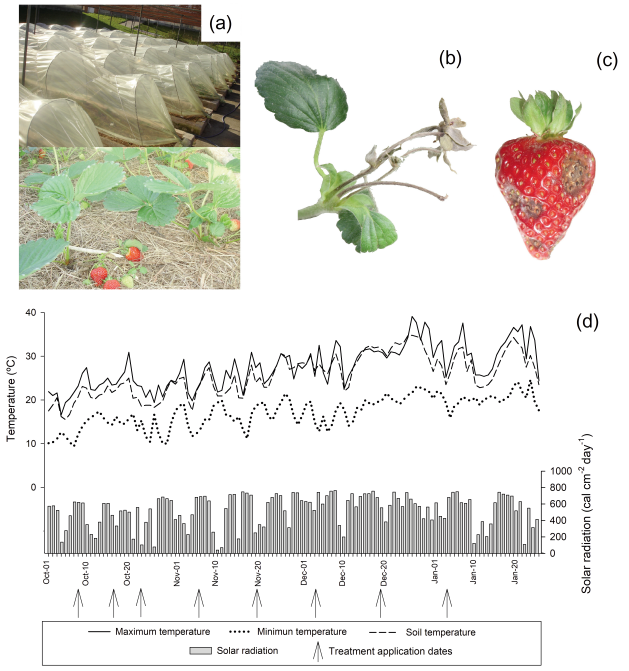

Fig. 1 - Experiment under field conditions (a), anthracnose symptoms in strawberry plants (b) and fruits (c), and climatic data from the experiment period (d).

Treatments

The treatments of induction of systemic resistance consisted of four concentrations of the Chi-Pyro-Film and a reference treatment with dibasic potassium phosphate (K_2HPO_4). The Chi-Pyro-Film was registered in the field of Green Chemistry at the National Institute of Intellectual Property in Brazil (PCT/BR2013/000597), United States (US201503 36854A1) and Germany (DE112013006230T5) as a phytoprotective for agriculture use.

The Chi-Pyro-Film with a concentration of 30 g L^{-1} (Fig. 2) was diluted with distilled water in different concentrations (0, 25, 50, and 100 mL L^{-1}). Treatment with K_2HPO_4 was applied at a concentration of 50 mM (Orober *et al*., 2002; Aleandri *et al*., 2010). This compound has shown efficacy against e.g. powdery mildew on barley, cucumber, pepper, and tomato (*Blumeria graminis* f. sp. hordei, *Sphaerotheca fuliginea*, *Leveillula taurica,* and *Erysiphe oronti*, respectively), anthracnose (*Colletotrichum lagenarium*) on cucumber, rust (*Puccinia sorgi*) and leaf blight (*Exserohilum turcicum*) on maize and rice blast (*Pyricularia oryzae*), mildew (*Sphaerotheca fuliginea*) (Reuveni *et al*., 1996; Reuveni and Reuveni, 1998; Manandhar *et al*., 1998; Reuveni *et al*., 2000; Ehret *et al*., 2002; Orober *et al*., 2002; Hamza *et al.,* 2017).

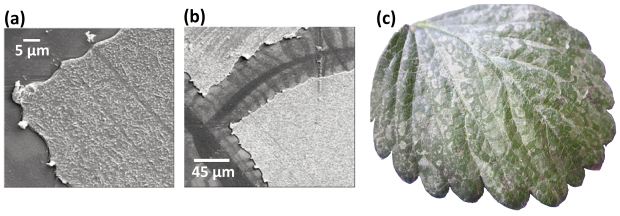

Fig. 2 - Electron micrograph of the phytoprotective film of Chi-Pyro-Film, after spraying on a smooth surface at a temperature of 18 to 25°C (a and b). Chitosan strawberry leaf covered with a film formed by Chi-Pyro-Film (c).

The spraying of treatments started at the beginning of fruiting, and the application dates were indicated in figure 1d. The spray volume was 4.5 mL per plant (500 liters per hectare), applied through conical jet nozzles, observing a total coverage of the plants until close to the runoff point.

Analysis of plant growth, damage by anthracnose, and fruit yield

Vegetative growth evaluations consisted of dry mass (g $plant^{-1}$) of crown, root, and leaf, by drying to constant weight (65°C) of three plants collected in each experimental unit at the end of the production cycle, 256 days after seedlings transplanting. The productive variables measured were fruit yield (g $plant^{-1}$) and fruit weight (g $fruit^{-1}$), obtained by evaluating the total fruits harvested in each experimental unit. The percentage of fruits with anthracnose was obtained by counting the fruits with symptoms at each harvest (Fig. 1c). The harvests were carried out three times a week between October and January.

Enzymatic activity analysis

Biochemical evaluations were realized by determination of the specific activity of peroxidase (POX),

polyphenoloxidase (PPO), and phenylalanine ammonia-lyase (PAL) enzymes, in strawberry leaf samples collected immediately before application (BA) and 48 hours after application (AA) of Chi-Pyro-Film concentrations and reference treatment (K_2HPO_4). For the extraction of POX and PPO, was used 500 mg of ground tissue below 4°C in 10 mL of 0.05 M phosphate buffer (pH 7.0) containing 1 mg of polyvinylpyrrolidone-10. Subsequently, centrifugation was performed at 4,000 *g* for 30 minutes under refrigeration. The supernatant was preserved on ice and used for determinations according to Campos *et al*. (2003). The POX and PPO extraction was carried out by grinding the leaves with 20 mg polyvinylpyrrolidone (Sigma-Aldrich). The enzyme extract obtained after filtration (Whatman 1) and centrifugation (5,600 g*n*, 15min) were used to test the activity.

POX activity was determined in the enzyme extract mix with a phosphate-citrate buffer composed of 0.2 M sodium phosphate solution and 0.1 M citric acid (pH 5.0).

The mixture was homogenized in vortexed for 15 seconds. POX activity was determined according to Campos *et al*. (2004).

PPO activity was determined in the enzyme extract with 3.6 mL of 0.05 M phosphate buffer (pH 6.0) and 0.1 mL of 0.1 M catechol. PPO activity was determined according to Campos *et al*. (2004).

PAL activity was determined in crude leaf extracts according to the methods described by Hyodo and Yang (1971) and Hyodo *et al*. (1978) modified by Campos *et al*. (2003).

For extraction, 500 mg of tissue was macerated (below 4°C) with 8 mL of 50 mM sodium borate buffer (pH 8.5) containing 25 g L^{-1} of polyvinylpyrrolidone-10 and 4 mL L^{-1} of mercaptoethanol. The protein extract obtained after filtration (Whatman 1) and centrifuged (5,600 g_n, 30 min) under refrigeration (below 4°C). was used to assay the PAL activity. Protein in the extracts was determined by the Bradford method (Bradford, 1976).

Experimental design and statistical analyses

The experimental design was randomized blocks with four replications of nine plants. The results were submitted to variance analysis and the means of the variables with significant effect were compared using the Tukey test (cultivars) or regression analysis (concentrations) at 5% error probability.

3. Results

Chi-Pyro-Film concentrations had a significant effect on the growth, yield, anthracnose resistance induction, and enzymatic activity of strawberry plants.

Growth and development

The dry mass of the plant showed factors interaction. The three cultivars showed a quadratic response to Chi-Pyro-Film concentrations (Fig. 3), but the highest efficiency concentration was different. 'Albion' and 'Portola' had the highest efficiency concentration estimated at 60 mL L^{-1} of Chi-Pyro-Film, while for 'San Andreas' the concentration was 50 mL L^{-1} (Fig. 3). The treatment with reference resistance inducer, K_2HPO_4, had also different according to cultivars. In 'Albion', it provided a leaf mass slightly higher than the control treatment and similar to the 100 mL L^{-1} Chi-Pyro-Film concentration, but it was lower than 25 and 50 mL L^{-1} (Fig. 3).

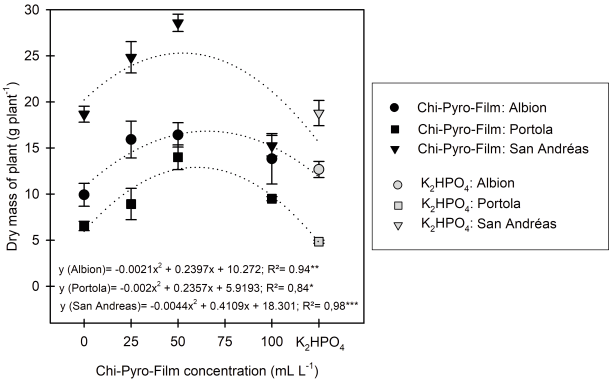

Fig. 3 - Dry mass of leaves of Albion, Portola and San Andreas cultivars, in response to Chi-Pyro-Film concentrations and reference treatment (K_2HPO_4, 50 mM). Interaction effect between factors (cultivar and film concentration). *, **, ***, significant at p<0,05, p<0,01, p<0,001, respectively.

The yield also was significantly influenced by Chi-Pyro-Film, with a quadratic response to isolated effect of the film concentration factor (Fig. 4). Regardless of cultivar, the highest efficiency concentration was estimated at 60 mL L^{-1}. Regarding the reference treatment (K_2HPO_4), it was observed that it had a performance similar to 100 Chi-Pyro-Film and higher than control (0 mL L^{-1} of Chi-Pyro-Film), but less than the 50 mL L^{-1} (Fig. 4). Between cultivars

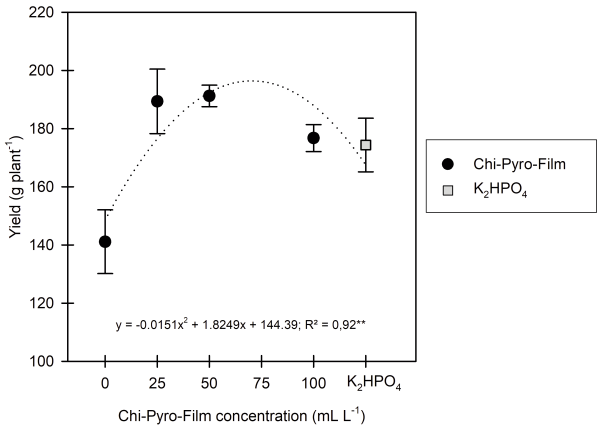

Fig. 4 - Yield of strawberry, in response to Chi-Pyro-Film concentrations and reference treatment (K_2HPO_4, 50 mM). Isolated effect of film concentration (average of cultivars responses). *, **, ***, significant at p<0,05, p<0,01, p<0,001, respectively.

effect, 'Portola' showed the highest yield than 'Albion' and 'San Andreas' (Table 1). Results similar to those observed by Carini *et al*. (2015) in a study of evaluation of strawberry cultivars in an organic system, in which 'Portola' was also more productive than 'Albion' and 'San Andreas'.

The fruit weight was not influenced by Chi-Pyro-Film treatments. However, there was an effect of the cultivar factor, with 'San Andreas' producing fruits of a higher mass (Table 1). Corroborating with Carini *et al.* (2015), which evaluated the same three cultivars, found a higher fruit weight of 'San Andreas', but with the same weight that 'Albion'.

The variable fruits with anthracnose showed an isolated effect of the factor Chi-Pyro-Film concentrations, that is, all cultivars had the same response to the application of Chi-Pyro-Film, with a quadratic reduction in the number of fruits attacked, with the maximum efficiency concentration estimated at 60 mL L^{-1} Chi-Pyro-Film (Fig. 5). The reference treatment with K_2HPO_4 was similar to the 100 mL L^{-1} of Chi-Pyro-Film, but lower than the 25 mL L^{-1} and 50 mL L^{-1} (Fig. 5). Among the cultivars, 'Portola' was the most sensitive to the occurrence of anthracnose in fruits, with no difference between 'Albion' and 'San Andréas' (Table 1).

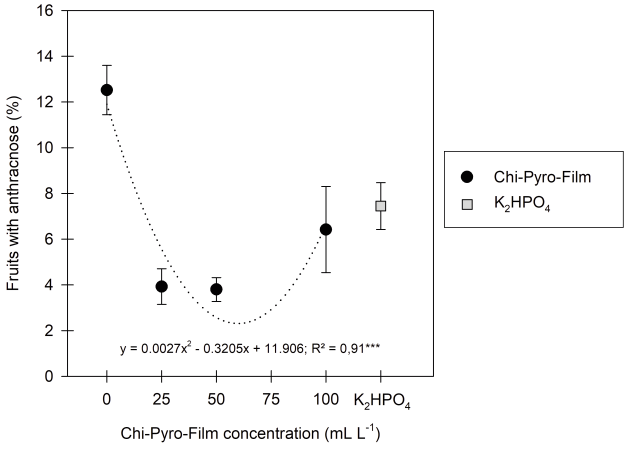

Fig. 5 - Percentage of fruits with anthracnose in response to Chi-Pyro-Film concentrations and reference treatment (K_2HPO_4, 50 mM). Isolated effect of film concentration (average of cultivars responses). *, **, ***, significant at p<0,05, p<0,01, p<0,001, respectively.

Enzymatic activity

In the present study, there was a significant effect of Chi-Pyro-Film concentrations on the activity of POX, PPO, and PAL enzymes (Figs. 6 and 7). An interaction effect between film concentration and sampling time indicated that 48 hours after application, there was a quadratic effect of film concentrations on the activity of the three enzymes studied. In the case of POX and PPO, an activity reduction effect was obtained up to the estimated concentrations of 54 mL L^{-1} and 50 mL L^{-1}, respectively, followed by an increase (Fig. 6a and 6b). However, PAL activity increased until the estimated concentration of 36 mL L^{-1}, with a subsequent reduction (Fig. 6c). About the

Table 1 - Yield, fruit weight and fruits with anthracnose in Albion, Portola and San Andreas cultivars ([z])

Variables	Cultivar			C.V. (%)
	Albion	Portola	San Andreas	
Yield (g $plant^{-1}$)	131.90 ± 7.93 c	221.35 ± 12.87 a	170.4 5± 9.30 b	20.18
Fruit weight (g $fruit^{-1}$)	8.60 ± 0.67 b	8.50 ± 0.57 b	13.33 ± 1.09 a	72.71
Fruits with anthracnose (%)	5.29 ± 1.71 b	9.13 ± 1.53 a	5.83 ± 1.49 b	107.57

[z] Means followed by different lowercase letters in the row, differ by Tukey test at 5% error probability. The averages correspond to the isolated effect of cultivar factor to studied variables.

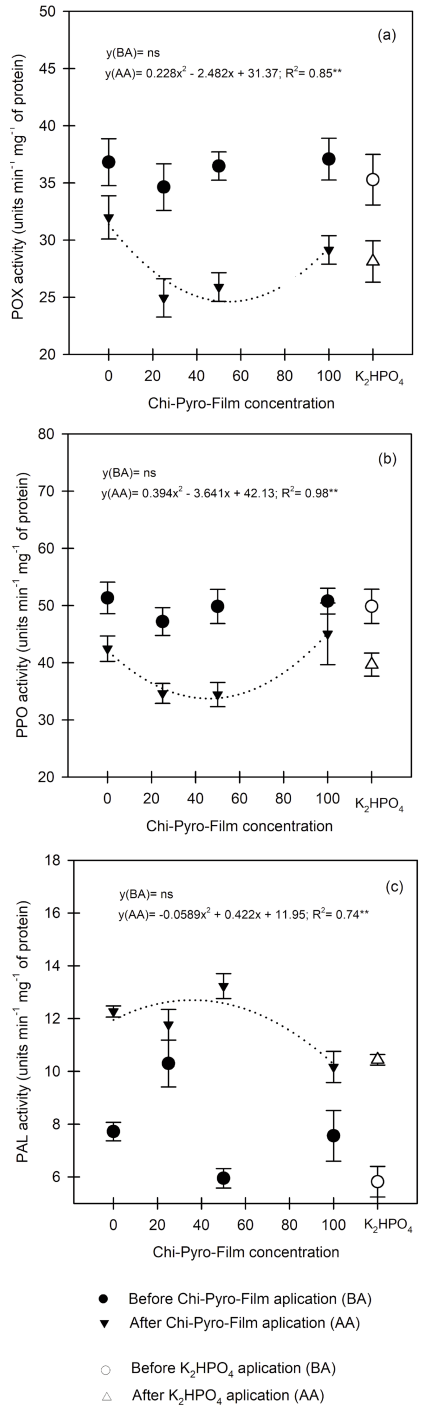

Fig. 6 - Specified activity of peroxidase-POX (a), polyphenoloxidase-PPO (b) and phenylalanine ammonia lyase-PAL (c) in the leaves, before (BA) and after application (AA) of Chi-Pyro-Film concentrations and reference treatment (K_2HPO_4, 50 mM). Interaction effect between film concentration and sampling time (before and 48 hours after application). *, **, ***, significant at p<0,05, p<0,01, p<0,001, respectively.

reference treatment, with K_2PHO_4, it induced activities similar to Chi-Pyro-Film in the concentration of 100 mL L^{-1} for POX, PPO, and PAL.

The enzymatic activity also had an interaction effect between treatments of resistance induction and strawberry cultivars. POX activity decreased to concentrations of 49 mL L^{-1} and 57 mL L^{-1} in Albion and Portola, respectively (Fig. 7a). For San Andreas, although there was a similar trend, it was not significant (Fig. 7a).

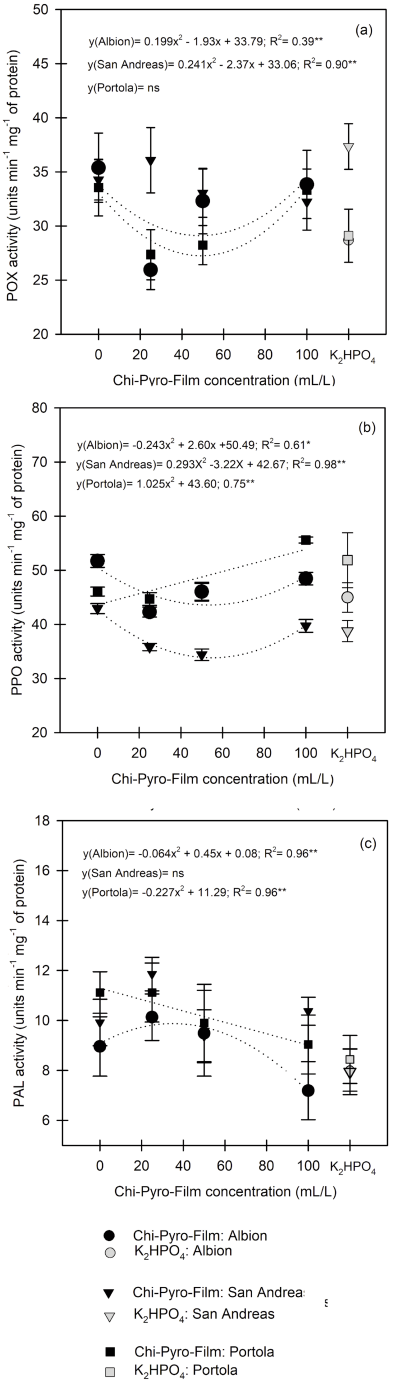

Fig. 7 - Specified activity of peroxidase-POX (a), polyphenoloxidase-PPO (b) and phenylalanine ammonia lyase-PAL (c) in leaves of Albion, Portola and San Andreas, in response to Chi-Pyro-Film concentrations and reference treatment (K_2HPO_4, 50 mM). Interaction effect between film concentration and cultivars. *, **, ***, significant at p<0,05, p<0,01, e p<0,001, respectively.

The cultivars Albion and San Andreas also had a quadratic response to Chi-Pyro-Film, with a decrease in PPO activity up to the estimated concentrations of 54 mL L^{-1} and 55 mL L^{-1} (Fig. 7b). Concerning Portola, there was a linear increase up to a 100 mL L^{-1} concentration of Chi-Pyro-Film (Fig. 7b).

Concerning PAL, there was a quadratic response in Albion, with an increase up to the 38 mL L^{-1} rate of Chi-Pyro-Film, as well as a linear reduction in Portola (Fig. 7c).

The reference treatment with K_2HPO_4, showed a response similar to the 100 mL L^{-1} film concentration for the enzymes PPO and PAL in the three cultivars studied (Figs. 7b and 7c). For the POX in Albion and San Andreas, the reference treatment was similar to the 25 mL L^{-1} concentration, while in Portola, it was similar to the 50 mL L^{-1} film (Fig. 7a).

4. Discussion and Conclusions

In general, the results indicate that Chi-Pyro-Film contributed to the increase of vegetative growth and yield, as well as, to the anthracnose damage reduction in strawberry fruits. Results that are in agreement with those found in the literature, where it is observed an increase in vegetative, productive, and health variables, such as height, number of leaves, leaf area, yield and reduction of incidence of diseases in plants that received chitosan or pyroligneous extract (El-Miniawy *et al*., 2013; Masum *et al*., 2013; Mungkunkamchao *et al*., 2013; Mukta *et al*., 2017; Kumaraswamy *et al*., 2018; Ventura-Aguilar *et al*., 2018).

The effect of Chi-Pyro-Film on strawberry plant growth can be attributed to the role of chitosan as a non-toxic and biodegradable plant growth promoter (Salachna and Zawadzińska, 2014; Ahmed *et al.*, 2020). Some authors suggest that foliar application of chitosan enhances the endogenous concentration of phytohormone such as gibberellic acid and auxin (Uthairatanakij *et al*., 2007; Ahmed *et al*., 2016). But the increase in macro and micronutrient accumulation and improved the content of photosynthetic pigments, provided by chitosan are also related to its influence on plant growth (Shehata *et al*., 2012; Ahmed *et al*., 2016). On the other hand, pyroligneous extract contributes to plant growth by its phytoprotective effect against pathogens, especially fungi. Was reported antipathogenic effects of the pyroligneous extract on plant pathogenic fungi like *Helminthosporium sativum*, *Cochliobolus sativus*, *Valsa mali*, *Colletotrichum orbiculare*, and *Alternaria mali* (Jung 2007; Wei *et al.*, 2010 a). This antifungal activity has been related to the presence of furaldehydes and phenols in pyroligneous extract (Grewal *et al.*, 2018).

The efficiency of spraying with Chi-Pyro-Film can be attributed to the effects of pyroligneous extract and chitosan individually, and to an interaction effect between both. According to Porto *et al.* (2019), who studied physicochemical properties of Chi-Pyro-Film, the film showed a semicrystalline structure, which is smooth and stable up to 50°C, being persistent in environmental conditions; it is permeable to water vapor and has high hygroscopicity, in addition to being able to efficiently block incident UVB and UVC radiation. The coverage presented by the Chi-Pyro-Film, as well as its persistence on the leaf surface (Fig. 2), probably provide several days of action, perhaps a large part of the application interval (15 days).

The main effect of chitosan and the pyroligneous extract is attributed to resistance induction by increased defense enzyme activity and accumulation of phenolic compounds acting on reactive oxygen species (ROS) (Wei *et al*., 2010 b; Katiyar *et al*., 2015; Pichyangkura and Chadchawan, 2015; Grewal *et al*., 2018). This effect is in line with the behavior verified by the PAL activity in this study (Figs. 6c, 7c). However, the activity of the POX and PPO enzymes responded differently, with a reduction in activity in the concentrations estimated between 50 and 60 mL L^{-1}, 48 hours after application of treatments (Figs. 6a 6b), especially in the Albion and San Andreas cultivars (Figs. 7a, 7b). This film rate (50-60 mL L^{-1}) had a better performance in the dry mass accumulation, yield, and incidence of anthracnose.

The results indicate that in addition to the increase in systemic resistance, suggested by the PAL response, there is a direct effect of phytoprotection and a reduction of stress condition. Some aspects that can be associated with this second aspect, maybe the block that the film exerts concerning UVA and UVB radiation, as well as its potential action on pathogens, a hypothesis that corroborates the results obtained by Porto *et al.* (2019).

In this way, it can be suggested that Chi-Pyro-Film has a complex performance, acting both on metabolism with increased plant resistance and reducing cellular damage caused by physical (radiation) and biological stress (pathogens), as well as forming a phytoprotective film that inhibits the direct action of stress

agents, such as UVA and UVB radiation and inhibiting the attack of pathogens.

In general, Chi-Pyro-Film showed greater efficiency in promoting growth, yield, and resistance to anthracnose than the reference treatment (K_2HPO_4), mainly in concentrations between 25 and 50 mL L^{-1}. K_2HPO_4 performed similarly to the 100 mL L^{-1} of the film. Different studies indicate the effect of K_2HPO_4 in the resistance induction of several species (Reuveni and Reuveni, 1998; Kashiap and Dhiman, 2009; Aleandri *et al*., 2010; Hamza *et al*., 2017; El-Tanany *et al*., 2018). According to Orober *et al*. (2002), foliar application of K_2HPO_4 results in the activation of systemic resistance mechanisms. The positive effect of K_2HPO_4 is associated with salicylic acid involved in triggering plant cell defense and sensitization responses for a faster and stronger response to subsequent pathogen attack (Mauch-Mani and Métraux, 1998; Orober *et al.*, 2002).

The 100 mL L^{-1} concentration of Chi-Pyro-Film may be high for the strawberry crop. Probably the increase in film thickness, which according to Porto *et al*. (2019), can significantly reduce the film's permeability to water vapor. An aspect that can make some physiological processes such as the flowing water, the absorption of nutrients, and photosynthesis less efficient.

This study provides indicates that the phytoprotective film (Chi-Pyro-Film) is effective as a growth promoter and inducer of systemic resistance to anthracnose, resulting in increased growth and fruit production in strawberry plants of different cultivars. The optimal concentration of Chi-Pyro-Film ranges between 50 and 60 mL L^{-1}, depending on the strawberry cultivar.

We believe that the tested film can be an important tool for production systems, especially agroecological and organic systems, which require alternatives for disease control. But also, due to its physical-chemical properties and great stability, we believe that the film can be combined with other components, such as nutrients, biostimulants, and plant hormones, to enhance its effect. However, such combinations need to be studied.

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Review paper

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Flower differentiation and fruiting dynamics in olive trees (*Olea europaea*): Eco-physiological analysis in the Mediterranean basin

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Key words: Anthesis, fruit setting, hermaphrodite and staminate flower, olive, pistil abortion.

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Abstract: The formation of flowers in sufficient number and quality is a prerequisite for a successful subsequent fruit set. Despite the abundant flowering, olive trees (*Olea europea)* are characterized by a very low fruit set, and a very severe yield alteration leading to market fluctuation over time. The goal of this paper is to explore and analyze eco-physiological driving factors behind the poor fruit set in Mediterranean olive groves. The key mechanisms causing floral differentiation and extreme yield alternate are functional of plant genetic variability, nutrient competition, and some ecological aspects as a response to climate change. Additionally, olive inflorescence architecture appears to be complex and can vary between cultivars; the olive flower differentiation results in a variable proportion of hermaphrodite, pistillate and staminate flowers among olive cultivars as well as across canopy positions and branches, enhancing nutrient competition between flowers. Self-pollination could be one of the limiting factors for increasing early fruit abscission and extreme alternate fruit-bearing. Hormonal treatments to reduce alternate production in olive trees should be explored. The current review analysis shall help to improve olive grove management, but also for breeding new cultivars more suitable for Mediterranean agro-ecological constraints. Ovule viability and fertilisation, and embryo sac development abnormalities should all be further investigated.

1. Introduction

Olive (*Olea europea*) farming began thousands of years ago in the Middle East and has since spread to the eastern Mediterranean; Spain and Italy produce more than 60% of the world's olive oil (Terral *et al.*, 2004; Herrera-Caceres *et al*., 2017). Some olive groves have been converted to alternative land uses, such as grazing, due to significant yearly production fluctuation and significant competition for nutrient supplies, such as water (Loumou and Giourga, 2003). For the Mediterranean region's delicate environment, olive grove protection is becoming a priority. Olea species are divided into four genetic groupings. According to traditional classification, three of them had close phylogenetic ties and genotypes

that were similar to wild olive genotypes (Angiolillo *et al*., 1999; Contento *et al*., 2002). *Olea* is a genus in the *Oleaceae* family with approximately twenty species that thrive in tropical and subtropical climates throughout five continents. Based on the taxonomic position of the genus *Olea* and of its components, four subgenera (subg.) can be distinguished: subg. *Olea* section *Olea*, subg. *Olea* section *Ligustroides*, subg. *Paniculate* and subg. *Tetrapilus* (Besnard *et al*., 2002; Green *et al*., 2004). Based on morphology and geographical distribution, *O. europea* should be divided into six subspecies based on morphology and geographic distribution, including: (1) Subspecies *europea*, which includes the two botanical varieties, which are *europea* (cultivated olive) and *Sylvestris* (wild olive), and is widely distributed throughout the Mediterranean region; (2) *Cuspidate* subspecies, which is found in Southeast Asia, southern China; and the Arabian Peninsula in the east and south (Contento *et al*., 2002; Rugini, 2016). The *O. europea L.* is a frost-sensitive subtropical evergreen indigenous to the Mediterranean region. It has adapted to a semi-arid temperate climate with well-drained soils, a moderate to low pH (below 8.5), and little salinity in the soil (Terral *et al*., 2004; Doveri and Baldoni, 2007).The olive tree is notable for its abundant bloom, which is followed by a low fruit set and a low yield. Apart from inflorescence structure, cultivars differ substantially in the proportion of a hermaphrodite (bisexual) to staminate flowers on inflorescences (Guevas and Polito, 2004). About approximately 10% to 15% of a mature tree's flowers set fruit, with only 2% to 5% of them developing mature fruits, depending on the location and type (Reale *et al*., 2006). Low fruit set and the shift from non-functional hermaphrodite flowers to fully functional staminate flowers seems to be the most important and limiting factors for olive tree productivity. The formation of functional staminate rather than entirely functional hermaphrodite flowers throughout development is one of the primary factors determining the fruit setting level of olive flowers (Reale *et al*., 2009). Although various studies have been done on the differentiation process of olive flowers, there have been very few specialised investigations to document and compare biological flower development and fruit set research among cultivars in this area. Olive farming has been an important part of Mediterranean nutrition, therapeutic body care, economics, and religious rites for millennia (Angiolillo *et al*., 1999; Rugini, 2016; FAO, 2020). The olive fruit is eaten and pressed for its seed oil, which has been shown to have health-promoting properties when consumed regularly and can be stored and consumed for up to three years if stored properly (Besnard and Bervillé, 2000). The oil is used to make soaps, hair conditioners, massage oils, and other therapeutic products. In terms of nutritional value, as well as economic importance to national economies, they are a necessary food. Olive fruits have been demonstrated to aid in the prevention of coronary heart disease and various cancers due to their high level of monosaturated fatty acids and phenolic compounds (Terral *et al*., 2004). Despite the fact that olives have been cultivated for many years and various cultivars have been domesticated for their fruit quality, morphological and physiological properties, the Mediterranean region's cultivar selection has not fully responded adequately to alternate fruiting and poor terminal fruit sets (Connor *et al*., 2014).

2. Physiological factors driving olive fruit setting

Pistil abortion during olive flowering

The production of functional staminate flowers rather than completely functional hermaphrodites, which are unable to yield fruit, is one of the primary reasons limiting fruit set in olive (*Olea europaea L*.) (Reale *et al*., 2009; Newton *et al*., 2014). Despite their extensive history, many key questions surrounding olives remain unanswered. While there are up to 2,600 different olive cultivars (Rugini and Lavee, 1992), many studies on diversity within *O.europea* have focused on morphology and agronomic behavior, with little research on variety within the Olea germplasm to yet. Low fruit set is frequent and varies between olive cultivars (Newton *et al*., 2014). Pollen flow (Guitian, 2006), resource availability (Terral *et al*., 2004), predation, environmental stress, or genetic stress are all possible causes (Newton *et al*., 2014). The olive tree has a low ultimate fruit set due to a high rate of undeveloped pistils later leading to its abscission (Chiappetta *et al*., 2015). The physiological differentiation processes are driven by competition for resources between growing vegetative and reproductive organs (Dixon, 2012; Erel, 2016). According to cytohistological observations of staminate and hermaphrodite flowers, after the megaspore mother cell develops, the pistil development in staminate flowers is halted (Guitian, 2006; Chiappetta *et al.*, 2015). Biochemical studies demons-

trated that starch granules were only discovered in the ovary, pistil, and stigma of hermaphrodite flowers at this time. The pistils of staminate flowers did not contain any substantial amounts of starch (Wiens *et al*., 1987; Seifi, 2015). The findings reveal a substantial connection between starch content and pistil formation (Chiappetta *et al*., 2015). The low chlorophyll content of the gynoecium, the absence of Rubisco activity in the pistils of these two flower types, and the ultrastructure of the plastids discovered by transmission electron microscopy research all point to a secondary source of starch within the flower(Reale *et al*., 2009; Erel, 2016). In olive varieties, the percentage of hermaphrodite to staminate flowers is also significant and varies (Cuevas and Polito, 2004). Fruit success appears to affect the gender of flowers in distal positions along the inflorescence, as seen by the gender pattern of flower buds in inflorescences with varying fruit placements. Other aspects of andromonoecy in *Caesalpinia* species, such as floral sex ability and fruit set impacts, are examined (Terral *et al.*, 2004; Seifi, 2015). Previous research used potency spectral analysis to look for all possible periodic patterns in yield data; and concluded that even with a fairly regular biennial succession, two or more "on" or "off" years can be observed. Staminate flowers are produced by andromonoecious species to boost reproductive success by increasing male function or redirecting resources away from useless pistils and onto fruits (Huang, 2003).

Biennial fruit-bearing in olive trees

Alternate fruiting and determining factors are critical in olive trees. Olive crop productivity has changed greatly over the years, due to the problem of alternating fruit-bearing that has been affecting the trade and consumption in the olive producing countries. Many fruit trees, such as olive trees, may not yield the same crop year after year, with mild to significant annual variations (Rallo *et al*., 1993; Seifi, 2015). In recent decades, the issue of alternating bearing in fruit trees has received a lot of attention, because of the huge swings in production, the alternating bearing is a common phenomenon in many fruit tree species, causing tremendous labor, marketing, and economic instability (Fig. 1). The concept of "alternate" or "biannual" bearing is defined as the production of heavy fruit "in" one year, followed by light or no fruit the next year (Monselise and Goldschmid, 1982). The development of trust worthy metrics to quantify biannual alternation, its severity

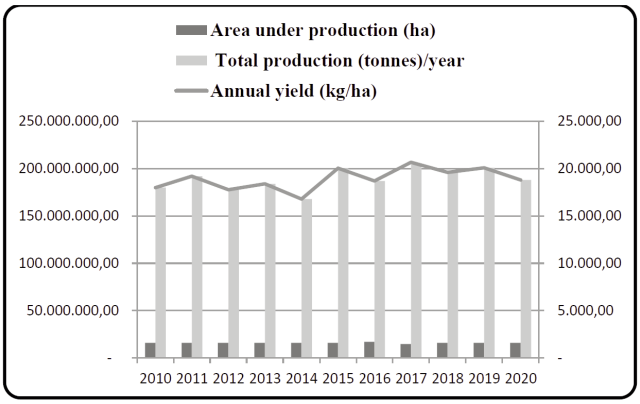

Fig. 1 - Variability of olive world production over 10 years. Adapted from FAOSTAT (FAO, 2020).

(maximum and average deviation from multi-year norms), synchrony at different sites, and other characteristics are clearly of importance. Monselise and Goldschmid (1982) presented two parameters for evaluating fruit production yearly alternation). The first parameter "B" denotes the proportion of biennial, while the second one "I" denotes the amplitude of volatility. Three aspects of olive reproductive biology were investigated by Rallon *et al*. (1993): the biannual cycle, the effect of cultivation on successive reproductive and vegetative processes, and the effect of temperature on bud dormancy are all discussed. Even though appropriate horticultural techniques such as sufficient pruning, thinning, irrigation, etc. are implemented, olive trees will gradually alternate their annual yield. Under good climate circumstances in the whole year, the alternate bearing will be very reduced at a given extent depending on cultivars (Lavee, 2007). A quantitative study relating cultivation to various indices of successive reproductive and vegetative processes revealed that while inhibition of floral induction by developing plentiful fruits is the main factor for biennial bearing, the successive reproductive processes are also important, especially fertilisation stage (Lavee and Avidan, 1993). Rosati *et al*. (2011) also investigated the effect of nutritional conditions on olive biennial fruiting. The researcher concluded that Flowers induction particularly hermaphrodite flowers formation are negatively affected by flowering load (Erel *et al*., 2016). Furthermore, biochemical studies have been conducted in an attempt to explain the alternate bearing in olive production. The protein content and their composition of "on" and "off" olive trees, especially the protein content in the leaves and bark of one-year-old shoots, have a critical effect on the olive annual produc-

tion (Lavee and Avidan, 1993; Eris *et al*., 2007), in Koronaiki, Uovo de Piccione, Manzanillo, and Barnea olive cultivars. The overall amount of useable protein in the "off" trees' leaves was substantially lower than in the "on" plants' leaves, while the bark revealed an antithetical relationship. The Koronaiki cultivar was the least alternative, presenting the smallest variance in its leaves. In contrast, other cultivars had a comparable percentage of proteins content in their bark. Additionally, Cuevas and Polito (2011) observed that the "off" trees of all cultivars had more crude protein in their bark than the "on" trees of the same cultivars. A 66 crude protein was more expressed in the bark of the "off" trees, rather than "on" years. However, Rosati *et al*. (2011), found that differences in some proteins were smaller in the leaves of "on" and "off" trees (Rosati *et al*., 2011).

Endo-genetic factors

The olive crop (*Olea europaea*) is a genetically diverse fruit in horticulture (Kour *et al*., 2018). Cytosine methylation is an essential epigenetic regulator of transposon silencing, heterochromatin organization, genomic imprinting, and gene expression, according to an investigation of endogenous factors (Zhang *et al*., 2006). Thus, all flowers in andromomonocioeus plants are claimed to be bisexually started, despite the presence of spatial patterns within inflorescences and plants (Hamanishi and Campbell, 2011). In addition to the biochemical difference, Cuevas and Polito (2004) confirmed that the dry weight of hermaphrodite flowers was 19% larger than the dry weight of staminate blooms begun at similar places on the panicle. Because there were no significant differences in stamen weight, the author hypothesized that this discrepancy was mostly attributable to pistil and petal weight. Delph (1997), on the other hand, found no significant differences between staminate and hermaphrodite flowers in pollen amount per anther or pollen quality, as evaluated by viability, germination, and ability to fertilize other flowers. Furthermore, no link between gender and anthesis timing was discovered. On the other hand, Sedgley and Griffin (2013), found that the flower's position inside the panicle was linked to anthesis timing and gender. The Blooms on the tip and major pedicels were hermaphrodite and opened first, whilst flowers on subsidiary pedicels were mostly staminate and achieved anthesis last. In summary, the majority of the findings support the notion that pistil abscission is linked to resource competition among ovaries, and that genetic differences in pistil abscission between olive cultivars can be explained by changes in pistil mass and sink strength (Zilberman *et al*., 2007; Song *et al.*, 2014). Addionnaly, flower bud induction is a long-term process in the olive tree that is controlled by a variety of internal and external stimuli. Marone and Fiorino (2010) conducted experiments to identify the meristems that give birth to various types of shoots, as well as the fundamental mechanisms influencing the evolution of the apical meristem and its lateral buds. Observations led to the discovery of a vertical succession of two types of buds in the same bud complex: the "main" bud and, in the upper position, the "accessory" bud, the former having a reproductive function and the latter specialized in environmental exploitation (vegetative role). They concluded that the generation of new bearing vegetation is confined to the central leader in "mature" shoots, and all branches arising from accessory buds are committed to burst new vegetation. The findings by Fabbri and Benelli (2000) also backed the theory of the two-step induction leading to flower bud differentiation, which appears to begin around the end of fall. On the otherhand, the impact of seasonal variations in the phenolic content of olive cultivar's leaves (*Olea europaea L*) on the cultivar's alternating bearing have been suggested to be associated to the alternatate bearing too. In 2008 (off year), Mert *et al*. (2013) reported a substantial variations in the amount and distribution of these phenolics in the leaves (on year). Chlorogenic and p-coumaric acids were abundant in the "on" year, but other phenolic compounds were scarce. The chlorogenic and p-coumaric acid levels were low during the "off" year, while the levels of the other phenolic acids were high. In the "on" and "off" years, the Same Authors discovered a negative connection between chlorogenic acid and caffeic acid concentrations: caffeic acid levels were high, while chlorogenic acid levels were low. Comparing the "on" and "off" years, they concluded that the contents of chlorogenic acid, caffeic acid, 3-h ydroxycinnamic acid, and p-coumaric acid were considerably different.

Ovary abortion and incomplete embryo development

The findings back up the theory that pistil abortion is linked to ovaries competing for resources, and they imply that genetic variations in pistil abortion between olive cultivars might be explained by changes in pistil mass and sink strength (Ji *et al*., 2010; Rosati *et al*., 2011). In andromonoecious species, pis-

til abortion is thought to be an evolutionary adaptation to save resources by balancing the quantity of pistils with the resources available. As a result, pistil abortion is likely to be higher in large-fruited varieties. Rosati *et al*. (2011) working with olive cultivars with varying ovary/fruit mass discovered that pistil abortion, represented as a percentage of staminate flowers, was positively associated with the average ovary mass at bloom. Furthermore, both ovary mass and pistil abortion were inversely linked with the number of perfect flowers per inflorescence, whereas both factors increased the number of staminate flowers per inflorescence (Famiani *et al*., 2019) (Fig. 2). Thus, the above researchers, among others, suggested that the leaf-bud ratio, as well as the amount of leaves present for each inflorescence bud, are two factors that influence pistil abortion in olives (Fig. 2). The olive flowers will develop poorly if the number of leaves decreases; and the number of aborted pistils will rise as the number of leaves decreases.

3. Ecological factors

Nutrients resources distribution and plant nutrition factors

A variety of alterations in the activation and inhibition of endogenous metabolic pathways are involved in the manifestation of alternative bearing. Pistillate abortion, known as andromonoecy, which refers to the generation of both perfect (hermaphroditic) and staminate blooms, is thought to be influenced by resource competition (Haberman, 2019). According to Rallo *et al*. (1993) and Cuevas and Polito (2011), the high variation in the proportion of staminate flowers observed in olive trees, branches, shoots, and even inflorescences within the same shoot, could be part of a general reproductive strategy that adjusts maternal investment in gender expression in response to available resources and environmental conditions. If nutrient shortages cause increased pistillate abundance and staminate flower development, Solomon (1985) and Emms (1993) suggest that the nutrition deficiency could also alter pollen output or pollen quality. If this is the case, staminate flowers would be regarded as a result of nutritional deficiency, and andromonoecy would be regarded as a process of partial flower abortion (Lavee and Avidan, 1993; Cuevas and Polito, 2011). In contrast, if staminate flowers benefit from the resources conserved by pistillate miscarriage, we should expect more pollen grains or higher pollen performance from staminate flowers than from hermaphrodite flowers (Song *et al*., 2012). Vining *et al.* (2012) and Guevas and Polito (2013) investigated the synthesis and use of carbohydrates linked with regular and alternation fruiting in the olive production cycle. Male flowers are more likely to appear on the less fed secondary pedicels, where fruit set is unlikely to happen; while hermaphrodite flowers are more likely to form on the apex and major pedicel of the inflorescences. However, some studies have found that flower position has no effect; for example, in the 'Mission cultivar', the fate of a certain floral meristem is not mixed, and gender cannot be firmly attributed to a certain inflorescence location (Delph, 1984;

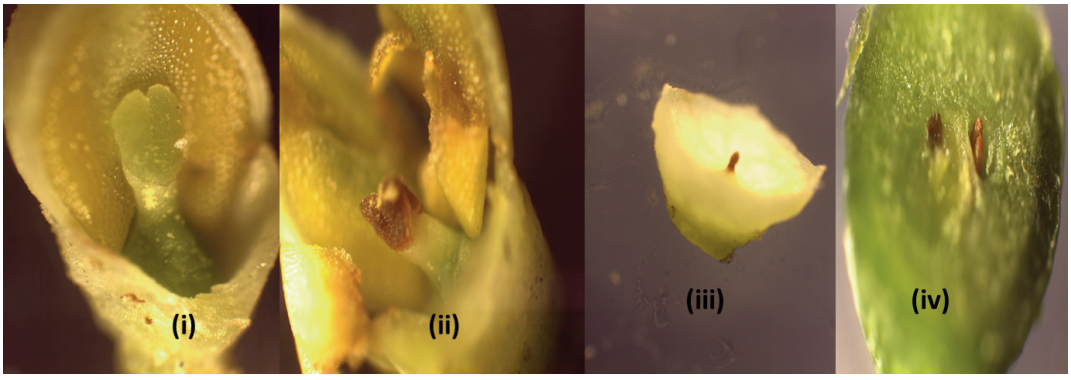

Fig. 2 - Illustration of some olive flowers development biological stage by a Light microscope: A longitudinal view of hermaphrodite flower before anthesis (i), A hermaphodite flower with a degenerating (ii), an aborted pistil (iii), a transverse view of an ovary expansion with one functional ovule and 3 others degenerating (iv) Electron micrograph of the phytoprotective film of Chi-Pyro-Film, after spraying on a smooth surface at a temperature of 18 to 25°C (a and b). Chitosan strawberry leaf covered with a film formed by Chi-Pyro-Film (c).

Haberman, 2019).

Despite the fact that staminate flowers originate in less desirable locations, the findings showed that the conditions that cause pistil abortion have no effect on their function. In fact, Nitrogen tended to improve blooming intensity but not floral quality; nevertheless, it consistently reduced fruit set. The amount of phosphorus in the soil was linked to the creation of beautiful blooms and fruit set. Potassium supplementation had a minor influence on olive yield (Erel *et al*., 2013). Pollen grains from staminate flowers, on the other hand, do not profit from the transfer of resources saved by pistil abortion, as Emms (1993) discovered. Because sexual reproduction resources are limited, it is sometimes argued, on theoretical grounds, that an increase in resources given to male function comes at the expense of resources dedicated to female function, and vice versa (Delph, 1984). Furthermore, Guevas and Polito (2003) observed that in hermaphroditic flowers, larger resource allocation to pistils occurs, while stamen dry weight does not rise in staminate flowers in response to reduced pistil allocation. The positive association discovered in hermaphroditic flowers between petal, stamen, and pistil dry weight shows that conditions that favor pistil development also favor resource investment in stamens and petals (Lavee and Avidan, 1993).Plants do not shift resources from the pistil to the stamens in staminate flowers, according to patterns of resource allocation to floral organs in other andromonoecious species (Lavee and Avidan, 1993; Emms, 1993). Reale (2009) showed that nutritional deficits can impact pollen production or pollen quality, in addition to increased pistil abortion and generation of staminate flowers. If this is the case, staminate flowers would be regarded as a result of nutritional deficiency, while andromonoecy would be regarded as a simple process of incomplete floral abscission. Even though, staminate flowers gain from the resources saved by pistillate miscarriage (Song *et al*., 2012), we should expect more pollen grains or greater pollen performance from staminate flowers compared to hermaphrodite flowers. In this situation, the ailment could be viewed as a precursor to monoeciousness (Vining *et al*., 2012). During the annual and bennial cycles, there are claimed to be dramatic changes in the carbohydrate components of leaves. Sugars and starches are substantially higher at the start of a bearing year than at the start of a non-bearing year, according to Duyvelshoff (2011), polysaccharides are extensively hydrolyzed throughout winter. Low temperatures and good flower induction seem to be linked to a high carbohydrate content (Rosati *et al*., 2006). The influence of seeds on floral induction in growing fruits has also been underlined. Seed-produced auxin has been seen moving from the seed to the fruit spur. In a biennial cultivar (Laxton's Superb), the mobility is larger than in a regular bearing cultivar ('Cox's Orange Pippin') (Zhang, 1993). Long ago, it was suggested that seeds may deprive a key metabolite essential for flower initiation (Duyvelshoff, 2011). The function of nutrition in pitillate abortion in olives revealed that high leaf/flower ratios and nitrogen fertilization increase hermaphroditic flower formation, which is consistent with feminization trends in andromonoecious plants developing under favorable climatic conditions (Solomon, 1985; Rosati *et al*., 2006). As a result, the wide range of staminate flower proportions observed in olive across years, trees, branches, shoots, and even inflorescences within the same shoot could be part of a general reproductive strategy that adjusts maternal investment in sex expression in response to available resources and environmental conditions (Rosati *et al*., 2006). The latter stated that high amounts of metabolites or photosynthates may be associated with high hormone levels, which is consistent with prior findings by Durand (1990) and Frankel and Galun (2012), who determined that hormone concentration and sex expression in plants had a close relationship. Female flowers grow near younger leaves, which have high auxin levels, according to the same authors. The favorable effect of phosphorous on female reproductive development was independent of total carbohydrate availability, according to Erel (2016) in his study on the influence of phosphorous nutrient levels on reproductive development. As a result, the researcher hypothesized that Phosphorous nutrition had a favorable influence on productivity measures that was unrelated to carbohydrate reserves or carbohydrate transit to the developing inflorescence. In addition, Fernández (2009) and Reale (2009) discovered that phosphorous nutrient levels were connected to the rate of reproductive bud burst, inflorescence weight, rate of hermaphrodite flowers, pistil weight, fruitlet persistence, fruit set, and the overall number of fruits produced. Pollen viability was consistently high in Phosphorus deficient trees, the authors reported, presumably due to higher carbohydrate availability,

in contrast to female reproductive organs.

Environmental factors

According to ecophysiology research, the degree of alternance bearing in fruit production is strongly reliant on environmental variables and can vary significantly between growing regions depending on climate (Terral *et al*., 2004; Fernández, 2015). A research done on the influence of climatic conditions on the ratio of hermaphrodite to male flowers and fruit set, in regular and alternating olive varieties, found out that different inductive circumstances are created by harsh climate conditions. In seasons with a lot of blooms, staminate flowers are plentiful and the number of flowers per inflorescence is fairly low (Erel *et al*., 2013; Erel *at al*., 2016).Even among the same cultivar at different locales, the effects of tree age have been studied as debatable and presumably not uniform (Song *et al*., 2014). The alternation phenomena can be triggered by environmental factors. Because of its self-sustaining features, cyclic activity can last for years after it is started (Emms, 1993). Different tree species, as well as the same tree species cultivated in various climates, may have different relevant conditions. The same tree species can be cultivated in a variety of environments, such as irrigated vs. arid culture, somewhat warm and humid vs. hot and dry summers, overcast vs. bright days, and so on. Conditions that do not trigger in one zone may become triggers in another or for different trees within the same zone (Hamanishi and Campbell, 2011). Monselise and Goldschmid (1982) investigated the impact of plant size and light intensity interactions on sex expression. The percentage of female and hermaphrodite flowers was highest when large plants were exposed to full sunshine. That is, when high light intensity was combined with a big plant size, the percentage of female and hermaphrodite flowers was larger than when each component was used alone (Karapatzak *et al*., 2012). Furthermore, it was discovered that both high light intensities and plant size increase female flowering. “Plants that are cultivated in full sunlight, with appropriate hydration, and embedded in an appropriate substrate are sturdy and produce female flowers in the majority of cases. Male flowers are produced by less vigorous plants that are frequently planted in the shade or lack enough nutrients (Fernández *et al*., 2009). When the olive is cultivated in a greenhouse at a minimum temperature of 16°C and a maximum temperature of 27-30°C, flower production is absolutely suppressed, although it does occur when cultivated in California during the winter. He also came to the conclusion that morphological alterations in the bud are linked to an increase in blooming, which is regulated by the treatment time (Terral *et al*., 2004).The classic example of the change in woody plants throughout a large climatic area relates to climatic factors that can trigger the biennial cycle. Spring frosts in deciduous trees, for example, or unusual drought stress during the set in warm locations, are examples of such triggers. Outside of the cycle, however, it is normal for woody plants and trees to swap places with their neighbors. Individual branches could even be out of sync with the rest of the tree (Monselise and Goldschmidt, 1982). To some extent, the effects of temperature on flowering, fruit set, and fruit development have been examined. Due to influences on pollen germination and pollen tube expansion in the fertilization process, high day and night temperatures (300°C/200°C) during the growing season might impair yield potential, resulting in flower death following anthesis (Karapatzak *et al*., 2012; Haberman, 2019).

4. Conclusions

Despite the fact that olive farming has been practiced in the Mediterranean agricultural ecosystem since ancient time, the productivity of some olive groves is hampered by the floral divergence leading to the low fruit set. On one hand, the pistil abscission can be viewed as an evolutionary response in the Mediterranean ecology that balances pistil numbers with available resources. On the otherhand, benefit of developing staminate flowers in olive trees can also be seen as adaptative strategy to increase male flowers activity and dispersal efficiency, as well as to boost pollinator attraction**.** Thus, in olive trees, the transition from hermaphrodite to staminate flowers can be regarded as a strategy to maximize the ability of male flowers by allocating biochemical plant resources to male and female tasks in the most efficient way possible. Pollination fitness and additional resources are provided for the reduced number of pistil growth, if the optimal number of staminate flowers exceeds the number of pistils. However, further research should be done on how to balance the ecological flower adapatability and the olive productivity. Thus, there is a needto adjust Fthe variability of the ratio between hermaphrodite and staminate flowers,

as well as the histological structural study that goes along with it. Hormonal treatments to reduce alternance production in olive trees should be also explored. Ovule viability and fertilisation, as well as embryo sac development during flower differentiation and fruit abscission, should all be further investigated too. Lastly, cross-effect pollination's on fruit value between olive cultivars, as well as the synchronization of flowering and fruit set, in the Mediterranean ecology could be also of great interested to improve olive productivity.

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Review paper

AHS
Advances in Horticultural Science

An overview of Betel vine (*Piper betle* L.): Nutritional, pharmacological and economical promising natural reservoir

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Abstract: With its magnificent green heart-shaped leaf, the betel vine (*Piper betle* L.) is also known as Paan in India. It is a member of the Piperaceae family. It is cultivated in the coastal regions of Odisha (Balasore, Jagatsinghpur, Puri, Khordha, and Ganjam). Paan is consumed by over 1 million people throughout the state, but they are unaware of its high nutritional quality. It is considered superior to pharmaceuticals and is one of the best remedies in nature. It has anti-microbial, anti-apoptotic, anti-cancer, antioxidant, and anti-inflammatory attributes. Furthermore, the leaves retain eugenol-rich essential oil (EO) (1-3%), which is the hotspot for medication, stimulants, antiseptics, tonics, and other ayurvedic compositions. This oil can also be used as an industrial raw material to make medications, fragrances, tonics, mouth fresheners, food additives, and other products. It contains anticarcinogens, which show potential for the development of medicines against cancer treatment. Betel plant farming is an agricultural activity that provides a source of income for remote farmers. Sometimes economic crises occurred due to the development of diseases such as foot rot, leaf spot, powdery mildew, and collar rot. Most farmers got seasonal revenue, whereas betel vine cultivation provided year-round income from a tiny plot of land.

1. Introduction

The betel vine (*Piper betle* L.) belongs to the Piperaceae family, which also contains pepper and kava. Paan leaves are produced in the Philippines, Malaysia, India, Sri Lanka, Taiwan, Thailand, and other Southeast Asian countries as a post-meal mouth freshener. It is primarily consumed in South Asia and by certain Asian emigrants worldwide as betel quid or paan, in combination with areca nut or tobacco (Saraswat *et*

al., 2020; Shah *et al*., 2021). A sheaf of betel leaves is typically presented in Odisha as a token of respect and auspicious beginnings in traditional culture. It belongs to the genus *Piper* of the Division Magnoliphyta, Class Magnolipsida, Order Piperales, and Family Piperaceae. It is a unisexual perennial evergreen climber with shiny cardio leaves and white catkins that bloom in the spring. Betel vine is categorized into odorous and non-pungent kinds depending on the form, length, and flavour of the leaf. The plant's leaves are basic and have an acuminated crown. Mostly, the leaves are smooth and shining. The leaves differ in color from light green to dark green. The leaves are long-stalked with 2-3 pairs of secondary veins (Swapna *et al.,* 2012). The betel plant's limbs usually bulge at the nodes and are completely smooth. Female spikes are cylindrical, whereas male spikes are pendulous. Female spikes measure 2.5-5.0 cm in length. In the humid environment of East India, female plants often generate blooms or fruit (Sengupta and Banik, 2013; Rahman *et al.,* 2020).

Throughout thousands of years, nature has provided a reservoir of medical substances, and current medications are derived from ecological resources. The betel leaves contain a variety of bioactive compounds and are employed in ancient medical methods. Such leaves are high in minerals, vitamins, enzymes, proteins, and essential oil (EO), and they are very nutritious (Nayaka *et al.,* 2021; Paswan *et al.*, 2021). They also include certain useful therapeutic components for the therapy of disorders of the brain, liver, and cardiac (Pradhan *et al*., 2013; Ullah *et al.*, 2020). Polyphenols, alkaloids, steroids, saponins, and tannins were also found. In the Indian subcontinent, medicinal plants are a resource of economical worth (Sen and Chakraborty, 2017; Madhumita *et al.,* 2020). Medicinal plants are the primary source of medicine for the bulk of the rural community in emerging nations, and consequently play a key role in their health systems (Patra *et al.,* 2014; WHO, 2019). Upto 80% of people in developing countries still use local medicinal herbs for their basic health care needs (Sen and Chakraborty, 2017; WHO, 2019). Furthermore, diastase and catalase activities are detected in the leaflets (Abrahim*et al.*, 2012; Shah *et al.,* 2021). It assists in curing and treating many conditions, including halitosis, boiling and absceeding, conjunctivitis, headache, constipation, hysteria, itching, mastitis, leucorrhoea, otorrhoea, mastoiditis, gum swelling, ringworm, rheumatism, abrasions, injuries, cuts, etc (Shukla *et al.,* 2018). Because of their antibacterial and antioxidant properties, these oils have a promising future in the novel food packaging industry (EI Asbahani *et al.,* 2015; Guha and Nandi*,* 2019; Nguyen *et al.,* 2021), as well as being a prospective and appealing flavouring component for the food and beverage sectors.

This plant is grown as a cash crop in the Balasore, Jagatsinghpur, Puri, Khordha, and Ganjam areas of coastal Odisha (Jena, 2021). In Assamese/Urdu/Hindi/Odia/Bengali, the betel leaf is recognised as Paan, whereas in Sanskrit it is considered as Taambuul and Nagavalli. The finest betel leaf is the "Magadhi" type cultivated near Patna in Bihar, India. The popular type of betel leaf in Kerala is called "Venmony Vettila" and comes from Venmony near Chengannur (Guha and Nandi*,* 2019). In Odisha, four distinct forms of betel leaf are grown. The Bhograi block in the Balasore district is known throughout the country for its betel vine farming (Patra and Pradhan, 2018). Cultivars with the prefix Desi in their names, on the other hand, always relate to the cultivars Desavari in Madhya Pradesh, Kapoori in Maharashtra, Bangla in West Bengal (Guha and Nandi*,* 2019), Bali and Chandrakana in Bhogarai (Fig. 1 A1-A2, B1-B2). The varieties are Nova Cuttak, GodiBangala (Fig. 1 C1-C2), Sanchi (Fig. 1 D1-D2), and Birkoli. Only one type, GodiBangala, is grown by the locals in the research region Bhainchigodi (Patra and Pradhan, 2018). Odisha is one of the states that produce the most betel vine. In the context of such scientific research, this review article tries to summarise all the possible information on betel leaf with its propagation, socio-economics, and bioactive compounds, justifying wider possibilities for its use as a natural source for people in Coastal Odisha.

2. Habitat and Ecology

Betel vine is primarily grown in Odisha's coastal districts such as Balasore, Jagatsinghpur, Puri, Khordha, and Ganjam (Jena, 2021). All the respective district locations are as follows. 20.27°N and 86.17°E, 19.48°N and 85.48°E, 20.18°N and 85.62°E, 19.38°N and 85.05°E (Fig. 2). North-Western highlands, the inner alluvial plain, and the coastal belt are the three geographical regions that can be found in all five districts. Those regions are flooded with brackish water from estuarine rivers, making them unsuited for agriculture in a regular manner. Those lands are current-

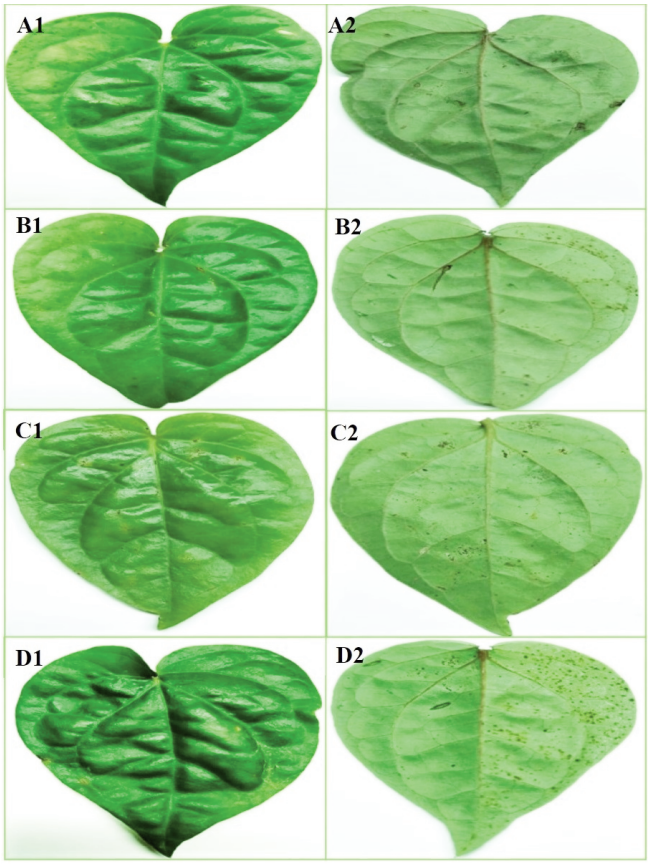

Fig. 1 - Major varieties of *Piper betle* (A1) ventral and (A2) dorsal side of 'Bali', (B1) ventral and (B2) dorsal side of 'Chandrakana', (C1) ventral and (C2) dorsal side of 'Godi Bangala', (D1) ventral and (D2) dorsal side of 'Sanchi' cultivated in coastal region of the Odisha.

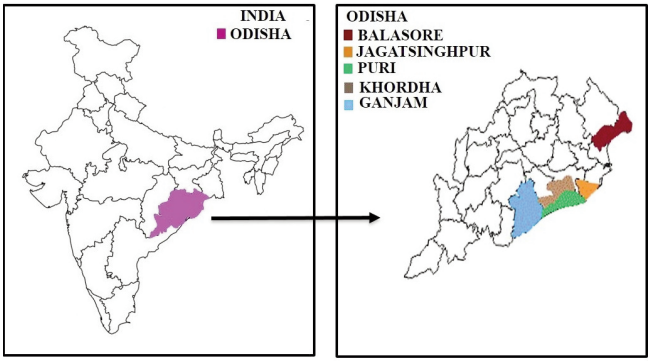

Fig. 2 - Map showing the study sites of coastal Odisha (Balasore, Jagatsinghpur, Puri, Khordha and Ganjam district).

ly used for betel and coconut agriculture (Ahuja and Ahuja, 2011). Recently, prawn culture and salt production units have sprouted in this region. Paddy, fish, and betel vine farming are important parts of the local economy in these districts. The soil in the central zone is mostly sandy loam and clay loam, making it perfect for producing rice and betel vines (Kaleeswari and Sridhar 2013). Coastal Odisha has an oceanic climate and experiences rainfall from the south-west monsoon (Gouda *et al.,* 2017). The coastline area has a pleasant temperature. The summers in such districts are hot, having excessive humidity during the monsoon season, a dry winter, and little diurnal temperature fluctuation over the year (Krishnan *et al.,* 2020). The district's average annual precipitation is 1591 mm, which is over 9% greater than Odisha's average. Tropical cyclones, which bring a lot of rain to the area, are responsible. Due to the district's proximity to the Bay of Bengal, cyclones are common. Cyclones, which begin in the Bay of Bengal over the Andaman and Nicobar Islands and proceed towards India's east coast, are a common occurrence in the district (Sahoo and Bhaskaran, 2016). The South-West monsoon brings more than 70.9% of the annual precipitation from June to September. The three distinct seasons observed in the area are summer (March-June), rainy (July-October), and winter (November-February) (IMD, 2020).

3. Cultivation and propagation in coastal Odisha

This plant's cultivation needs consistent soil wetness, adequate humidity, and moderate heat, which are not usually present in the ecological environment (Filipovic, 2020). Variations in these environmental variables were found to be harmful to the plant. As a result, it is grown in an artificially manufactured hut-like structure that keeps the plant's growth parameters within a reasonable range, simulating natural ecological conditions (Raza *et al.,* 2019). The building is known as a 'Baraja,' and is made up of various plant materials like (*Bambusa vulgaris* Sch. ex Wendl.) and Bamboo stems (*Bambusa bambos* L. Voss.), jute (*Corchorus capsularis* L.), paddy straw (*Oryza sativa* L.) (Fig. 3 A-B), Khadi or Chaee stick, and coconut leaves (*Cocos nucifera* L.) make up the Baraja architecture (*Arunda donox* L.) (Haider *et al.,* 2013). The structure's proportions are limited to 2 to 3m in height, 10 to 20 m in length, and 5 to 15 m in breadth. The plant is grown through vegetative proliferation from cuttings of 3 to 5-year-old vines. Plantlets with one or two nodes and connected leaves are commonly used as propagating specimens. The planting season varies depending on the area to area. It takes roughly a month for roots to develop and grow after planting. Plants are supported when

they have 5-7 leaves. After one year of plating, harvesting begins by plucking the leaves. The harvests are known locally as Maghei paan, Jhanji paan, Vejua paan, Nua paan, and Jagannath paan. Nua paan is harvested during the months of February and April. Cultivators remove dry or damaged leaves during this plucking period. Jhanji paan is harvested between May and mid-July. This is the season with the highest yield. Cultivators are removing some broken Khadi or Chaee sticks this season. During the months of August and September, Jagannath paan is collected. Farmers strategically gather the leaves from the lowest section of the branches during this plucking. During the months of October and November, Vejua paan is collected. Cultivators pick the tops of branches in this plucking technique. Maghei paan harvesting begins in December and continues until February (Patra and Pradhan, 2018). All total leaves are plucked in this collection. Farmers sprinkle water on leaves and use cotton sheets or paddy straw to keep them fresh. A "Pono" is made up of eighty paan leaves. After that, the leaves are distributed in local markets or transferred to other towns (Haider *et al.,* 2013).

Fig. 3 - Cultivation and propagation of *Piper betle* inside the Baraja at Bhograi area of Balasore district, Odisha (A-B).

4. Nutritional composition of betel leaf

Ayurveda, or traditional Indian medicine, has long been a part of Indian culture and 'Herbal Materia Medica. The ancient Sanskrit books 'Charaka and Sushruta Samhita' (600-400 B.C.) document betel leaf chewing, establishing its identity as an old use. "Charaka and Sushruta Samhita," "Astanga Hridayam," "Bhabaprakasha," "Harivamsa," "Varahapurana," and "Panchatantra and Jataka Stories" are among the Sanskrit writings that allude to it as "tamabool" (Kumar, 1999). In Asian countries, this plant is a frequent element in the creation of various indigenous medicines. In comparison to related Indian traditional plants such as "tulsi" (*Ocimum sanctum*), for example, this plant has received very little research aside from strong ethnomedicinal promises (Cohen, 2014; Kurepa and Smalle, 2021). Nayaka *et al.* (2021) revealed that the betel leaves contain many ingredients such as water (80-90%), EO (0.05-0.2%), fat (0.5-1.0%), protein (3-4.5%), riboflavin (4.5-15.5 µg/100 g), fibre (2-2.5%), minerals (2.5-3.5%), chlorophyll (0.05-0.25%), tannin (0.1-1.3%), vitamin C (0.005-0.01%), carbohydrate (0.5-6.5%), vitamin A (2-3 mg/100 g), nicotinic acid (0.65-0.9 mg/100 g), potassium (1.5-4.5%), thiamine (10-80 µg/100 g), iron (0.005-0.01%), phosphorus (0.05-0.06%), nitrogen (2.0-7.0%), calcium (0.2-0.5%), iodine (3-3.5 µg/100 g) respectively (Madhumita *et al.,* 2019). Fresh betel leaves contain minerals 2.3-3.3%, protein 3-3.5%, fat 0.4-1.0%, moisture 85-90%, carbohydrate 0.5-6.10%, fibre 2.3%, calcium 0.2-0.5%, phosphorus 0.05-0.6%, iron 0.0050-007%, vitamin C 0.005-0.01%, and energy 44Kcal per 100 g (Mazumder *et al.,* 2016; Vernekar and Vijayalaxmi, 2019). After dehydration, the betel leaf samples became a concentrated source of nutrients, according to the results of the nutritional analysis. The findings support those of (Subhash and Neeha, 2014), who found that after sun drying and cabinet drying, the leaves preserved high levels of protein, fibre, and calcium. The moisture material of dehydrated betel leaves flour was 9.45%, fat 1.10%, protein 3.30%, ash 6.87%, carbohydrate 63.92%, fiber 10.15%, vitamin C, calcium, and iron were 1.11%, 2.57%, and 1.53% (Chauhan and Aishwarya, 2016; Akshata *et al.,* 2018).

5. Phyto-chemicals found in betel leaf

The phytochemicals screening was analyzed on the ethyl alcohol extract of betel vine using standard protocol for identification of the constituents. The leaf extracts have various pharmacological activities which are prepared by using different solvents such as aqueous, ethanol, powder, and hot water (Kumari and Rao, 2015) (Table 1 and Table 2). By this analysis, it was concluded that it consists of Tannins, Anthraquinones, Flavanoids, Alkaloids, Terpenoids,

Table 1 - Components of *Piper betle*

Sl No.	Components of *Piper betle*	Percentage of components (%)	References
1	1.8-cineol	0.04	Rajamani *et al*., 2016
2	a-pinene	0.21	Pradhan *et al.*, 2013
3	Allypyrocatechol diacetate	0.71-6.2	Mazumder *et al*., 2016; Sakinah *et al*., 2020
4	Allypyrocatechol monocetate	0.23	Junairiah *et al*., 2018
5	Campene	0.48	Junairiaha *et al*., 2020
6	Caryophyllene	3.71	Pradhan *et al*., 2013; Junairiaha *et al*., 20
7	Chavicol	0.4	Pradhan *et al*., 2013; Sakinah *et al*., 2020
8	Chavibetol	53.1-80.5	Junairiaha *et al*., 2020; Azahar *et al*., 2020
9	Chavibetol acetate	11.7-15.5	Pradhan *et al*., 2013
10	Chavibetol methyl ether	0.48	Mazumder *et al*., 2016; Junairiaha *et al*., 2018
11	(E)-caryophyllene	0.4	Pradhan *et al*., 2013; Sakinah *et al*., 2020
12	Eugenol	0.32-0.4	Junairiaha *et al*., 2020
13	f-pinene	0.21	Pradhan *et al*., 2013; Junairiah *et al*., 2018
14	Methyl eugenol	0.4	Rajamani *et al*. 2016; Junairiaha *et al*., 2020
15	Methyl salicylate	0.05	Pradhan *et al*., 2013
16	Saprobe	0.11	Shameem *et al*., 2013
17	U-limonene	0.14	Akther *et al*., 2014

Saponins, Caediac glycosides, Glycosides, Reducing sugars (Rajamani *et al.,* 2016). The compounds reported in betel plants include Bisabolene, Cavacrol, Isoeugenyl acetate, Eugenol methyl ether, 2-Mono palmitin, Allypyrocatechol, Eugenol, Piperitol, Chavibetol, Chavibetol acetate, Myrcene, Quercetin, Hydro-xychavicol, Stearic acid, Allyl catecol, Germacrene-A, α-terpineol, Luteolin, Caffeic acid, Limonene, Piperlonguminine,α-cadinol, β-sitosterol, 4-allyl phenyl acetate, D- limonene, m-Cymen-8-ol, 2-noanone, Ocimene, N-decanal, 2-undecanone, Allo ocimene, Sabinene, 3-allyl-6-methoxyphenol, Humlene, Cymene, Terpinolene, β-pinene, α-Myrcene, Allyldiacetoxy benzene, Vanillin, Thymol, Cispiperitol, Tarpinolene, Propcatechuic acid, Eucalyptol, Gallic acid, Iso eugenyl acetate, (E)-ß Damascenone, Linalool, Camphene, 4 cineole, α-pinene, Germacrene-D, Piperol-B, Piperol-A, Estragol, Arecoline, Isoeugenol, Isoascaridole, Chavicol, Ellagic acid, Safrole, Eugenyl acetate, 4-allyl phenol, 5- Indanol, 4-allyl resorcinol, α-bergamotene, (E)-ß-ocimene, Ferulic acid, Carryophyllene, Anethole, α-farnesene, Cepharadione-A, Piperine,4E-decadienamide, 4-Allyl anisole, Cuparene, ß-iso safrole, αmuurolene, Cadinene, α-copaene, α-cubebene, Benzene acetic acid, α-selinene, Methylpiperbetol (Pradhan *et al.,* 2013; Mazumder *et al.,* 2016; Junairiah *et al.,* 2018; Junairiaha *et al.,* 2020; Sakinah and Misfadhila, 2020; Azahar *et al.,* 2020) (Fig. 4, Table 1).

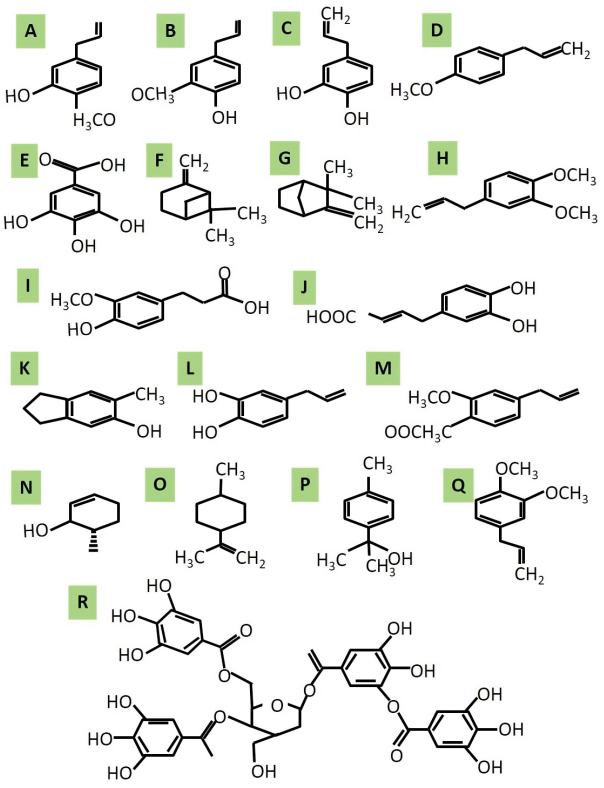

Fig. 4 - Structure of major bioactive constituents of *Piper betle* a) Chavibetol, b) Eugenol, c) Hydroxychavicol, d) Estragol, e) Gallic acid, f) β-pinene, g) Camphene, h) Allyl diacetoxy benzene, i) Ferulic acid, j) Caffeic acid, k) 5-Indanol, l) Allopyrocatechol, m) Cahvibetol acetate, n) Piperitol, o) D-limonene, p) α-terpineol, q) Eugenol methyl ether, r) Quercetin (Flavonoids).

6. Pharmacological activities

Paan is healthy and significant because of its medical, religious and ceremonial history (Rai *et al.,* 2011). Asthma is also prevented, vocalisation is improved, and gums are strengthened. Indigestion, constipation, congestion, cough, and asthma are treated (Peddapalli *et al.,* 2020). The anti-alzheimer bioactive compounds were found in various varieties of betel vine (De *et al.,* 2021). The following biochemical roles of phytochemicals from leaves are depicted in figure 5.

Possible source of wound healing agents

Betel vine component at 0.025 ml/l concentrations boosted fibroblast reproduction and encouraged umbilical cord-mesenchymal stem cells (UC-MSCs) proliferation at 0.03 ml/l, increasing *in vitro* model wound healing in accordance with empirical evidence. The homogenate reduces the presence in cells of oxidative stress factors such as VCAM, CD248, and IL-33, so that recovery of umbilical cord cells can be accelerated (Thi *et al.,* 2021). Researchers discovered that natural lysates of leaves containing phenolic compounds can reduce the activity of inflammatory factors and oxidative stress, which could be useful in regenerative medicine.

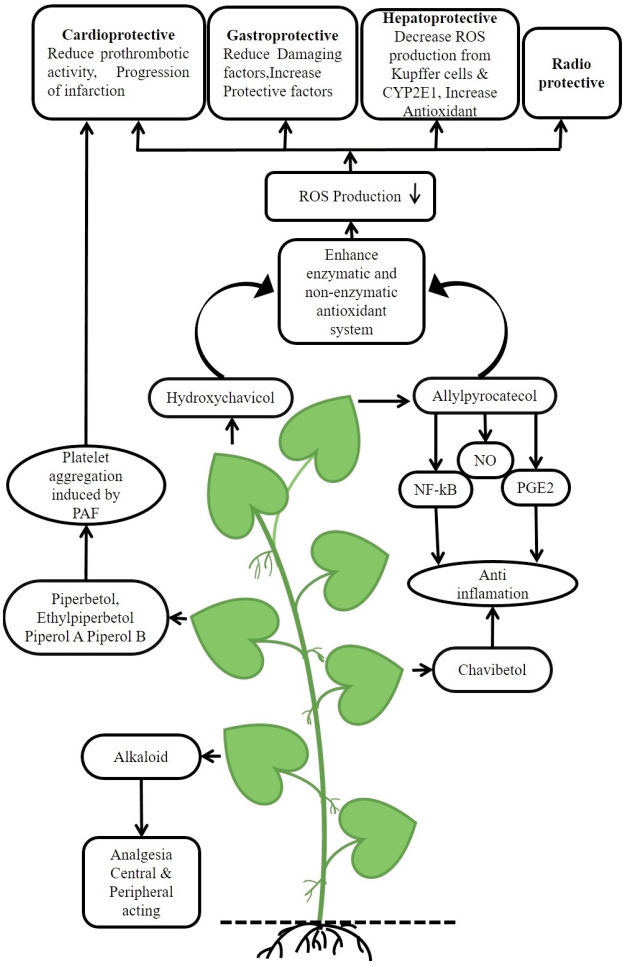

Fig. 5 - Various biochemical activities of phytochemicals extracted from *Piper betle*.

Insecticidal activity

The crucial oil extracted from the sheets of betel vine was examined on the corn weevil (*Callosobruchus maculatus* F.) and the beetle (*Sitophilus zeamais M.*) and has been suggested as a potential crop protective agent (Nair and Kavrekar, 2017). Piperaceae EO has been extensively researched for its larvicidal role in mosquito larvae control (Huong *et al.,* 2019; Alves *et al.,* 2021). *Piper permucronatum, Piper arboreum, Piper gaudichaudianum, Piper marginatum, Piper longum, Piper humaytanum, and Piper aduncum* EO have the ability to regulate *Aedes aegypti*, *Anopheles gambiae*, and *Culex quinquefasciatus* (Santana *et al.*, 2015; Takeara *et al.,* 2017; Silva *et al.,* 2019; Durofil *et al.,* 2021). *Piper aduncum* was found to be larvicidal, killing *Aedes aegypti* mosquito larvae at concentrations of 500 and 1000 ppm (Oliveira *et al.*, 2013; Martianasari and Hamid, 2019). Within a few days of being exposed to similar amounts of *Piper marginatum* EO, 100% of *Aedes aegypti* larvae died (Santana *et al.*, 2015; Marques and Kaplan, 2015). The EO of betel vine has regulated the population as well as various stages of mosquito (*Aedes aegypti*) and acts as an alternative bioinsecticide (Martianasari and Hamid, 2019).

Antimicrobial activity

The antibacterial properties of the EO obtained from the leaves are impressive (Prasetya *et al.,* 2021). They prevent bacteria from adhering to initial tooth plaque (Punuri *et al.,* 2012). The antifungal activities of magahi variant betel leaf EO were investigated by (Madhumita *et al.,* 2019; Madhumita *et al.,* 2020). Towards *Aspergillus flavus*, the minimum level of EO inhibition (MIC) from leaves was found to be 0.8 ml/l Chavicol, allylpyrocatechol diacetate, chavibetol acetate, propenylphenols, hydroxychavicol, and chavibetol are some of the aromatic compounds discovered in the chloroform extracts (Aliahmat *et al.,* 2012). Antibacterial properties against *Proteus vulgaris, Escherichia coli*, *Staphylococcus aureus,* and *Streptococcus pyogenes*

were investigated (Chakraborthy and Shah, 2011; Patra *et al.*, 2014; Nayaka *et al.,* 2021) (Table 2). The drug was used as a solution against chosen pathogens such as *Streptococcus pyogenes*. Additionally, antifreeze action was evaluated on pathogenic microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Lubis and Marlisa, 2020; Nayaka *et al.,* 2021; Nguyen *et al.,* 2021) for several species of dried betel leaves (Desawari, Desi, Bangladesh, and Jaleswar). Cold aqueous, methanol (80%), ethanol (70%), and ethyl acetate (80%) solvent removal techniques were achieved for the dried leaf extract. It has been shown that the variety of Bangladeshi and Jaleswar betel leaf extracts are a potent and efficient source of antibacterial herbal medicines (Agarwal *et al.,* 2012; Valle *et al.,* 2021). The ethanol compound from betel leaves appeared to be quite active at preventing the proliferation of harmful pathogens such as *Staphylococcus aureus, Vibrio cholerae*, and *E. coli* Hoque *et al.,* 2011; Valle *et al.,* 2021). The combination of betel and red betel exhibited reduced action against *Staphylococcus aureus*, *Escherichia coli, Stephylococcus epidermidis* (Hartini *et al.,* 2018). The model influence of betel leaf EO of 'Meetha' was explored by (Basak, 2018) on the *Aspergillus flavus* germination period and the population spore of *Penicillium*.

Antigiardial assay

Giardiasis is regarded by humans worldwide as the most frequent protozoan diarrheal illness. Researchers have sought out novel, herbal medicines that might replace commercial pharmaceuticals with unpleasant potential side effects in the treatment of giardia (Nazer *et al.*, 2019). The betel vine extracts

Table 2 - Different therapeutic activities of *Piper betle* extract

Sl No.	*Piper betle* extract	Function	Result	References
1	Aqueous extract	Antimicrobial activity in different microorganism by disc diffusion method. Antihemolytic and Antioxidative activity in *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Escherichia coli*).	The bacteria were effectively inhibited by aqueous extracts. The high content and combined action of flavonoids and polyphenols were linked to the antioxidative and antihemolytic properties.	Rai *et al*., 2011; Shameem *et al.,* 2013
2	Spray dried powder extract	Antidiabetic activity in diabetes mellitus patients.	Piper betle, as a nutraceutical, has been identified as a possible therapy for type 2 diabetic patients.	Chakraborty and Shah, 2011
3	Aqueous and ethanol extract	Antibacterial Activity in gram positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus* and also gram negative bacteria like *Pseudomonas aeruginosa* and *Escherichia coli* by Agar diffusion method.	According to the findings, both aqueous and alcoholic extracts are potent against bacteria types that are major causes of illnesses.	Arawwawala *et al.,* 2011
4	The hot water extract	Gastroprotective activity.	Due to its antioxidant and mucin-protecting characteristics, the study found that it can protect against indomethacin-induced stomach ulcers.	Kaveti *et al.,* 2011
5	The methanolic extract	Anti-inflammatory and analgesic efficacy in Swiss albino mice and Wistar rats in a carrageenan-induced hind paw edoema model, as well as hot plate, writhing, and formalin tests.	The dosage considerably increased the pain threshold in the hot plate technique, significantly decreased the writhing generated by acetic acid, and dramatically inhibited carrageenan-induced paw edoema.	Pradhan *et al.,* 2013
6	The *Piper betle* plant extract	Antifertility activity in female rats.	According to the findings, betel extract had antifertility and antiestrogen effects in female rats.	Akther *et al.,* 2014

containing methanol, tetrahydrofuran, and water have the potential of anti-diarrheal activities (Peckova *et al.,* 2017). It has been examined for its physiological and pharmaceutical properties, but its significance for *Giardia intestinalis* has yet to be proven. Various solvents for extract preparations have been utilized in investigations of these effects. They discovered a significant reduction in cyst shedding in the group of gerbils administered with the aqueous solution that matched the most relevant settings. The anti-adhesive effects on vasodilatory activities (Runnie *et al.,* 2004), antioxidant effects, early settlers (Aara *et al.,* 2020), and antihepatotoxicity have already been demonstrated with aqueous excerpts of *Piper betel*.

Antidiabetic activity

More troublingly, the increase in the incidence of diabetes has an impact not only on advanced countries but also on emerging nations that have less money to deal with another serious illness burden again. Present diabetes therapies can have side effects, so the move now to herbal remedies is more effective, cheaper, less risky, and less side-effective. The present information reveals that the juice of the betel vine has assurance for antidiabetes. Betel vine leaves have been examined in induced diabetes rats (CEE), normoglycaemic, and strepozotocin (STZ) with anti-diabetic action assessed by oral hot water and ethanol extract (HWE) (Khatun *et al.,* 2016; Arawwawala *et al.,* 2011). Blood glucose levels were dose-dependent in both HWE and CEE in normoglycaemic rats. During the glucose tolerance process, both extracts considerably lower the external glucose load. HWE has an antidiabetic action comparable to CEE. Both extracts have not been harmful and tolerated since prolonged oral administration (No open evidence of renotoxicity, and hepatotoxicity). Moreover, the weight of the spleen was increased in treated groups, indicating lymph regenerative effects (Khatun *et al.,* 2016). Betel leaf extraction is a useful anti-diabetic feature that enables blood sugar levels to be regulated. This investigation discovered betel vine extracts lowering the blood glucose level by the activation of insulin/biomimetic action and have a possible therapy for type-2 diabetic patients (Arawwawala *et al.,* 2011) (Table 2). Increased transaminase activity (SGPT and SGOT) has been found in several investigations in liver and serum diabetic rats (Ramachandran *et al.,* 2012).

Gastroprotective activity

Extraction of betel vine was also found to be mediated gastro defensive activity, resulting in (i) an increase in the production of mucus and/or bicarbonate, (ii) a decrease in the amount of stomach acid secreted, or (iii) a decrease in gastric acidity (Arawwawala *et al*., 2014; Ahmed *et al*., 2021).The leaf extracts have marked gastroprotective properties which are shown by the strong ($P \leq 0.05$) inhibition of gastric lesions caused by absolute ethanol (in terms of duration and number) (Arawwawala *et al.,* 2014). CEE gastric activity was comparable with HAE activity. The existence of alkaloids, flavonoids, steroids, saponins, and tannins has been seen by a phytochemical sampling of betel vine (Syahidah *et al.,* 2017; Altemimi *et al.,* 2017). Polyphenols, especially tannins, are antioxidant phytochemicals and can protect against indomethacin-induced stomach ulcers (Neyres et *al.,* 2012; Zakaria *et al,* 2015; Sharifi-Rad *et al.,* 2018; Ahmed *et al.,* 2021) (Table 2). The antiulcerogenic properties of these compounds were also shown by their protein-prone and blood pressure effects. Powerful gastroprotective behaviors were demonstrated (Berenguer *et al.,* 2006). Thus, the gastroprotective effect can also be influenced by secondary metabolites, such as alkaloids, saponins, tannin, flavonoids, and other phenolic compounds (Barbosa *et al.,* 2019).

Anti-asthmatic effect

Betel vine's antioxidant, anti-inflammatory, and antihistamine activity have been linked to a wide range of diseases (Alam *et al.,* 2013; Aara *et al.,* 2020; Ahmed *et al*., 2021; Clemen-Pascual *et al.,* 2022). The anti-asthmatic activity of betel vine in guinea pigs has been tested (Misra *et al.,* 2014). Asthma is the tracheobronchial smooth muscle's hyper reactance to a multitude of stimuli (Chapman and Irvin, 2015). Bronchitis is a chronic inflammatory disease. Bronchial asthma may be caused by free radicals and superoxide (Phaniendra *et al.,* 2015; Boukhenouna *et al.,* 2018). Histamine has the potential to produce bronchoconstriction (Yamauchi and Ogasawara, 2019). The extract can substantially minimize the impact of bronchial asthma, but it has fewer effects than di-phenylhydramine. But other mediators such as leukotriene play a vital part in asthma in humans. Betel vine has been reported to have the ability to reduce bronchial asthma in guinea pigs, despite its weak influence on human asthma (Darvhekar *et al.,* 2011; Misra *et al.,* 2014; Rekha *et al.,* 2014; Ahmad *et*

al., 2021).

Role of betel leaf extract in thyroid disease

Ethylacetate *Piper betle* L. (EPBL) extract administration reverted the T4-induced rise in serum thyroid hormones, heli marker enzymes, MDA, and LOOH, but improved antioxidant enzyme activity and decreased the content of glutathione. Lighter results from liver histology show that the EPBL administration has enhanced twisted hepatic tissue architecture in hyperthyroid species. Analysis of the mass-spectroscopy of high-resolution liquid chromatography showed the presence of four primary glycosides, including quercetine, rutin, kaempferol, and luteoline. The antithyroidism of EPBL was seen to be caused by hyperthyroidism induced by T4. EPBL's antithyroid and antioxidant properties may be attributable to the existence of extracted flavonoid glycosides in hyperthyroid animals which may have blocked the secretion of the thyroid hormone and translation of T4 into T3 by 5'DI inhibitors (Panda *et al.,* 2018).

Anticancer activity

Anti-cancer agents with antioxidant activities may provide their beneficial effects by balancing ROS, such that cancer cells do not proliferate when apoptosis is not allowed to occur (Abrahim *et al.,* 2012). Chronic inflammation is the root cause of many human diseases, including cancer and tumors, according to experimental and clinical research (Kangralkar and Kulkarni, 2013). The betel leaf was used for irritation in the mouth cavity as a typical folk medication. Mouth cancer is among the ten most common cancers, with 90% of cases occurring in Southeast Asia, where cigarette and smoking behaviors are common (Jiang *et al.,* 2019). One of the earliest studies (Toprani and Patel, 2013) discovered that topical treatment with leaf extracts inhibited -pinene-induced oral cancer in hamsters. It was also discovered that combining leaf extracts and turmeric into the dietary supplements was beneficial. *Curcuma manga*, *Dendrophthoe pentandra*, *Piper betle* L., and *Catharanthus roseus* extracts in breast cancer cell lines were explored as anti-cancer and radical free scavenging power (Widowati *et al.,* 2013; Rekha *et al.,* 2014). Betel vine has reported cancer preventative effects (Kudva *et al.,* 2018; Shukla *et al.,* 2018; Malkani *et al.,* 2021; Chowdhury and Markus, 2022). Supplementing leaf extract with potable water significantly reduced the concentration of benzo (a) pyrene-induced neoplasia of the forestomach. The leaf extracts contain anti-proliferative and preventative chemical potential and can thus be utilized to treat several conditions, including human lung cancer (Banerjee and Shah, 2014).

Cardioprotective action

Acute myocardial infarction is caused by an imbalance between raised oxygen demand and decreased blood supply caused by prolonged ischemia (Smilowitz *et al.,* 2015). During ischemia, the heart is further damaged by the formation of ROS. As a result, increasing antioxidant levels may help to prevent future infarction (Munzel *et al.,* 2017; Zhou *et al.,* 2018). Pre-treatment with betel vine enhanced hemodynamic and ventricular function parameters in rats with isoproterenol (ISP)-induced myocardial infarction. It restored SOD, CAT, GSH, and GPx levels, decreased lipid peroxidation of the heart, and therefore lowered CK-MB isoenzyme and LDH leakage into the blood (Arya *et al.,* 2010). Platelet hyperactivity, which leads to intravascular thrombosis, is a key component in the development of cardiovascular disorders (Kaur *et al.,* 2018; Alkarithi *et al.,* 2021).

Anti-malaria activity

As compared to the well-known insect deterrent citronella oil, EO provided greater resistance against mosquito bites from *Anopheles stephensi* and *Culex fatigans* (Johirul *et al.,* 2016). The oil provided greater than 4h of resistance from *Anopheles stephensi* and *Culex fatigans* when sprinkled at a rate of 20l/cm^2, whereas citronella oil provided only 2.2 and 2.6h of protection, correspondingly. As a result, the power of paan to resist mosquitos has been established (Pal and Chandrashekar, 2010; Ibrahim *et al.,* 2017; Cang *et al.,* 2020).

Antioxidant activity

Many researchers have investigated the antioxidant activity of extracts using a variety of solvents and extraction times (Alam *et al.,* 2012; Aliahmat *et al.,* 2012). The properties of the leaf extracts from various solvents were determined using high-performance liquid chromatography to calculate the oil-water partition coefficient (HPLC). Due to their high oil-water partition coefficient, leaf phenolics have been shown to be less polar than other phenolic antioxidants. The investigation showed that the solvent extraction and the period for preparing betel leaf extract (petroleum ether, methanol, water,

and ethyl acetate) for use as a natural antioxidant are crucial acts against four different harmful bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Proteus vulgaris,* have been investigated by Chakraborty and Shah (2011), Lubis and Marlisa (2020) Nayaka *et al.* (2021), and Nguyen *et al.* (2021). Others were isolated from these extracts, with few recognized and unknown metabolites. Various analytical methods such as NMR, Mass Spectroscopy, and IR Spectroscopy have been used for structural investigation. TBARS and DPPH methods conducted anti-oxidative investigations. Abdullah *et al.* (2015) found antioxidant betel vine extract activity and its components. 1-1-diphenyl-2-picrylhydrazyl (DPPH) demonstrated that the Bangla variety of betel vine is the best antioxidant that can be combined with total phenol contents and lower the strength of these respective extracts in a test for ethanol extracts of three varieties (Bangla, Sweet, and Mysore) of *Piper betle* L. (Swapnil *et al.,* 2014; Sarma *et al.,* 2018). Bangla extract column chromatography resulted in chavibetol (CHV), allylpyrocatechol (APC) separation, and corresponding glucosides. Similar chemical characteristics of three *Piper betles* were identified after HPLC analysis (Abdullah *et al.,* 2015).

7. Marketing and socio-economic status

Around 15 to 20 million Indians regularly eat betel leaves, and there are an estimated 2 billion betel leaf consumers worldwide, proving the crop's enormous economic potential (Jeng *et al.,* 2002). As for national employment generation, an estimated 15million people in India depend on betel leaf production, processing, handling, transportation, and selling as a source of income (Jana, 1995). It has been reported that these varieties of betel leaf, such as Nova Cuttak, Godi Bangala, Sanchi, and Birkoli, are grown in coastal Odisha. Barajas' construction, annual maintenance, leasing costs, and input costs like labor and fertilizers are included in the cost of betel leaf production, which are essential for their crop propagation. Throughout the observation phase, each seasonal and annual money expenditure in a Barajas in coastal Odisha was examined. Patra and Pradhan (2018) reported that US$ 474.20 and $ 596.70 were invested for watering and irrigation for one year. However, it is too costly for a cultivator. As a result, some cultivators are unable to hire labor and must rely on their own manpower to irrigate their crops. As a result, they are no longer charged for watering labor, and their annual financial investment is merely $ 122.50. As a result, excluding irrigation and watering costs, the cultivator paid $ 21.00, $ 21.08, $ 51.37, and $ 7.90 for each harvesting in a year. Cultivators have spent a lot of money on their traditional agriculture in the hopes of getting a high return on their investment from the local market. However, the area is well-known for its betel vine agriculture, and the market price is quite low. As a result, growers sell their crops through a middleman. Thus, Cultivators can earn money from two different sources. Paan's annual income from the local market is $ 131.72, with a middleman fee of $ 172.92, and income from the local market per harvesting is $ 16.47, $ 24.70, $ 32.93, $ 32.93, and $ 24.70. Annual market prices varied depending on the purity of the paan. The local market rate was higher in the winter season, at $ 32.93, in the rainy season, at $ 24.70, and in the summer season, at $ 16.47, i.e. cheaper in all seasons. People worked 360 days a year in a paan Baraja, plus 26 days of minor work within the Baraja. Laborers take five vacations each year, all of which are self-initiated. Its economic operations are now restricted to the local and national levels (Patra and Pradhan, 2018). In tropical nations, cash crops such as cocoa, cola nuts, coffee, citrus fruits, and other high-income-generating crops were more highly valued than the production of betel vine. Several unidentified infections and insects harm betel vine cultivation, resulting in significant losses for growers (Vishwakarma and Purohit, 2020). Another issue is seedling transportation. It happens when seedlings are damaged. Transportation, too many middlemen, a lack of grading, price fluctuations, and a lack of financial resources were all issues in marketing. The intensity of pests and illnesses, lack of water, soil quality, and the frequency of rains and winds were all agro-biological issues that limited productivity (Bar *et al.,* 2020). The primary reasons for the low betel leaf output are conventionally handled operations, uneducated laborer, and inferior planting materials.

8. Diseases

"Foot rot," "leaf spot," "powdery mildew" and"collar rot" are major diseases that affect betel vines

(Vijayakumar and Arumugam, 2014).

Foot Rot

Phytophthora spp. causes the most common fungal illness. The plantation suffers from foot rot (Haider *et al.,* 2013; Meszka and Michalecka, 2016). *Phytophthora* species was discovered in 1927, which was eventually recognised as *P. nicotianae* var. parasitica (Meng *et al.,* 2014). Foot rot induced by *P. parasitica* and *Phythium vexans* de Bery (*Phythium piperinum* Dastur) was documented by (Haider *et al.,* 2013). The lamina of the leaves begins to drop gradually, while the petiole remains upright. This sickness is known in the area as 'Khada Kala', 'Khada Pacha', or 'Madua'. Local growers are spraying Blitox and Tegron drugs to avoid the sickness from spreading (Haider *et al.,* 2013). *Sclerotium rolfsii* causes foot and root rot which is the most devastating disease that decreases the production of betel leaf (Rahman *et al.,* 2021).

Leaf spot

Patra and Pradhan (2018) reported a leaf spot disease induced by *Fusarium semitectum*. Berk. et Rav. Singh and Shanker (1971) described infections induced by *Cladosporium pipericola*, *Drechslera rostrata*, *Corynespora cassicola,* and *Cercospora piperisbetle* in Madhya Pradesh and Uttar Pradesh, India (Maiti and Sen, 1979). The sickness is called "Champa Fulia" or "Champa Tipa" in the area. The leaves' tips are tiny and curling. Farmers must cut sick leaves as soon as they are spotted, or the virus may migrate to the main stem of betel vines in a few days. It was also carried by ants and insects from one vine to another. This disease is more prevalent during the wet season. Farmers apply Diethen-M 45 and urea water to these diseases to eradicate them (Maiti and Sen, 1979).

Powdery mildew

In India, the disorder was discovered in Mysore by Narasimhan in 1933. *Oidium piperis* Uppal is the major cause of powdery mildew disease (Park *et al.,* 2012). This ailment is referred to as 'Jhalma' in the local community. On the bottom of the leaves, the infection appears as white to light brown powdery patches. At this phase, little white and black particles can be seen in the upper and lower regions of the leaves. This is a very communicable disease. There is no way to prevent or treat this illness. Farmers use a combination of dried fruit dust and Neem tree (*Azadirachta indica* A. Juss.) leaf juice mixed with water to dust betel vine leaves (Patra and Pradhan, 2018).

Collar rot

Sclerotium rolfsii Sacc., is a soil-borne fungus, infecting the collar and root parts of various crops, its growth, development, and pathogenicity were dependent on environmental factors like temperature, relative humidity, and rainfall (Garibaldi *et al.,* 2013). It is confirmed that *S. rolfsii* caused collar rot disease in betel vine which is reported to Garain *et al*. (2021) and cause 17-100% crop loss in West Bengal.

One hundred twenty million kg of EO is produced globally from approximately 300 crops, which are worth about $4 billion, including 4% production from India (Shukla, 2015). The EO extracted from coastal areas of Odisha and obtained from different varieties such as Chandrakala (0.42%), Godi Bangla (0.37%), Balia (0.35%), Desibangla (0.32%), Maghai (0.30%), Dandabalunga (0.20%), Nahua (0.15%), and Karpada (0.15%) (Das *et al.,* 2016). The leaves have indispensable oils which are known for their anti-allergic, anticancer, insecticidal, antibacterial, and antioxidant properties (Seow *et al.,* 2014), making them healthy food preservatives with significant customer interest. The application of an alternative food preservative to prevent microbial spoiling is necessitated by the growing customer desire for natural products (Mandal *et al.,* 2014; Roy and Guha, 2021). Besides being possible natural food preservatives, these oils could also have a future in novel food packets due to their antibacterial and antioxidant properties (EI Asbahani *et al.,* 2015; Roy and Guha, 2021), as well as being a viable and appealing flavoring component for the food and beverage sectors. The EO ingredients added in ice cream, chocolate, suji halwa, cupcake, lozenge, rosogolla, etc. (Guha and Nandi, 2019). Basak (2018) reported that the EO of betel vine (var. *Tamluk Mitha*) has many potentials as a natural preservative in the food sector due to its safety and antimicrobial effectivity without affecting the sensory qualities of the food products. As a result, around Rs 30-40 million worth of leaves are sold to nations like Italy, Great Britain, Bahrain, Hong Kong, Pakistan, Canada, Kuwait, Saudi Arabia, Nepal, and many other European countries each year (Pandey *et al.,* 2018). Clearly, this shows the crop's ability to generate foreign money, which should be enhanced in the national interest. Export policy decisions may be modified to increase betel leaf exports, in addition to an adequate study on export systems and intelligence. Rural

and urban populations are increasingly using the plant for culinary and ethnomedicinal purposes, which has led to an increase in demand. Cultivation and preservation of betel vine in rural areas should receive renewed attention in order to prevent the extinction of these species and to provide economic benefits to rural populations.

9. Future studies

The leaves are frequently used as remedies because they contain important bioactive components. Due to their inexpensive cost and ease of usage, they are widely utilized in India and abroad. To cure alcoholism, bronchitis, asthma, leprosy, and dyspepsia, it can be taken as a dietary additive or taken separately (Peddapalli *et al.,* 2020). Because betel leaves decompose quickly, they cannot be preserved for long periods of time. As a result, extra leaves are dumped aside or sold as cow feed in the marketplace. In this scenario, improved research approaches should be used to extend the shelf life of leaves. Usually, several diseases and insects attack the betel vine throughout production, causing significant losses to the growers. Microbes, parasites, and other contaminants can readily contaminate the leaves during preservation and transit. As a result, garbage utilization in the manufacturing industry is one of the most significant and difficult occupations on the globe. There are only a limited number of studies on the use of leaf isolates and EO. Because there is a dearth of study in this field, we should concentrate on its possible technologies in a variety of procedures such as food applications, pharmaceutical industries, cosmetic industries, and so on. Betel leaf post-harvest damages can be reduced by using superior storage techniques. The functioning of each unit (manufacturing, processing, packaging, handling, transportation, and marketing) of betel leaves affects the majority of individuals directly or indirectly.

Based on the foregoing, the federal and state governments should collaborate more effectively to fund diverse initiatives. A Research and Development Board should also be established by the government. This could help keep the price of betel leaves stable. This also aids in the expansion of cultivation and export-oriented operations, the development of conservation methods, and the reduction of waste and by outflow, among other things. If farmers, scientists, technicians, and researchers work together to resolve the limits, the economy and job prospects will grow.

10. Conclusions

This review article looked at the cultivation, nutritional components, pharmaceuticals compounds, pharmacological activities, antimicrobial compounds, diseases, and economy. Betel vine has ample of various metabolites such as phenolic tannins, anthraquinones, flavanoids, alkaloids, terpenoids, saponins, caediac possess more number of pharmacological activities and responsible for health benefits. As a result, there is a rising demand for using betel leaf extract and EO in a variety of industrial uses, including food supplements, cosmetics, and pharmaceuticals. Therefore, farmers of coastal Odisha have completely depended on the economy of betel vine cultivation and marketing at the national and international level. Sometimes, the betel vine is plagued by many diseases during cultivation, which generates a huge loss for farmers. The betel vine production was devastated due to being infected with several fungal pathogens resulting in foot rot, leaf spot, powdery mildew, and collar rot. In addition, this study discusses the recently accepted extraction technologies and characterization, both of which are critical for the growth of the product's economic worth. Bioactive chemicals from betel vine should be isolated, extracted using common and advanced technologies, and characterized for further bioactive properties, such as antibacterial activity, antioxidant activity, antidiabetic activity, anticancer activity, and so on. Such potential of betel vine made itself a green medicine in nature. It concludes that the betel vine keeps promising as a natural reservoir with regard to its nutritional, pharmacological and economical aspects for the rapidly growing human population.

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Short note

Influence of 6-benzylaminopurine spray time after pinching on growth and flowering of *Veronica dahurica* Steven

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Key words: Cytokinin, foliar application, foliar spray, multiple shoots.

Abstract: *Veronica dahurica* Steven (family *Scrophulariaceae*) is an ornamental plant from Korea. The aim of the present study was to produce multiple-branched plants by using 6-benzylaminopurine (BA) spray application at several time intervals after pinching. All 10 cm long plants were sprayed with 0, 500, 1000, and 2000 mg·L^{-1} BA at 0, 7, and 14 days after pinching. Growth characteristics were examined 10 weeks after pinching and flowering time was recorded. The number of branches was highest in the group sprayed with 1000 mg·L^{-1} BA at 0 d after pinching. The greatest plant height was observed in the group treated with 1000 mg·L^{-1} BA 14 d after pinching and the minimum plant height was observed in the group sprayed with 500 mg·L^{-1} BA at 0 d after pinching. The groups sprayed with higher BA concentrations and with longer intervals between pinching and spraying showed greater delay in the time to first flower. The flower length was decreased in the pinched and BA-treated group compared with the control. Thus, BA application and pinching could promote multiple branch induction and control flowering time in *V. dahurica* Steven.

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1. Introduction

The genus *Veronica* L. (family *Scrophulariaceae*) comprises about 500 species. It is distributed across most of the Northern Hemisphere and in many parts of the Southern Hemisphere and has a wide ecological range, from alpine to coastal vegetation and dry to aquatic vegetation (Albach *et al*., 2004; Albach *et al*., 2005). *Veronica* spp. have the characteristics of graceful and bountiful flowers, long-blooming, easy-care perennials, and they are commercially used in gardens and as cut-flowers (Areal *et al*., 2008; Hawke, 2010).

Veronica dahurica Steven is distributed across East Asia, in Siberia, Far East Russia, China, North and South Korea, and Mongolia (Choi, 2016). The stems of *V. dahurica* are erect and upright, with simple hairs, and occur individually or in small groups. The plant grows to 30-50 cm in height. The opposing leaflets are 18-25 mm in length and 15-22 mm in width and have a deltoid shape with deeply cut tips on the edge of the leaf. The flowers are racemule, white or pink colored, with short hairs on

the apical part. The corolla is 5-7.5 mm in length (KPNI, 2019).

Multiple branching has commercial advantages for flower production, because it improves yield and quality, controls plant size, and inhibits loathing (Zieslin *et al.*, 1975; Barbier *et al.*, 2017; Kim *et al.*, 2020). Pinching and BA foliar spray are the most useful methods for production of multiple-branched plants (Lee *et al.*, 2006).

Pinching is the removal of the apical bud to release the lower axillary buds from apical dominance. Pinching increases branching and stimulates axillary bud development by decreasing auxin production in the apical bud and inhibiting lateral bud growth (Barbier *et al.*, 2017). Pinching is commonly used to improve yield by inducing branches, decreasing plant height to produce dwarf plants, and controlling flowering time. This method also increases the cytokinin content. Exogenously applied cytokinin, like foliar application, has the same effect as pinching.

Plant growth regulators (PGRs), such as cytokinins, play important roles in the control of herbage type and flowering. Cytokinin regulates gynoecium formation and female gametophyte development, embryo development and seed size, pavement cell morphogenesis, axillary bud release, and nutrient uptake. This promotes vascular cambial development, nodulation, chloroplast development, cell division, and phloem development. It also inhibits lateral root formation, senescence, and cell proliferation in the root apical meristem (Kieber and Schaller, 2018).

In the present study, the use of foliar sprays of different concentrations of BA and pinching to improve the branching and flowering of *V. dahurica* are evaluated for commercial use on ornamental plants. In addition, the interaction and use of different growing environments is examined to further manipulate the production of *V. dahurica*, particularly for use as flowering pot plants.

2. Materials and Methods

Plant materials

Seeds of *V. dahurica* were collected from the Useful Plant Resources Center, Korea National Arboretum on 12th September 2018 and stored in a 4°C chamber. *V. dahurica* seedlings were grown on 128-cell trays filled with a commercial soilless substrate (Baroker; Seoul Bio, Eumseong, Korea) on 7th May 2019, and transplanted into 11.5 cm diameter containers filled with the same substrate on 26th June 2019. The Baroker consisted of 68% coir dust, 15% peat moss, 7% perlite, 6% vermiculite, and 4% zeolite, at pH 5.5-6.0. Two weeks after transplanting, plants were fertilized with 1000 mg·L^{-1} Peters Professional 20-20-20 (Everris, Geldermalsem, The Netherlands). All plants were grown in a greenhouse.

Pinching and BA foliar spray application

V. dahurica plants of 10 cm length, with 2-3 nodes and 2 leaves, were selected. All plant materials were pinched at every second internode from the basal stem, and then foliar-sprayed with 10 mL of 0, 500, or 2000 mg·L^{-1} BA (Duchefa, Haarlem, The Netherlands), using a hand-pump sprayer, at 0, 7, or 14 spraying days after pinching (SDP). The BA solutions were prepared in 10 mL of 99 % ethanol by diluting with distilled water.

Growth conditions

The experiment was conducted between 5th July 2019 and 13th September 2019. Pinching and BA treatments were applied once at 10 weeks of culture under greenhouse conditions. The mean values of relative humidity and temperature in the greenhouse are shown in figure 1. Irrigation was carried out by drip watering at 500 mL daily in September and twice daily from July to August.

Plant growth and flowering

The variables considered consisted of height and width of plant, number of nodes, length and width of leaf, number of leaves, lateral branch length, number

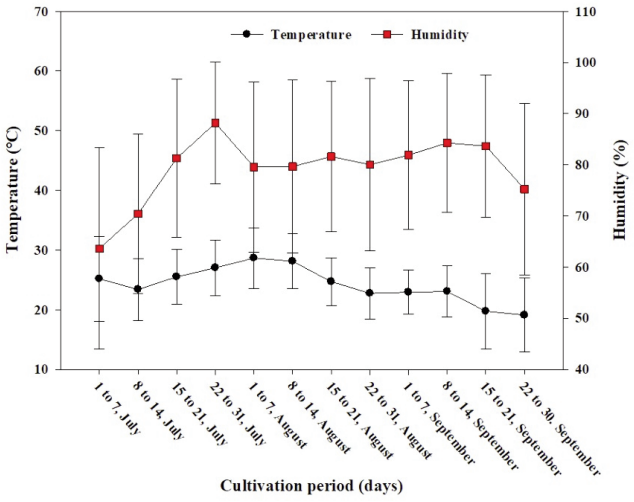

Fig. 1 - Principal component loading pattern of six traits of *Amaranthus* accessions.

of lateral branches and flower characteristics. These parameter were evaluated 10 weeks after treatment. The time to first flowering was recorded.

Statistical analysis

The experiment was conducted with twelve plantlets per three replicates and three replications per treatment, in a completely randomized block design, unless specified otherwise. Significant differences were determined using Duncan's multiple range test (DMRT) with SAS software (Version 9.4; SAS Institute, Cary, NC, USA).

3. Results and Discussion

Effects of BA and SDP on growth characteristics

V. dahurica was cultivated with 0, 500, 1000, or 2000 mg·L^{-1} BA foliar spray application at 0, 7, or 14 SDP, and plant growth was evaluated 10 weeks after treatment. BA foliar application groups, which were co-treated with pinching, successfully formed lateral branches, while the effect of pinching treatment alone was similar to that of the control. Maximum lateral branch formation was observed in the group treated with 1000 mg·L^{-1} BA spray on 0 day after pinching, with a mean of 13.92 secondary branches per plant and a branch production efficiency approximately 2.93-fold that of the control (Table 1). Both pinching and BA foliar spray application can promote branch production by controlling apical dominance through cytokinin signaling (Barbier *et al*., 2017; Kieber and Schaller, 2018). BA foliar spray application is recommended to promote branch production in many ornamental plants such as *Ardisia pusilla* (Lee *et al*., 2006), *Echinacea* cultivars (Latimer and Freeborn, 2009), and Kalanchoe species (Currey and Erwin, 2012). Pinching is also recommended for branch production in *A. pusilla* (Lee *et al*., 2006), *Targets exeta* L. (Meena *et al.*, 2015), and *Elsholtzia* (Sohn and Kim, 2003). However, even when the concentration of BA was increased with increasing SDP, the multiple-branch production effect was not greater than that of the treatment immediately after pinching. Lee *et al*. (2006) reported that 0 to 7 SDP increased branch production in *Ardisia pusilla*, but that branch production decreased at SDP>7. In our treatments, plant height was similar to or less than that in the control; however, lateral branch length was decreased in all treatment groups compared with that in the control (Table 1, Fig. 2). Leaf characteristics were also investigated in our experiments. Compared with the control, leaf number and leaf width were increased in almost all or all treatment groups, respectively, and leaf length was decreased in almost all treatment groups (Table 1). All treatments showed a decrease in the number of nodes compared with that in the control. Pinching and cytokinin application can be economically used to enhance production of plant stems, flowers, and

Table 1 - Growth effects of pinching and spray application on *V. dahurica* 10 weeks after treatment

SDP (days)	BA (mg·L^{-1})	Plant		No. of nodes (per/plantlet)	Leaf			Secondary branch	
		Height (cm)	Width (cm)		Length (cm)	Width (cm)	Number (per/plantlet)	Length (cm)	Number (per/plantlet)
Control		29.08 ab[z]	38.42 a	14.00 a	6.27 a	3.57 c	177.94 d	29.43 a	4.75 f
Pinching		28.69 ab	37.39 a	11.67 bc	6.33 a	3.76 bc	197.17 cb	28.52 a	6.78 ef
0	500	20.65 c	23.87 c	10.53 bc	6.99 a	4.24 ab	298.97 b	12.44 e	11.64 abc
	1000	24.86 bc	30.54 b	11.61 bc	7.08 a	4.48 a	407.36 a	15.60 cd	13.920 a
	2000	27.25 ab	23.44 c	7.00 d	4.71 b	3.86 bc	175.67 d	18.86 bcd	11.06 abc
7	500	26.59 ab	29.56 b	6.69 d	4.61 b	3.76 bc	215.61 cd	16.47 b	10.59 bcd
	1000	24.60 bc	27.68 bc	7.8 d	5.01 b	3.79 bc	191.08 cd	16.11 bc	11.48 bcd
	2000	28.22 ab	31.97 b	13.00 ab	4.23 b	3.75 bc	239.28 bcd	12.56 bcd	8.00 de
14	500	24.76 bc	30.57 b	12.28 ab	4.70 b	4.34 ab	256.72 bc	13.37 e	8.92 cde
	1000	30.56 a	32.53 b	12.77 ab	4.82 b	4.28 ab	432.14 a	12.86 de	12.86 ab
	2000	24.55 bc	30.98 b	11.36 bc	7.04 a	4.13 ab	370.58 a	18.10 bc	12.67 ab

SDP= spraying days after pinching;
BA= 6-benzylaminiopurine;
[z] Mean separation within columns by Duncan´s multiple range test at $p<0.05$.

seeds by decreasing the planting density and increasing the number of branches. Small dwarf plants were grown as pot plants by reducing the plant height (Table 1, Fig. 2).

Effects of BA and SDP on flowering

Pinching treatment alone resulted in the highest flowering percentage per plantlet after 10 weeks of culture (86%), followed by BA 2000 mg·L^{-1} spray application at 0 SDP, BA 1000 mg·L^{-1} at 0 SDP, BA 1000 mg·L^{-1} at 7 SDP, BA 1000 mg·L^{-1} at 14 SDP, BA 500 mg·L^{-1} at 7 SDP, and the control.

There were significant differences in days to first flowering among treatment groups. Pinching delayed the first flowering (24.69 days after pinching) compared with the control (14.14 days after pinching). BA 500 mg·L^{-1} (0 SDP: 30.94 days/ 14 SDP: 29.17 days) and 1000 mg·L^{-1} (0 SDP:30.72 days/ 14 SDP: 26.06 days) at 0 SDP and 14 SDP increased the number of days to first flowering compared with the control and BA 0 mg·L^{-1} with pinching groups. However, other treatments decreased the number of days to first flowering compared with the BA 0 mg·L^{-1} with pinching and control groups. In the flower development stage, the first increase in cytokinin occurred in the bud induction stage, followed by a dramatic increase in the transition stage. Exogenous cytokinin treatment can promote floral transition through transcriptional induction of the floral activators TWINSISTER OF FT (TSF), FLOWERING LOCUS D (FD), and SOCI (D'Aloia *et al*., 2011; Winterhagen *et al*., 2020).

The number of flowers and buds also differed among treatments. The BA 2000 mg·L^{-1} at 0 SDP treatment produced the highest flower number, followed by BA 1000 mg·L^{-1} at 14 SDP. However, the highest number of buds was observed in the BA 2000 mg·L^{-1} at 14 SDP treatment group, followed by BA 1000 mg·L^{-1} at 7 SDP, pinching, and BA 1000 mg·L^{-1} at 14 SDP (Fig. 2, 3). The observed increases in flower number and buds are due to the increase in branch number and the promotion of flowering by cytokinin (Kumar *et al*., 2002; D'Aloia *et al*., 2011).

Both the flower length and the peduncle length decreased with BA foliar spray application and pinching treatments. The effect of the interaction between pinching and BA on the flower length was found to be significant. The smallest flower length (10.49 cm) was recorded with application of BA 1000 mg·L^{-1} at 7 SDP compared with a flower length of 21.62 cm in the control group. With BA 2000 mg·L^{-1} treatment at

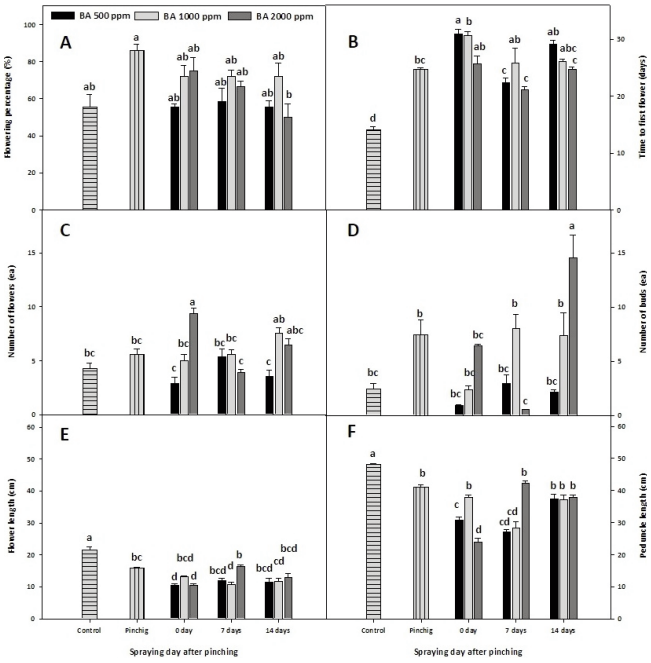

Fig. 2 - Flowering characteristics of *V. dahurica* with pinching and BA spray application.

0 SDP, the peduncle length of *V. dahurica* Steven was significantly lower than that of the control (Fig. 3). Ferrante *et al.* (2006) reported that a 500 µM BA spray application reduced inflorescence length in *Salvia splendens* Kerr Gawl 'Flamex 2000'. However, the effect of BA on flower size depends on BA concentration, method of application, and plant species (Pobudkiewicz, 2008) and can also affect other variables such as tepal length, flower diameter, and inflorescence length (Pobudkiewicz and Nowak, 1994; Ferrante *et al*., 2006; Pobudkiewicz and Treder, 2006).

4. Conclusions

The present study revealed the commercial utility of pinching and BA foliar spray application for induction of multiple branching and control of flowering in *V. dahurica.* Treatment with 1000 mg·L^{-1} BA at 0 days after pinching produced the highest branch number after 10 weeks. Pinching delayed the first flowering time compared with that in the control, but some BA foliar applications accelerated the first flowering time. Pinching and BA foliar application also had the effect of reducing flower length and peduncle length. We anticipate that this knowledge will be beneficial for enhancing branching and controlling flowering

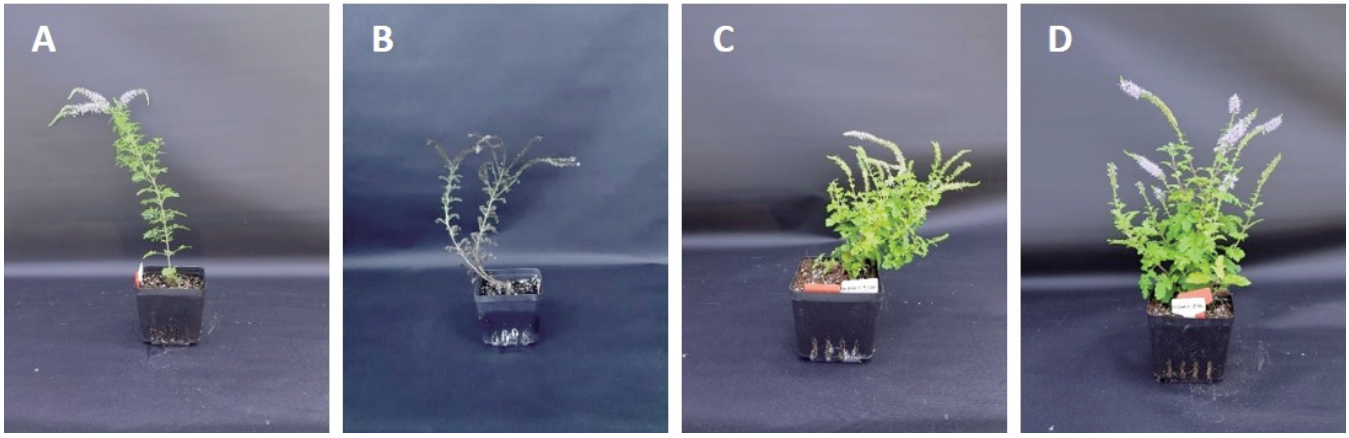

Fig. 3 - Growth and flowering characteristics in pinching and BA application groups 10 weeks after treatment. A) Control. B) Pinching. C) BA 500 ppm 0 week after pinching. D) BA 1000 ppm 0 week after pinching.

time and characteristics of *V. dahurica* in commercial applications.

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