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Superior sweet oranges for varietal diversification of tropical rainfed orchards

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Abstract: Citrus orchards in northeastern Brazil are mostly rainfed and comprised basically of 'Pera CNPMF D-6' sweet orange budded on 'Rangpur' lime, for the drought tolerance and productivity imparted by this rootstock. Therefore, the selection of new varieties is needed to broaden the genetic basis of citrus cultivated in this region. Accordingly, this study compared vegetative, productive, and fruit quality traits of eight sweet orange scions grafted on 'Rangpur' lime over eleven years under the tropical rainfed conditions of northeastern Brazil. 'Kona' trees excelled in yield performance associated with bulk canopy, precocity, sweet fruit with intermediate acidity, and high vitamin C contents in spite of proneness to alternate yields and low ratio (maturity index). 'Valencia Montemorelos' and 'Rubi' trees, in turn, had high yield performances coupled with intermediate canopies, sweet fruit, intermediate acidity ('Rubi') and vitamin C contents, low propensity for yield fluctuation ('Valencia Montemorelos'), and high precocity ('Rubi'), albeit low ratio. Overall, our results emphasize 'Kona,' 'Valencia Montemorelos,' and 'Rubi' as superior sweet orange varieties for diversification of tropical rainfed orchards for their outstanding yield performance and good fruit quality.

1. Introduction

Brazil is the largest producer of sweet oranges [*Citrus sinensis* (L.) Osbeck] worldwide, with 578,057 ha and 16.21 million tons of fruit harvested in 2021 (FAO, 2021). The country is also the world's top exporter of orange juice. Most orchards are rainfed, and the southeast and northeast regions are the main producers nationwide, with 421,171 ha and 98,475 ha, respectively. However, the yields in the northeast (11.40 t·ha^{-1})

are only a third of those in the southeast (IBGE, 2019).

Lower yields in northeastern Brazil stem mainly from soils with fertility restrictions and hardsetting layers that impair drainage and root development in addition to water deficits due to irregular rainfall distribution, low technology adoption, improper management practices, and aging plants (Gomes *et al.,* 2017; Carvalho *et al*., 2020; Martins *et al*., 2020). Despite water deficit negatively affecting citrus yields, citriculture in Brazil is predominantly rainfed (Carvalho *et al*., 2019 a, 2020, 2022). Water stress linked to climate change is predicted to increase and affect the citrus industry worldwide (Fares *et al.*, 2017). In addition to these constraints, most citrus orchards in this region comprise 'Pera CNPMF D-6' sweet orange (referred to as 'Pera') budded on 'Rangpur' lime (*C. limonia* Osbeck), which is a graft-compatible rootstock that confers good tolerance to drought, quality to fruits, and yield to scions (Carvalho *et al.*, 2020). 'Pera' has a medium-sized canopy and is classified as a mid-season maturating variety beginning in July with fruit suited for both *in natura* consumption and juice production (Carvalho *et al*., 2019 b).

However, broadening the genetic diversity of scions could increase the much-needed fruit yield and quality to enhance the competitiveness of farms in this region. Accordingly, in 2008, the Brazilian Agricultural Research Corporation (Embrapa) established a comprehensive research project aimed at varietal diversification with scion-rootstock combinations for rainfed citrus orchards in the coastal tablelands of northeastern Brazil. As a result, new combinations of sweet oranges and rootstocks have been selected for cultivation in this area (Carvalho *et al*., 2019 b, 2020, 2022). Herein, we recommend new varieties of sweet oranges for the diversification of tropical rainfed orchards. To this end, we conducted comparative vegetative, productive, and fruit quality trait assessments among eight sweet orange scions grafted on 'Rangpur' lime over eleven years in Brazil's northeastern region.

2. Materials and Methods

Site description and experimental design

The study was conducted from 2008 to 2019 at the experimental station of Embrapa in Umbaúba (11° 22' 37'' S, 37° 40'' 26'' W; 109 m a.s.l.), Sergipe State, in the coastal tablelands of northeastern Brazil. The study site soil is an Haplic Acrisol (Ultisol), which is a reddish-yellow acid soil with medium texture, a clay-rich B horizon (Kaolinite). According to soil analyses at 0-20 cm depth, the pH is 6.72, phosphorus 13.5 mg·dm^{3}, organic matter 21.2 g.kg^{1}, potassium 0.23 g.kg^{1}, Calcium 2.22 g.kg^{1}, Magnesium 0.86 g.kg^{1}, and base saturation (74%). The climate is classified as "As" according to Köppen-Geiger, with a rainy period from May to September. Rainfall was recorded over the study period, with an annual mean of 1309 (±275) mm. The yearly rainfall and potential evapotranspiration patterns over the study period are shown in figure 1 a.

The experimental orchard consisted of eight sweet orange scions grafted onto 'Rangpur' lime in a randomized complete block design, with three replicates and three trees per plot. The orchard was planted at a density of 416 plants ha^{-1} (6.0×4.0 m) under rainfed conditions, but plants received 6 l of water weekly in the driest months. The orchard was annually fertilized according to the recommendation for sweet oranges in this region (Carvalho *et al.,* 2022). Pest and weed control treatments with registered pesticides as well as pruning were also conducted whenever necessary. The orchard was fertilized twice a yer based on soil analysis and soil acidity was corrected by the application of dolomitic limestone. The scions were 'Kona', 'Rubi', 'Valencia Montemorelos', 'Pera', 'Natal CNPMF-112', 'Sukkari', 'Lima Verde' and 'Lima', and were obtained from the Embrapa Mandioca e Fruticultura breeding program. These varieties hold potential for diversifying

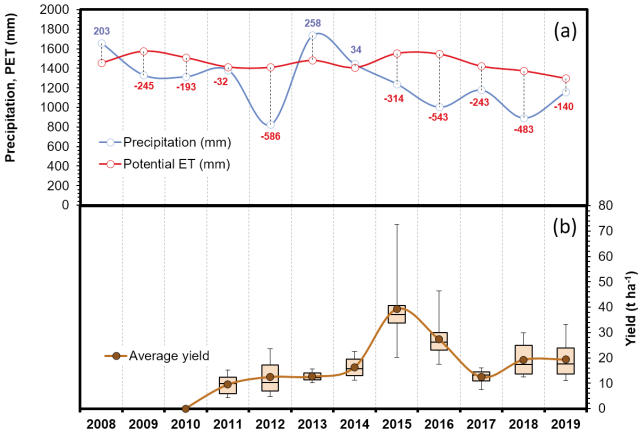

Fig. 1 - Water balance measured by the difference between rainfall and potential evapotranspiration (PET) (a) and boxplot chart of citrus yield for all observations during the experimental period (b) (2008-2019).

orchards in the study region that are dominated by 'Pera' sweet orange, therefore their agronomical performances under rainfed field conditions were assessed.

Vegetative performance and fruit yields

Vegetative growth of scions was evaluated in 8-year-old trees in 2016 by recording plant height (PH, in m), rootstock (RD, in m) and scion diameters (SD, in m), and by estimating canopy volume (CV, in m^3) as per Zekri (2000).

Productive performance was assessed by fruit yield (FY, in $t \cdot ha^{-1}$) from the first harvest in 2011 to 2019, and yield efficiency (YE, in $kg \cdot m^{-3}$) was estimated in 2016 by the quotient between per plant fruit production and canopy volume. The alternate bearing index (ABI) was estimated using FY from 2011 to 2019 using the following formula (Monselise and Goldschmidt, 1982):

$$ABI = \frac{\sum_{(i=2)}^{n} \frac{|Y_i - Y_{i-1}|}{(Y_{i+1} + Y_{i-1})}}{n-1} \quad \text{Equation (1)}$$

where n denotes years and Y_i is the yield in year i. Precocity (Prec., in %) estimates considered the ratio between FY in the first two harvests and the cumulative yield (CY; 2011 to 2019).

Fruit quality

The quality was appraised in nine randomly chosen fruits per plant from the 2015 and 2016 harvests as follows: fruit weight (FW, in $g \cdot fruit^{-1}$), diameter (FD, in mm), and height (FH, in mm), as well as rind thickness (RT, in mm) as measured by a caliper, juice content (JC, in $g \cdot 100\ g^{-1}$ of fruit mass), total titratable acidity (TTA) was measured with 0.1 mol L^{-1} NaOH as titrant and given in g of citric acid per 100 mL of juice, total soluble solids (TSS, in °Brix) using a refractometer, and vitamin C content (Vit. C, in $mg \cdot 100\ mL^{-1}$ of juice) as measured by the oxidation-reduction volumetric technique using potassium iodate solution. The ratio, or maturity index, was estimated as the quotient between TSS and TTA. All measurements followed the methods described by França *et al*. (2016).

Statistical procedures

Data were subjected to ANOVA, and means were grouped by Scott-Knott analysis. Multivariate analyses were also performed using XLSTAT to identify homogenous groups of scions, considering only the variables that were significant in univariate analyses. Briefly, a principal component analysis (PCA) was used to shorten the dataset into synthetic and uncorrelated variables, that is, the first principal component (Carvalho *et al*., 2019 a). Afterwards, the scions were grouped by agglomerative hierarchical clustering analysis (AHC) applied to the PCA scores that complied with the Kaiser criterion, that is, those whose eigenvalues were ≥1.0. Euclidean distance was used as a measure of dissimilarity, and Ward's minimum variance was used to identify clusters. The automatic truncation option was used for cluster splitting. This approach creates homogenous groups based on the largest decrease in Shannon's entropy between a node and the next one. The resulting clusters were interpreted using PCA results and put into perspective with the results of univariate analyses of variance (Carvalho *et al*., 2019 a).

3. Results

Vegetative performance

PH, RD, SD, and RD/SD girth ratio were not influenced by scions (data not shown, $p < 0.05$). Although not significantly different, PH varied from 2.5 m ('Lima') to 3.5 m ('Kona'), and RD/SD girth ratio from 1.19 ('Valencia Montemorelos') to 1.68 ('Lima'). However, 'Kona' trees were characterized by the largest CVs (26.3 m^3); 'Valencia Montemorelos' (19.0 m^3), 'Rubi' (18.5 m^3), and 'Sukkari' (17.1 m^3) had intermediate values, while 'Pera' (15.5 m^3), 'Lima Verde' (15.1 m^3), 'Natal CNPMF-112' (14.3 m^3), and 'Lima' (11.4 m^3) presented the smallest canopy volumes (p<0.0001).

Fruit yields

The annual water balance over the experimental period was predominantly negative, reaching deficits of 586 and 543 mm in the driest years of 2012 and 2016, respectively (Fig. 1 a). Considering yield performance, the production peak of sweet oranges was generally achieved in the fifth harvest (2015), decreased until 2017, and stabilized thereafter (Fig. 1 b, Table 1). 'Kona' trees exhibited the highest mean yields over nine years, followed by 'Valencia Montemorelos' and 'Rubi'. 'Pera' which is the main sweet orange grown in the study region, had intermediate yields while 'Lima' and 'Lima Verde' were the least productive (Table 1). Except for 'Lima Verde' with the lowest values, all varieties had similar yield efficiencies. 'Lima Verde', Natal CNPMF-112',

Table 1 - Yield performance of eight sweet orange varieties budded on 'Rangpur' lime (2008-2019)

	Fruit yield (t·ha^{-1})										YE[y]	ABI[x]	Prec.[w] (%)
	2011	2012	2013	2014	2015	2016	2017	2018	2019	Mean			
Kona	12.1 c[z]	23.5 a	11.7 b	19.1 b	72.5 a	46.4 a	14.0 b	29.8 a	33.2 a	29.2 a	4.27 a	0.33 c	13.6 c
Lima	4.3 f	4.7 e	10.1 b	12.0 d	34.4 c	17.6 d	7.4 d	13.8 d	13.9 d	13.1 e	3.73 a	0.27 b	7.6 e
Lima Verde	4.2 f	4.8 e	12.2 b	11.2 d	31.8 c	17.5 d	8.9 d	12.6 d	10.9 d	12.7 e	2.79 b	0.24 a	7.9 e
Natal CNPMF-112	13.5 b	7.6 d	10.3 b	15.2 c	20.1 d	24.8 c	11.6 c	13.2 d	23.2 b	15.5 d	4.26 a	0.20 a	15.2 b
Pera CNPMF D-6	8.0 d	15.4 b	13.8 a	15.9 c	39.4 b	25.4 c	15.5 a	14.0 d	12.8 d	17.8 c	3.98 a	0.19 a	14.6 b
Rubi	11.6 c	23.0 a	15.6 a	20.8 a	40.7 b	26.8 c	12.7 c	24.7 b	15.2 d	21.2 b	3.53 a	0.26 b	18.1 a
Sukkari	6.5 e	11.8 c	12.2 b	13.3 d	34.8 c	29.9 b	13.6 b	25.3 b	20.0 c	18.6 c	4.37 a	0.22 a	10.9 d
Valencia	15.2 a	8.9 d	15.0 a	22.5 e	40.8 b	30.1 b	15.9 a	20.7 c	25.9 b	21.7 b	3.83 a	0.21 a	12.3 c
CV (%)	10.9	14.7	15.4	8.9	9.9	5.8	8.7	10.7	17.4	4.3	13.5	8.6	7.8
F	51.3	50.4	3.32	24.8	44.2	99.7	23.7	32.3	15.6	135.0	2.99	14.9	40.5
p-value	<0.0001	<0.0001	0.027	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.038	<0.0001	<0.0001

[z] Means in the same column followed by the same letter are not significantly different according to the Scott-Knott analysis (p<0.05).
[y] Yield efficiency; [x] Alternate Bearing Index;
[w] Precocity.

'Pera', 'Sukkari' and 'Valencia Montemorelos' were less prone to alternate bearing. 'Rubi' appeared to have the highest precocity (Prec.) for the ratio between the first two harvests and the CY. However, 'Kona' and 'Rubi' presented the highest absolute yields in 2011-2012 with more than 30 t·ha^{-1} (Table 1).

Fruit quality

Regarding fruit quality, 'Lima' had a smaller fruit than the other scions. 'Rubi' fruit had the thickest rind in contrast to 'Lima Verde', which had the thinnest. Fruit of 'Natal CNPMF-112' and 'Valencia Montemorelos' showed the highest citric acid content, followed by those of 'Kona', while 'Lima', 'Lima Verde' and 'Sukkari' exhibited the least acid fruit. 'Kona', 'Lima', 'Natal CNPMF-112', 'Rubi', 'Sukkari' and 'Valencia Montemorelos' produced sweeter fruit than the remaining scion varieties (Table 2). Additionally, 'Kona' and 'Lima' had the highest vita-

Table 2 - Attributes of fruit quality of eight sweet orange varieties budded on 'Rangpur' lime (Average 2015-2016)

Orange varieties	Mean FW (g·fruit^{-1})	Fruit diameter (mm)	Fruit height (mm)	Rind thickness (mm)	Juice content (g·kg^{-1})	TTA (g·100 mL^{-1})	TSS (°Brix)	Vitamin C (g·100 g^{-1})	Ratio TSS/TTA
Kona	190	76.3	71.0 a[z]	3.81 b	545	0.798 b	11.5 a	60.0 a	14.5 c
Lima	193	70.0	60.8 b	3.34 c	544	0.128 d	11.3 a	58.3 a	88.0 b
Lima Verde	190	74.6	72.7 a	2.60 d	579	0.097 d	8.9 b	40.6 d	93.2 a
Natal CNPMF-112	180	72.9	69.8 a	3.59 b	572	1.208 a	12.3 a	52.3 b	10.4 c
Pera CNPMF D-6	205	74.9	75.6 a	3.12 c	548	0.670 c	8.9 b	43.5 d	14.7 c
Rubi	187	75.9	70.0 a	4.15 a	539	0.617 c	11.3 a	49.2 c	18.7 c
Sukkari	189	72.9	67.0 a	3.08 c	544	0.114 d	10.5 a	54.1 b	93.7 a
Valencia Montemorelos	188	72.1	69.6 a	2.99 c	549	1.180 a	11.0 a	53.4 b	9.6 c
CV (%)	8.2	3.5	5.9	6.0	4.2	11.0	7.2	7.1	8.2
F	0.63	2.01	3.38	18.62	1.15	142.1	7.56	10.00	397.2
p-value	0.723	0.126	0.025	<0.0001	0.385	<0.0001	0.0007	0.0002	<0.0001

[z] Means in the same column followed by the same letter are not significantly different according to the Scott-Knott analysis (p<0.05).
TTA= Total titratable acidity;
TSS = Total soluble solids.

min C contents, whereas 'Lima Verde' and 'Pera' had the lowest values. 'Sukkari' and 'Lima Verde' followed by 'Lima' had the highest values of ratio, a proxy for fruit maturity (Table 2). FW, FD, and JC were not affected by scions (Table 2).

Multivariate analyses

Multivariate analysis helped to identify scion groups that performed homogeneously, considering the universe of all significant attributes. The first two PCA principal components explained more than 66% of the total observed variability, and the square cosine of the variables showed that, while average yield, CV, TTA, Prec., RT, TSS, and ratio were mostly associated with PC1, FH, alternate bearing, and vitamin C contributed to most of the variation along PC2 (Fig. 2 a).

Multi-correlation analysis indicated that average yield correlated positively with CV and that Prec. exhibited a positive correlation with total acidity, but both correlated negatively with ratio. In addition, while alternate bearing correlated positively with vitamin C, it had a negative correlation with FH (Fig. 2 a).

Agglomerative hierarchical clustering analysis (AHC) using Shannon entropy for grouping all observations that showed similar results for the entire set of variables identified three distinct clusters (Fig. 2 c) with the following characteristics, as observed through the visual inspection of the observation cloud projection in the plane of the first two principal components of the PCA (Fig. 2 b). The heatmap of the results for all variables is shown in figure 2 c.

The first cluster grouped all observations of 'Kona' and its main characteristics were high and alternate yields associated with bulk canopy and high vitamin C content; intermediate values for TSS, RT, Prec., acidity, FH, and low ratio.

The second cluster included the observations of 'Valencia Montemorelos', 'Rubi', 'Pera' and 'Natal CNPMF-112'. In general, these varieties showed intermediate values for all evaluated variables except ratio, which were mostly low for this cluster.

Finally, the third cluster encompassed all observations of 'Sukkari', 'Lima Verde', and 'Lima'. In contrast to the first and second clusters, the main characteristic was that the ratio was high for all observations and average yield, CV, RT, and acidity were predominantly low. Intermediate values were observed for alternate bearing, vitamin C, TSS, and FH.

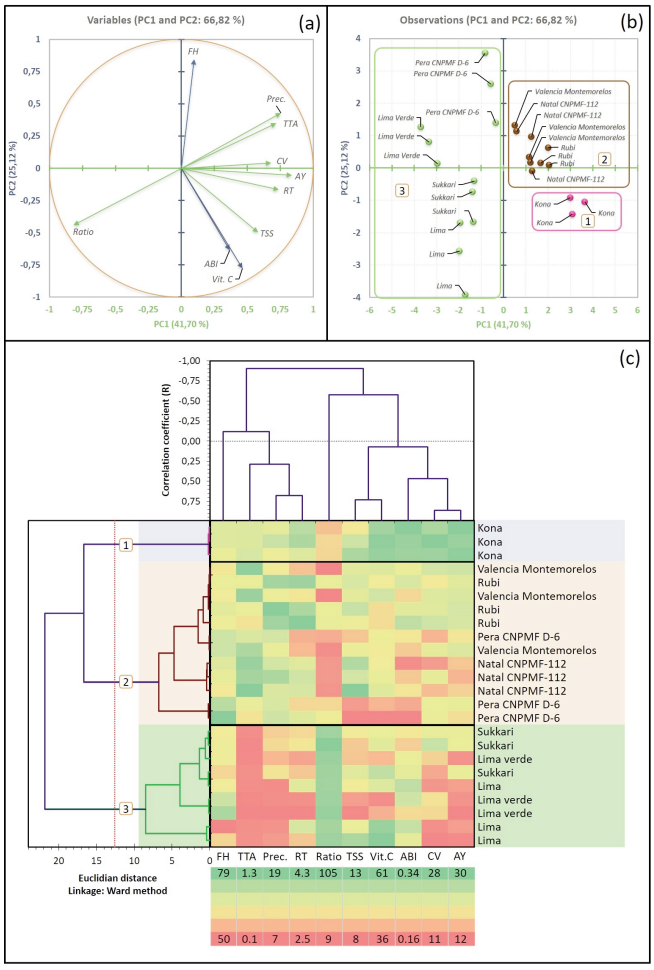

Fig. 2 - Principal Component and Agglomerative Hierarchical Clustering Analysis. (a) Correlation Circle of the variables; (b) Score plot of the observations in the plane of the two first Principal Components and (c) Heatmap of the relative values of all observations for all variables and group separation by the Agglomerative Hierarchical Clustering analysis (AHC). The first two axes (PC1 and PC2) accounted for 66.82% of the total variance. Arrows in green represent variables associated with PC1 whereas those in blue are associated with PC2 in figure a. Observations belonging to different groups by the AHC analysis were delineated (boxes) in figure b. CV: Canopy volume; AY: Average yield; ABI: Alternate Bearing Index; Vit.C: Vitamin C content; TSS: Total soluble solids content; RT: Rind thickness; Prec.: Precocity; TTA: Total titratable acidity; FH: Fruit height.

4. Discussion and Conclusions

Here, we comparatively assessed vegetative, productive, and fruit quality traits among eight sweet orange scions grafted on 'Rangpur' lime for enhancing orchard varietal diversification under the tropi-

cal rainfed conditions of northeastern Brazil. Overall, the varieties reached peak yields in the fifth harvest, and subsequently decreased. The same pattern was observed for 'Sincora,' 'Valencia Tuxpan,' and 'Pineapple' sweet oranges in the same study region (Carvalho *et al*., 2019 a; Martins *et al*., 2020). As the experimental orchard was cultivated under rainfed conditions, predominantly negative yearly water balances possibly interfered with the productive potential of the different varieties. Major drought spells occurred in 2012, 2016, and 2018, with water deficits exceeding 480 mm. Water stress strongly impairs growth and development of citrus trees and sweet orange varieties commonly present water deficiency symptoms throughout the study region even when scions are grafted on drought-tolerant 'Rangpur' lime, which emphasizes the severe seasonal drought rainfed orchards face (Soares *et al*., 2015; Carvalho *et al*., 2016). As we did not specifically evaluate drought tolerance, further studies are needed to shed light on the susceptibility of sweet orange varieties to drought.

Comparatively, 'Kona' was the most productive variety, with an annual average of 29.2 t·ha^{-1} of fruit. 'Valencia Montemorelos' (21.7 t·ha^{-1}) and 'Rubi' (21.2 t·ha^{-1}) also had remarkable yield performances. The high fruit yield of 'Valencia Tuxpan' grafted on 'Santa Cruz Rangpur' lime was also verified by Carvalho *et al*. (2019 a). Similarly, 'CNPMF 003 Rangpur' lime and 'Santa Cruz Rangpur' lime rootstocks conferred high yields to 'Valencia' in Brazil's southeastern state of São Paulo (Fadel *et al*., 2018). The dominantly grown 'Pera', however, was characterized by intermediate yields. 'Kona' trees produced 72.5 t·ha^{-1} at its peak, and alongside 'Rubi' excelled in precocity, with yields that surpassed 23 t·ha^{-1} in the second harvest. Apart from 'Lima Verde' with lower values, all varieties had similar yield efficiencies despite the sharp differences in canopy volumesCVs. For instance, 'Kona' trees had the largest canopies (26.3 m^3) in contrast with 'Lima' (11.4 m^3) and 'Pera' (15.5 m^3). This is consistent with similar yield efficiencies of 'Pineapple' sweet orange irrespective of the grafted rootstock in the same study region (Martins *et al*., 2020). It is noteworthy that after the two driest years (2012 and 2016), the most productive varieties were 'Pera' and 'Valencia Montemorelos'. Melgar *et al.* (2010) showed that 'Valencia' sweet orange trees [grafted on 'Swingle' citrumelo *C. paradisi* Macfad. x *Poncirus trifoliata* (L.) Raf.] that experienced drought for a hundred days and were well irrigated in subsequent months produced more fruit than those that were not subjected to water stress.

Alternate bearing is a widespread phenomenon among fruit trees in which high yield in one harvest is followed by low production in the subsequent harvest (Monselise and Goldschmidt, 1982). Alternate bearing is an undesirable trait from an economic standpoint, especially for mandarins and tangerines, but generally a minor to moderate problem for sweet oranges such as 'Valencia' (Monselise and Goldschmidt, 1982; Abobatta, 2019). Alternate bearing in citrus is caused by fruit load inhibiting return flowering (Abobatta, 2019) and seemed here to be variety-specific, as 'Kona' possessed high propensity for yield alternation in contrast with less-prone 'Lima Verde', 'Natal CNPMF-112', 'Pera', 'Sukkari' and 'Valencia Montemorelos'. 'Lima' and 'Rubi', in turn, had an intermediate degree of susceptibility to yield alternation. Nutrition, hormones, and abiotic stresses ranging from soil fertility and physical restrictions to drought susceptibility might also have played a role in yield fluctuation (Monselise and Goldschmidt, 1982; Abobatta, 2019; Carvalho *et al*., 2019 a, 2020).

Fruit quality is expressed by several parameters including the amount of juice, TSS content, acidity level, and the amount of vitamin C (França *et al*., 2016; Lado *et al*., 2018; Tirado-Corbalá *et al*., 2020). Moreover, the TSS content is the basis for the payment of a premium price differential for high-quality fruit (Zhang and Ritenour, 2016). The ratio, or maturity index, expresses fruit ripeness and is also an indicator of flavor (Lado *et al*., 2018; Ribeiro *et al*., 2020). These fruit quality traits may be influenced by the scion variety, management practices, maturity level, climate, and rootstocks (Al-Mohuei and Choumane, 2014; Carvalho *et al*., 2020; Ribeiro *et al*., 2020; Tirado-Corbalá *et al*., 2020). Here, we showed that FW, FD, and juice yield did not differ among the sweet orange varieties. However, 'Lima' trees produced smaller fruit than the other varieties. 'Rubi' produced the thickest and 'Lima Verde' the thinnest fruit rinds. 'Lima Verde' and 'Pera' produced the least sweet fruit, while 'Natal CNPMF-112' and 'Valencia Montemorelos' were the most acidic. The high fruit acidity of 'Valencia' grafted on 'Santa Cruz Rangpur' lime was also demonstrated by Rodrigues *et al*. (2019).

There is evidence that rainfed orchards generally produce sweeter fruit, as lower juice yields related to water deficits favor sugar concentration (Lado *et al*., 2018). This is in line with higher fruit sugar contents

of 'Valencia' trees subjected to deficit irrigation treatments in Italy (Mossad *et al.*, 2020). The ratio, or maturity index, was highest for 'Lima Verde' and 'Sukkari', which can be related to their lower acidity levels. 'Kona' and 'Lima' fruit had the highest contents of vitamin C as opposed to the lowest values for 'Lima Verde' and 'Pera'. Generally, the juice yields, TSS, and ratio values obtained here were within the minimal requirements for Brazilian fresh orange markets.

Multivariate analyses showed that fruit quality traits were mostly associated with PC1 (RT, TTA, TSS and ratio) while FH and Vit. C were strongly related to PC2. Ratio was negatively related to RT and especially to TTA. TSS, in turn, was associated with the majority of fruit quality traits, being negatively related to FH (smaller fruit, higher TSS) and positively with RT, TTA and Vit. C. However, TSS was not related to ratio. PC2, which accounted for 25% of variability, was negatively related with FH and Vit. C contents, suggesting that smaller fruit concentrate more Vit. C.

Collectively, our results obtained in northeastern Brazil highlight 'Kona', Valencia Montemorelos' and 'Rubi' as superior sweet orange varieties for diversification of tropical rainfed orchards. 'Kona' excelled in productive performance combined with a voluminous canopy, precocity, sweet fruit with moderate acidity, and high levels of vitamin C despite the propensity for alternate yields and low ratio. 'Valencia Montemorelos' and 'Rubi' had high yield performances coupled with intermediate canopies, sweet fruit, moderate acidity ('Rubi') and vitamin C levels, low proneness for yield fluctuation ('Valencia Montemorelos'), and high precocity ('Rubi'), despite a low ratio.

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Application of essential oils and optimizing storage conditions for control of postharvest diseases in apple

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Abstract: Postharvest loss in fruit and vegetables accounts for more than one third of the world production. On the other hand, using chemicals has raised food health concerns, and grown the demand for eco-friendly materials. Given the promising results of essential oil use to control and prevent postharvest decay, we conducted this research. In the present study, a two-step statistical method was used to determine and optimize application of essential oils along with parameters of storage conditions. Significant level of essential oils and storage conditions (temperature, ventilation, and relative humidity) were screened by using PBD method and the best concentration were determined by central composite design of response surface method. The results showed that 1000 and 1500 microgram/l (µg/l) of basil essential oils, and 1500 µg/l of peppermint essential oils reduced the lesion diameter in apple fruits infected with *Penicillium expansum*. All storage conditions had significant effect on postharvest decay. Based on the CCD of RSM method, the best concentration of essential oils and the optimal level of the storage condition were determined. Furthermore, basil and peppermint treatments reduced rate of the ethylene production during 56 days after treatment. The results of this study revealed and confirmed that basil essential oils as a postharvest treatment under optimized storage conditions can be utilized as a low-cost substrate for controlling postharvest decay in apple.

1. Introduction

It is estimated that more than one third of harvested fruit and vegetables are lost due to pathogen infections in the field or after harvest, resulting in a serious economic loss (Romanazzi *et al.*, 2016). The average loss is about 29% in North America and Europe, and more than 35% in Asia (Romanazzi *et al*., 2016). Given severity of economic loss worldwide, many researchers have employed various strategies to decrease loss and control the conditions after harvest. The first generation of attempts was to use chemical materials to control diseases, however, health and safety

concerns turned attentions on natural products (Rajestary *et al.*, 2021). Nowadays, essential oils, used as additives, color intensifiers, and antioxidants, are extracted from plant sources have gained attentions because of advantages over synthetic materials such as a good quality, biodegradable, economical characteristics, eco-friendly easy large-scale production, and especially a lack of safety concerns (Sivakumar and Bautista-Baños, 2014). A study suggested that fungal strains could develop a resistant when synthetic fungicide are continuously used, while the different components of essential oils make the process of the resistant more slowly during the application (Sivakumar and Bautista-Baños, 2014).

Recently, many studies have explored the use of essential oils to control postharvest losses. For instance and to mention some of these recent studies, Antonioli *et al.* (2020) reported the use of nano-capsules containing plant essential oil to control bitter rot of apples and showed that the fruits treated with nano-capsules have a smaller lesion. Another study showed that mint and thyme essential oils reduced Rhizopus rot on strawberry and peach fruits. Kontaxakis *et al.* (2020) evaluated several essential oils to assess their effect in preserving fruits during postharvest phase. Many other studies have showed that essential oils are promising to control postharvest decay in fruit and vegetables, however, the question of this study is to (1) find the best concentration in which essential oil could act, and (2) to determine the best environmental conditions of the storage place in which essential oils applied. We think that these points are worth to be explored. Therefore, the objective of the present study was to test efficiency of plant essential oils and optimize storage conditions in apple postharvest period. In this study, two steps statistical method was employed. Moreover, ethylene concentration was evaluated on the optimized conditions.

2. Materials and Methods

Materials and the experiment design

This study was conducted to enhance apple storage through application of plant essential oils as well as optimizing storage conditions. The culture was in submerged ferment and three parameters were assessed to reach an optimal production process. Apples (*Malus domestica*, cv. Red Delicious), harvested from orchards in northern-west Iran, Ardabil, Iran, were divided in groups of 27 fruits/treatment.

The first parameter was basil essential oil (Eugenol 10.04%, Linalool 68.52%, a-trans-Bergamotene 6.94%, and b-Cadinol 3.20%). The second parameter was peppermint essential oils (1,8-Cineole 5.89%, Menthofuran 4.59%, Menthol 38.29%, and Menthyl acetate 29.39%). Basil and peppermint essential oil were used in three level (500, 1000, and 1500 microgram/l) as a treatment. The third parameter was storage conditions including temperature (-2, -1, and 0°C), ventilation (3, 4.5, and 6 LPM), and relative humidity (85, 90, and 95%). A 15 percent emulsion (15% essential oil, 83% water and 2% Tween) was prepared from basil and peppermint essential oils. The resultant emulsion was shaken for 45 s before spraying.

A two-step statistical strategy (Embaby *et al.*, 2018) was anticipated to optimize and determine relationship among postharvest control of apple fungal diseases with basil essential oils, peppermint essential oils, and storage conditions (temperature, ventilation, and relative humidity). Plackett-Burman Design (PBD) (Plackett and Burman, 1946) was employed to monitor the linear effect of three parameters using 27 experimental trials designed in 28-run design. PBD is an efficient way to screen the most important variables when there are multiple variables. A three-level optimization (+1, 0, -1) arranged in central composite design (CCD) of response surface methodology (RSM) was used to specify the optimal levels of each key parameters deduced from PBD approach.

Treatments

We used *Penicillium expansum* to create the disease in apples. The fungal spores were inoculated on Potato Dextrose Agar (PDA) slants and incubated for 7 days at 30°C. *Penicillium expansum* was maintained on PDA at 4°C for further use. To prepare a suspension of the fungal spore, spore-inoculated PDA was washed using sterile water and added into seed culture medium. Seed medium was prepared by using modified minimal medium (g/L: yeast extract, $NaNO_3$ 2, KH_2PO_4 1, $MgSO_4.7H_2O$ 1, KCl 0.5, and $FeSO_4.7H_2O$ 0.01).

Apple fruits were disinfected in 1% sodium hypochloride dried at room temperature. With sterile and small plastic tip, some wound made on the fruits. Pathogen and essential oil suspensions were dropped into each wound. All samples, control and the inoculated, were stored in chambers with optimized storage conditions. The diameter of the rot around each wound was measured after 18 and 26 days.

Ethylene measurement

Ethylene analysis was performed using HPLC analysis. The analysis was performed using a Waters 244, column: Shoex C18, 4 µm, 250 mm×4.0 mm, temperature: 28°C, injector volume: 20 µl. Fluorescence detector (Waters 470) set at 331 nm excitation and 500 nm emission wavelength. The mobile phase consisted of methanol/acetonitrile/water (3:3:4 v:v:v), the pH value of the mixture was 2.5, and the flow rate was the 0.15 mLPM. The concentrations of ethylene of samples were calculated by the equation: Cs ¼ Cp As=Ap; where Cp and Cs are the concentration of ethylene solution and sample solution, respectively, Ap and As are the area of peaks of ethylene solution and sample solution respectively.

Statistical analysis and calculations

Each experiment was performed in triplicate. To optimize the production of monascus pigments, the software SPSS Statistics 19.0 was used to generate PBD matrices and carry out multiple linear and non-linear regression analyses. All tables and diagrams were prepared in Microsoft Excel 2010. The effects of the treatments on pigment composition, ethylene, and biomass were analyzed by one-way ANOVA, and tests of significant differences were determined by using Student's t-test at $p<0.05$.

3. Results

Application of essential oils

To study essential oils (basil and peppermint) and storage conditions (temperature, ventilation, and relative humidity), twenty-four groups were analyzed based on PBD design shown in Table 1. Lesion diameter (mm) ranged from 50.4 to 75.8. Based on confi-

Table 1 - Effect of three independent sources including basil essential oil, peppermint essential oils, and storage conditions in postharvest controlling of apple decay caused by *Penicillium expansum* based on PBD. The parameters were Basil and peppermint essential oil in three level (500, 1000, and 1500 microgram/l), temperature (-2, -1, and 0°C), ventilation (3, 4.5, and 6 LPM), and relative humidity (85, 90, and 95%)

Independent variable					Lesion diameter (mm)
Basil essential oils	Peppermint essential oils (µgL)	Temperature (°C)	Ventilation (LPM)	Relative humidity (%)	
1500	1500	0	6	95	69.2
1500	1000	0	6	90	72.3
1500	500	0	6	85	67.2
1500	1500	0	4.5	95	71.2
1500	1000	0	4.5	90	75.8
1500	500	0	3	85	63.2
1000	1500	-1	6	95	62.2
1000	1000	-1	6	90	65.3
1000	500	-1	6	85	63.7
1000	1500	-1	4.5	95	67.7
1000	1000	-1	4.5	90	72.3
1000	500	-1	4.5	85	56.1
1000	1500	-1	3	95	64.9
1000	1000	-1	3	90	70.1
1000	500	-1	3	85	59.7
500	1500	-2	6	95	56.5
500	1000	-2	6	90	59.6
500	500	-2	6	85	58.3
500	1500	-2	4.5	95	62.6
500	1000	-2	4.5	90	66.4
500	500	-2	4.5	85	50.4
500	1500	-2	3	95	59.2
500	1000	-2	3	90	64.3
500	500	-2	3	85	54.1

dence level (>95%), the results showed that basil essential oils have a more significant effect on preventing apple postharvest control of the fungal disease. Of storage conditions, results showed that temperature, ventilation, and relative humidity have a significant effect on controlling the fungal diseases in apple postharvest period. All storage conditions (temperature, ventilation, and relative humidity) were found to have a significant effect (p-value < 0.05). The results of multiple linear regression indicated that basil essential oils as the best treatment under the condition set for storage (intercept effect) have a significant influence on postharvest controlling (Table 2).

When basil essential oils used as treatment, it decreased lesion diameter about 60% and 200%, respectively, compared to those observed in the fruits treated with peppermint essential oils (Fig. 1). A significant percentage of increase 15% and 35%, respectively, was obtained, when basil essential oils used in association with -2°C of temperature, 4.5 LPM of ventilation speed, and 90% of humidity compared to other storage conditions (Fig. 1). With regard to peppermint essential oils, the highest level of postharvest control was achieved in -2°C of temperature, 6 LPM of ventilation speed, and 85% of humidity.

Pertaining to storage conditions, temperature set to -2°C increased postharvest control over the disease through declining lesion diameter, especially when basil essential oils was used as treatment, by

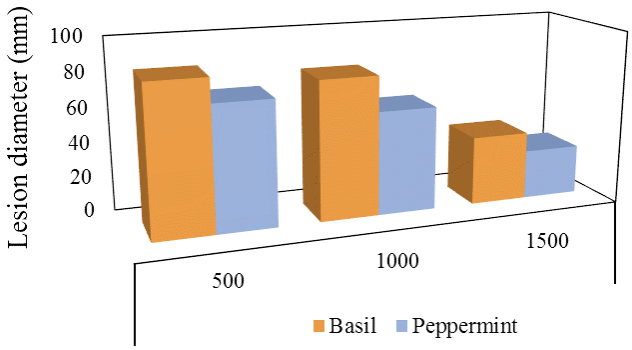

Fig. 1 - Application of basil and peppermint essential oils on postharvest controlling of apple decay caused by *Penicillium expansum* between treated fruits with basil and peppermint essential oils. Values represented in the figure are expressed in percentage (%).

an average decrease of 55% compared to the samples stored at -1 and 0°C (Fig. 2). Based on data of PBD and RSM, sample fruits with application of basil essential oils maintained at -2°C and ventilated with 4.5 LPM speed resulted in significant decrease of lesion diameter (Fig. 3, 4).

Effect of essential oil application on apple ethylene concentration

To determine the effect of optimized storage conditions and essential oil treatment (basil and pepper-

Table 2 - Multiple linear regression of PBD data for postharvest controlling of apple decay caused by *Penicillium expansum* using three independent sources including basil essential oil, peppermint essential oils, and storage conditions

Model	B-coefficient			t-value			p-value			Confidence (%)		
	A	B	C	A	B	C	A	B	C	A	B	C
Intercept	53.642	48.155	46.49	8.642	9.392	9.962	1.32E-04*	5.4E-06*	4.8-01*	99.99*	99.99*	99.9*
X_1	30.458	24.971	23.306	4.542	5.292	5.862	0.175001	0.80123	0.16471	-	-	-
X_2	-10.254	-11.741	-7.406	-1.254	-0.504	0.066	0.163501	0.17999	0.29019	-	-	-
X_3	-12.429	-8.916	-6.581	-1.429	-0.679	-0.109	0.192001	0.14872	0.41567	-	-	-
X_4	-5.398	-6.885	-8.55	4.398	5.148	5.718	0.180501	0.29746	0.54115	-	-	-
X_5	34.783	29.296	27.631	1.217	1.967	2.537	0.0194*	0.0362*	0.0402*	98.06*	96.38*	95.98*
X_6	20.854	15.367	13.702	2.146	2.896	3.466	0.0249*	0.0102*	0.0313*	97.51*	98.98*	96.87*
X_7	15.16	9.673	8.008	2.84	3.59	4.16	0.0342*	0.0199*	0.0146*	96.58*	98.01*	98.54*
X_8	18.243	12.756	11.091	6.757	7.507	8.077	0.0322*	0.0168*	0.0497*	96.78*	98.32*	95.03*

A= Yellow pigments.
B= Orange pigments.
C= Red pigments.
* A = Significant P-value<0.05. R2:066. Adjusted R2: 061. P-value for the model = 0.0038; B = Significant P-value <0.1. R2:071. Adjusted R2: 074. P-value for the model = 0.002; C= Significant P-value <0.1. R2:075. Adjusted R2: 064. P-value for the model = 0.0029.

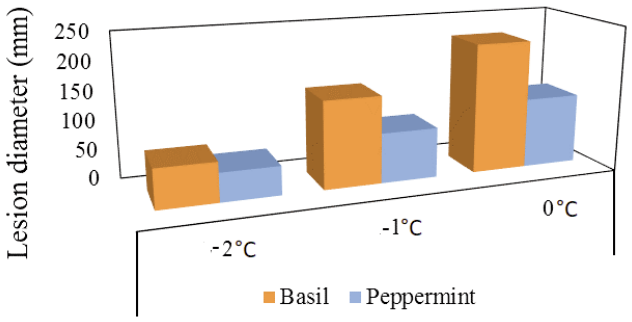

Fig. 2 - Effect of temperature on postharvest controlling of apple decay caused by *Penicillium expansum* between treated fruits with basil and peppermint essential oils. Values represented in the figure are expressed in percentage (%).

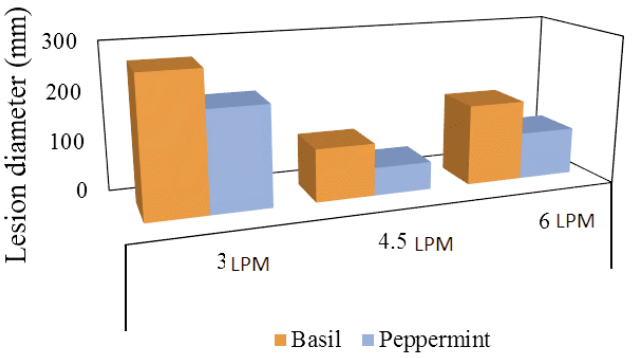

Fig. 3 - Effect of ventilation on postharvest controlling of apple decay caused by *Penicillium expansum* between treated fruits with basil and peppermint essential oils. Values represented in the figure are expressed in percentage (%).

mint), we analyzed the rate of the ethylene production for 56 days. Every 7 days, the ethylene rate was measured for control, basil, and peppermint treatment. The production rate of ethylene was significantly increased from 28 days and reached its highest level at the day 56 (p-value<0.05). The fruits treated with peppermint showed significant increase in the ethylene production from the 28th day. At the days 35 and 46, the level of ethylene in treated fruits was significantly low compared to control fruits (p-value<0.05). Basil treatment reduced significantly rate of the ethylene from 35th day to 56th day (p-value<0.05). Fruits under the optimized conditions plus basil essential oil showed the lowest amount of the ethylene (Fig. 5).

Fig. 4 - Effect of relative humidity on postharvest controlling of apple decay caused by *Penicillium expansum* between treated fruits with basil and peppermint essential oils. Values represented in the figure are expressed in percentage (%).

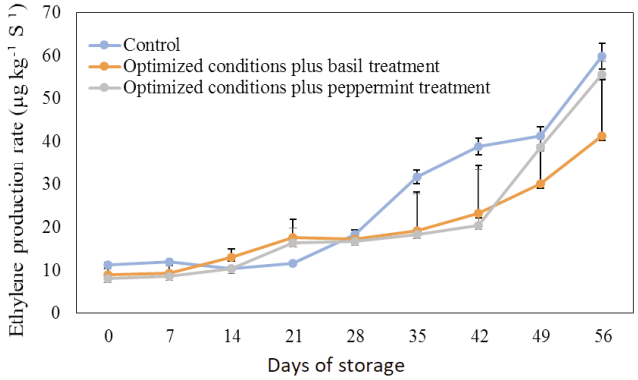

Fig. 5 - Ethylene production rate of apple stored at control and optimized conditions along with the treatment with basil and peppermint essential oils. The control condition was -2°C, 4.5 LPM, and 85% relative humidity. The best levels, determined for basil and peppermint in the previous section of the experiment, were used to asses ethylene production rate during postharvest storage of apple infected with by *Penicillium expansum*.

4. Discussion and Conclusions

In this study, basil essential oil in three levels as well peppermint essential oil in three levels were evaluated to find the best treatment for postharvest control of apple decay caused by *Penicillium expansum* using statistical methods, then the best treatments were investigated under various storage conditions (temperature, ventilation and relative humidity, each in three levels). The aim was to optimize and

enhance postharvest control of apple using cost-effective, safe, and eco-friendly plant materials. There is a few report that showed effect of basil and peppermint essential oil for controlling postharvest decay (Domínguez-Espinosa and Webb, 2003; Lopez-Reyes *et al.*, 2010). In the context of studying the effect of basil essential oil as controller of postharvest decay in apple, a study by Mohammadi *et al.* (2021) showed that basil essential oil can be utilized as a low-cost material for preventing button mushroom (*Agaricus bisporus* L.) during postharvest period. Lopez-Reyes *et al.* (2013) reported the efficiency of use of peppermint essential oil in controlling rot in stone fruits. Recently, the optimization and a combination of several factors to control postharvest decay, has been the object of several study. For instance, essential oils of Citrus species as mandarine (*Citrus reticulate,* Blanco), lemon (*Citrus aurantifolia* (Christm.) Swingle), and orange (*Citrus sinensis* L) have been reported as an effective inhibitor against some bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*) and yeast (*Candida albican*) (Fisher and Phillipps, 2008).

Herein, controlling apple decay after harvest was subjected to two-step statistical method (PBD and CCD of RSM) in order to reduce capital cost of postharvest control, maximizing quality of apple and minimize safety concerns. It has been indicated that statistical approaches are a reliable method towards enhancing and optimization of the yield in various biological processes (Jirasatid *et al.*, 2013; Embaby *et al.*, 2018). In this regard, the optimal level of each essential oils from basil and peppermint influencing postharvest control of decay in apple was determined to reduce decay effectively.

With regard to essential oils, the highest level of controlling and the lowest decay observed in samples upon using basil essential oils as the treatment. Basil essential oils enhanced inhabitation of the decay in apple more than the peppermint essential oil (Fig. 1). This might be attributed to the nature of basil essential oil component in degrading fungus-caused diseases (Grande-Tovar *et al.*, 2018) compared to peppermint essential oil. Action of the mono- and sesquiterpenes and mono- and sesquiterpene hydrocarbons in basil essential oils could inhibit fungal diseases (Caccioni and Guizzardi, 1994). Reports regarding essential oils as pesticides and antimicrobial agent have shown that the inhibitory effect of essential oils to prevent fungal diseases during postharvest period strongly associated with the monoterpenic phenols, notably thymol, carvacrol and eugenol in the oils (Antunes *et al.*, 2010). Hence, the rich compounds of basil essential oil would be enhance fungistatic activity and as a result the effective control over the fungal disease (Prakash *et al.*, 2015; Namiota and Bonikowski, 2021). It seems that essential oil content of peppermint has a lower level of fungicidal activity compared to basil, although it reduces the growth of the disease when relatively high amount of essential oil used as treatment.

Pertaining to peppermint essential oils, this study showed that peppermint essential oil could reduce decay caused by the fungal disease in apple samples (Fig. 2). Previous studies have revealed the effect of peppermint essential oils as postharvest treatment in reducing decay and maintaining quality of fruits and vegetables (Sellamuthu *et al.*, 2013; Qu *et al.*, 2020). Since menthol is the main constituent of peppermint essential oil, this compound could be mainly responsible for the effects. Furthermore, several studies suggest that the effect of peppermint might be indirect and through raising the activity level of superoxide dismutase (Jin *et al.*, 2009).

The results of the study showed that the best level of basil essential oil as treatment is 1000 and 1500 µg/l, while the best level for peppermint was 1500 (Fig. 1). The difference between 1000 and 1500 µg/l of basil was not significant. Lopez-Reyes *et al*. (2010) reported that 10% emulsion of essential oils could be more effective than 1% of the emulsion in controlling decay. However, scientific evidences are limit in the field of concentration effects on the inhibitory ability of essential oils during postharvest phase. Here, our findings suggest that 1000 and 1500 µg/l essential oil decrease the lesion diameter in apple compared to 500 µg/l, while the effect of basil essential oil with 1000 and 1500 µg/l is similar. In the case of peppermint, there is significant difference between 500, 1000, and 1500 µg/l. This might result from the nature of essential oil constitutes. More study is needed to support the power of constitutes in controlling postharvest diseases.

Temperature of the storage place is one of the main factor for optimal maintain of fruit and vegetables during postharvest phase (Agboyibor *et al.*, 2018; Patrovsky *et al.*, 2019; Silbir and Goksungur, 2019; Liu *et al.*, 2020). Several study have already showed that different isolates of fungal diseases grow best under an optimum temperature range of 30°C to 37°C (Mannaa and Kim, 2017; Liu *et al.*, 2018). These findings support the results of this study

where higher temperature showed a higher lesion diameter. Alternatively, an optimum relative humidity of the storage place is the point of a discrepancy. A study on some strains of fungal-cause diseases revealed that relative humidity of 85% promotes the fungus growth and thereby a higher decay (Nabi *et al.*, 2017). Another study reported that the greatest diameter of lesions achieved at relative humidity of 95 to 98% 5 (Arah *et al.*, 2015). In our study, sole effect of relative humidity was not significant between groups. However, it seems that high temperature trigger relative humidity effect on the growth the fungal disease. According to our results, in the absent of essential oil, fruits stored at high temperature and relative humidity showed higher lesion diameter. Regarding ventilation, Partridge-Hinckley *et al.* (2009) reported the highest occurrence of the decay in fruits stored in the poor ventilation conditions. Moreover, Carmona-Hernandez *et al.* (2019) showed that aeration in terms of agitation at 150 rpm promotes the growth and distribution of fungal diseases.

With regard to ethylene production rate (µg kg^{-1} S^{-1}) in the control and optimized storage conditions, results indicated that the level of ethylene in the control increases and reaches its highest level at 56 days after treatment. The same pattern meets for optimized conditions (Fig. 5). On the other hand, the finding suggests that basil and peppermint treatment significantly decrease the ethylene production rate at 32 and 42 days. After the day 42, basil treatment continues its inhibitory effects compared to peppermint treatment, although the ethylene production rate is significantly high and damage apple storage. Our results is consistent with those findings that have shown application of exogenous material such as melatonin, polyamines, and calcium limits rate of ethylene production (Wang *et al.*, 1993; Bulens *et al.*, 2012; Onik *et al.*, 2021). Jhalegar *et al.* (2015) showed that essential oil treatment could lower ethylene rate in fruits affected with fungal diseases during postharvest storage. Ethylene production is the main factor in deterring postharvest life of any fruits, especially apple. Data from various studies have shown that there is almost linear correlation between the ethylene production and fruit damage during storage phase (Cristescu *et al.*, 2002). Meanwhile, fruits affected with postharvest diseases show the higher level of the ethylene, thereby higher decay and damage (Jhalegar *et al.*, 2012). There are limited scientific reports on application of essential oils to control ethylene production during the postharvest period. Two studies by Moline and Locke (1993) and Sharafi *et al.* (2011) showed the decrease in the ethylene production in fruits treated with plan essential oils. Here, we represented that basil and peppermint decrease the ethylene rate in apple, as well as we optimized storage conditions with the treatment.

This study used the comprehensive combine of treatments along with well-designed experiment to show an efficiency of plan essential oils to control apple decay during postharvest phase, and mainly to find the best treatment and storage conditions. It is well understood that postharvest conditions depend on several factors and we showed that treatments need to be aligned with storage condition. Thereby, we showed that 1000 µg/l of basil essential oil is significantly effective under -1 and 0°C, 4.5 LPM, and 90% of relative humidity. While 1500 µg/l of peppermint need to be under -1ºC, 6 LPM, and 85% humidity to be effective. Furthermore, basil essential oil decreased the ethylene production rate in longer days after treatment. The findings suggest the basil essential oils as a cost-effective and eco-friendly substance that could be more applicable in a large scale.

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Exogenous application of biostimulators alleviates water deficient stress on *Azadirachta indica* plants

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Key words: Brassinolide, chitosan, drought stress, neem.

Abstract: Pot experiment was conducted to evaluate the effect of chitosan or brassinolide applications on morphology and physiology parameters of *Azadirachta indica* grown under water deficient stress. The plants received different irrigation intervals, and were sprayed monthly with either chitosan or brassinolide each at concentrations of 50, 100 and 200 ppm, while the control plants were sprayed only with tap water. The results showed that water stress reduced all growth parameters, chemical constituents of pigments content, total carbohydrates, N, P, and K%, total indoles, while proline and total phenols content were increased. Instead, the plants sprayed with the higher concentrations of chitosan or brassinolide resulted in significant increase in growth parameters, pigments content, total carbohydrates, proline content, N, P and K%, total indoles while reduced total phenols content. Based on the obtained results it can be concluded that, foliar application of chitosan or brassinolide at 200 ppm can alleviate the adverse effects of water deficient stress on the growth and physiology parameters of *Azadirachta indica*.

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1. Introduction

Azadirachta indica A. Juss is a tropical evergreen tree that belongs to the family of Meliaceae, commonly known as neem, nimtree or Indian lilac. It is native to India, its fruits and seeds are the source of neem oil (Koul and Wahab, 2004). In addition to use of neem for landscape activates as an ornamental tree, all parts of the tree have been utilized medicinally and now being used in pharmaceutical and cosmetics industries. Neem fruits, seeds, oil, leaves, bark and roots used as antiseptics, antimicrobials, anti-inflammatory, anti-cancerous and anti-diabeticv (Islas *et al*., 2020).

Water deficiency is the major environmental factors, as a biotic stresses limiting agricultural production in most countries, especially in the arid and semi-arid regions, affecting the quality, growth and production of pants. Water stress induces various physiological, biochemical changes in ornamental plants such as a reduction in the growth parameters (Abd-Elmoneim *et al.*, 2018; Yousaf *et al.*, 2018; El-Shanhorey and Sorour,

2019; Shaltout *et al.*, 2022), reductions in total chlorophyll and carbohydrate contents (Sarker and Oba, 2018), reductions in nutrient accumulation (Singh and Singh, 2020), as well as increasing in prolin, phenols content (El-Gamal and Khamis, 2021).

Recently, study for biological methods to avert utilization of chemical products and alleviating the harmful effect of water deficient in agriculture has led to utilize of bio-stimulators. Among the various kinds of bio-stimulators are chitosan and brassinolide.

Chitosan (CHT) is a deacetylated derivative of chitin. It is a natural polymer and biodegraded by biological agents and it is environment-friendly used in agriculture (Shafiei-Masouleh, 2019). Under stressed conditions, CHT has the efficiency to mitigate the harmful effects of drought by promoting chlorophyll, carbohydrates, proline content and the capacity of antioxidant activities (Pirbalouti *et al.*, 2017; Vosoughi, *et al.*, 2018; Elansary *et al.*, 2020). The favorable influence of CHT induces photosynthetic rate, stomatal closure through ABA synthesis; stimulates antioxidant enzymes via nitric oxide and hydrogen peroxide signaling pathways, and promotes production of organic acids, sugars, amino acids and other metabolites that are required for the osmotic adjustment, stress signaling, and energy metabolism under stresses (Hidangmayum *et al*., 2019).

Brassinolide is the first brassinosteroids (BRs) isolated in plants. BRs are type of plant hormone which have a vital effect on plant growth and development. Earlier workers have elucidated that BRs can reduce the harmful effects of water deficient stress via boosting the activity of antioxidant enzymes and non-enzymatic antioxidant contents to remove the damage of reactive oxygen species (ROS) (Hosseinpour *et al.*, 2020; Cai *et al*., 2021; Omidian *et al.*, 2022) or by increasing endogenous abscisic acid (ABA) to induce stomatal closure (Bhandari and Nailwal, 2020). Additionlly, BRs can enhance the energy metabolism balance between the chloroplast and mitochondria, increase the initial activity of Rubisco, and boost the use of light energy absorbed by plants, thus enhancing photosynthetic efficiency under water deficient stress (Cai *et al*., 2021).

Although the beneficial roles of bio-stimulators on ornamental plants and their helpful effect on boosting growth and flowering parameter, there are no enough available data about their action on alleviating the harmful effect of drought on ornamental trees. Therefore, this research is aimed to evaluate the response of *Azadirachta indica* grown under water deficient stress to foliar application of chitosan or brassinolide.

2. Materials and Methods

The present study was undertaken in the experimental nursery (30-32°C temperature, 14 h light conditions and 39-61% relative humidity) of the Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza, Egypt during the two successive seasons of 2019 and 2020. The latitude, longitude and altitude of the experimental site were 30° 03’ N, 31° 13’ E and 19 m ASL.

Plant material

On 1st of March in 2019 and 2020 seasons, uniform seedling of *Azadirachta indica* plants were obtained from a commercial nursery with an average plant height of 25-30 cm, 2-3 leaves/plant and transplanted individually in 25 cm diameter plastic pots filled with clay+ sand (2:1: v/v), some physical and chemical properties of soil mixture used in the study were determined according to (Jackson, 1973), the results are presented in Table 1.

Table 1 - Some physical and chemical characteristics of the soil mixture used for growing *Azadirachta indica* (mean of two seasons)

Soil characteristics	Data
Physicical characteristics	
Soil Texture	Clay
Clay (%)	39.80
Coarse sand (%)	3.59
Fine sand (%)	21.46
Silt (%)	35.15
Chemical characteristics	
Soluble cations (meq/l)	
Ca^{++}	7.09
Mg^{++}	2.86
K^{+}	0.30
Na^{+}	6.05
Soluble anions (meq/l)	
Cl^{-}	3.53
SO_4^{-}	2.42
N (ppm)	26.57
P (ppm)	22.00
Organic matter (%)	1.61
Electrical conductivity (dS/m)	1.64
pH	7.46

pH= soil acidity.

Experimental procedures

On 15th of March in first and second season, respectively the plants were irrigated every 3, 6, 9 and 12 days for imposing water stress. Amount of water used each time was equal to the field capacity (pot capacity) which was determined empirically as follows: three pots (25 cm) filled with about 2 kg of the soil mixture were watered thoroughly to saturation and weighed. Pots were covered with aluminum foil to prevent evaporation before they were left in a cool shaded place to drain freely for 4 hours. They were weighed again to calculate weight of water held by the soil mixture. Mean of the three pots representing the field capacity was found to be 1350 g, equivalent to 1350 cm^3 of water/pot (1.35 L /pot) (Abd-Elmoneim *et al*., 2018). Thus, 1.35 L of water were given to each pot in due time according to the irrigation schedule. This means that at the end of the experiment (after 7 months), plants irrigated every3, 6, 9 and 12 days interval were given 94.5, 47.3, 31.5 and 23.6 liters of water, respectively.

Starting from 31st March till to 15th October (in both seasons), the plants received different irrigation intervals were sprayed every 4 weeks with either chitosan (CHT, 500 mg, composed of β-(1-4)-linked d-glucosamine and N-acetyl-d-glucosamine randomly distributed within the polymer) or brassinolide (BRs, 0.01% steroid compounds) each at concentrations of 50, 100 and 200 ppm, while the control plants sprayed only with tap water. Both CHT and BRs were obtained from Tecknogreen company, Egypt. Tween 20 as wetting agent was added to bio-solution at concentration of 1 mL L^{-1} and the plants foliage were sprayed using automatic atomizer until run off point (80 ml of bio-solution/plant).

All the plants were fertilized every month with kristalon™ (NPK 20:20:20) at a rate of 3 g/pot, manual picking of weeds, disease and pest control has also been carried out.

Layout of experimental

The layout of the experiment was randomized complete blocks design with 28 treatments [4 irrigation intervals x 7 plant bio-stimulators (including the control)] each treatment consisting of 9 pots arranged in 3 replicates, each replicate containing 84 pots (3 pots from each treatment).

The data recorded

Vegetative growth parameters. At the end of the experiment, On 30th October in both seasons (after 7 months), vegetative growth parameters were recorded, two samples of plants were randomly taken from each replicate to determine the parameters including plant height (cm, measured with a ruler from soil surface to its highest point), fresh and dry weights of shoots, roots/plant, stem diameter (mm, at 5 cm above soil surface), root length (cm), number of leaves/plant and leaf area (cm^2, measured using CI-202 Portable Laser Leaf Area Meter, CID Bio-Science, Inc., USA). Dry weight of shoots and roots /plant was evaluated by drying plant in an electric oven at 70°C until constant weight.

Chemical analysis

Chlorophyll and carotenoid contents. Chlorophyll pigments including Chl a, Chl b and carotenoid contents (mg g^{-1} FW) in leaves were determined according to Lichtenthaler and Buschmann (2005): leaves extracted in 5 ml of 95% aqueous acetone was centrifuged at 4000 g for about 10 min. The aqueous acetone supernatant was then taken for spectrophotometric measurement. A blank of acetone was taken at wavelengths of 663, 645 and 452.5 nm respectively, and data were then calculated using the following equations:

Chlorophyll a.(mg g^{-1}) = 0.0127 A663 − 0.00269A645
Chlorophyll b.(mg g^{-1})= 0.0029A663 − 0.00468A645
Carotenoids (mg g^{-1}) = 4.2 E 452.5 − 0.0264

Total carbohydrates. Total carbohydrates content in leaves (percentage of dry matter) was determined in dried samples according to Dubois *et al*. (1956). A known weight (0.1 g) of the dried samples was completely hydrolyzed with 10 ml sulphuric acid (67%) in a test tube on a boiling water bath for one hour. The solution was decolorized and the filtrate was diluted to 100 ml with distilled water. A known volume (1 ml) of the extract was taken in a test tube, to which 1 ml phenol solution (5%) was added, followed by 5 ml of concentrated sulphuric acid. The optical density of the resulting color was measured at 490 µm, using a spectrophotometer, against a blank reagent. The standard curve of glucose was used to calculate the total carbohydrates concentration in the extract.

Proline content. Proline content in fresh leaves (µ moles/g fresh matter of leaves) was determined using the method of Bates *et al*. (1973). Leaves were homogenized in 3% aqueous Sulphosalicylic acid, then centrifuged 5,000 *g* for 20 min at 4°C. The amount of 2 mL of this homogeny solution react acid-ninhdrin and 2 mL of glacial acetic acid in a tube for 1 hour at 100°C and the reaction is torn up in an ice

bath and then extracted with 4 mL of toluene. It was kept at room temperature to stabilize. Proline content was measured by spectrophotometer (UV-160A, Shimadzu, Tokyo, Japan) at 520 nm.

N, P and K content in shoot. Dried shoot samples were digested to extract nutrients and the extract was analyzed to determine concentrations of N, P and K (as percentage of D.W) which were determined according to Estefan *et al*. (2013). Nitrogen concentration was determined by using the micro-Kjeldahl method. Phosphorus was determined calorimetrically by using the chlorostannous molybdophosphoric blue colour method in sulphuric acid. Potassium was determined by using the flame photometer apparatus (CORNING M 410, Germany).

Total indole and phenol contents. Total indole and phenol contents were determined in fresh shoots (3 g) of shoots, which were crushed and extracted with 80% ethanol at 0°C for 72 hours, the ethanol being changed every 24 hours, as described by Selim *et al*. (1978).

Statistical analysis

The means of all obtained results were subjected to two-ways analysis of variance (ANOVA) in randomized complete blocks design. Combined analysis of the two growing seasons was carried out. Means of data were compared by using Duncan's multiple range tests at 5% level Snedecor and Cochran (1989).

3. Results and Discussion

Growth parameters

Data recorded on *Azadirachta indica* plants, all growth parameters (including, plant height, fresh and dry weights of shoots and roots, stem diameter, root length, number of leaves and leaf area) were significantly affected by irrigation intervals, bio-stimulators treatment and interaction effects (Table 2). The data in Table 3 and 4 showed that within each level of the two bio-stimulators (CHT or BRs), all growth parameters were decreased significantly ($p<0.05$) with prolonged irrigation intervals daily from 3 to 6, 9 or 12 days. This reduction was steadily in most cases compared to the short intervals (3 days). The reduction of growth parameters in response to water deficient may be due to adverse effect of drought around the roots, lower soil moisture availability due to water stress, lead to reduce water and nutrients absorption by roots which in turn leading to reduction in vegetative biomass (Rouphael *et al*., 2012). This is greatly in harmony with numerous researches (Ashour and El-Attar, 2017; Abd-Elmoneim *et al*., 2018; Khatana *et al*., 2018; Toscano *et al*., 2018; El-Shanhorey and Sorour, 2019; Najihah *et al*., 2019; Tribulato *et al*., 2019; Al-Arjani *et al*., 2020; Singh and Singh, 2020; El-Gamal and Khamis, 2021; Papú *et al*., 2021; Sorour, 2021; Shaltout *et al*., 2022).

Data in same Tables also indicated that within

Table 2 - Mean square for the effect of irrigation intervals and bio-stimulators treatments and their interaction on vegetative growth parameters of *Azadirachta indica*

Traits	Source of variation				
	Treatment			Error	CV
	Irrigation intervals (A)	Bio-stimulators (B)	(A × B)		
Plant height (cm)	1281.31 ***	967.98 ***	34.50 *	19.48	4.69
Fresh weight of shoots (g/plant)	268.86 ***	731.60 ***	24.01 **	14.07	9.14
Dry weight of shoots (g/plant)	98.12 ***	279.41 **	4.87 *	3.38	9.53
Fresh weight of roots (g/plant)	225.15 ***	904.88 ***	15.76 ***	3.82	6.29
Dry weight of roots (g/plant)	47.79 ***	71.71 ***	1.83 *	3.12	13.22
Stem diameter (mm)	5.74 ***	8.85 ***	0.25 *	0.53	12.29
Root length (cm)	553.08 ***	662.22 ***	7.00 *	11.17	8.13
Number of leaves	105.61 ***	567.61 ***	7.17 *	4.32	9.65
Leaf area (cm^2)	33.01 ***	87.65 ***	0.54 *	2.83	13.57

*, **, *** significant at P≤0.05, P≤0.01, P≤0.001, respectively.

Table 3 - Plant height, fresh and dry weight of shoots and fresh and dry weight of roots of *Azadirachta indica* as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons)

Irrigation intervals	Bio-stimulators	Plant height (cm)	Fresh weight of shoots (g/plant)	Dry weight of shoots (g/plant)	Fresh weight of roots (g/plant)	Dry weight of roots (g/plant)
3 days	Control	79.63±1.94 j-l	34.18±1.97 jk	17.10±1.21 f-h	30.99±0.94 g-i	10.97±0.41 j-m
	CHT (1)	98.58±1.97 de	42.2±1.60 d-g	21.70±0.95 cd	32.97±0.14 d-h	16.11±1.47 a-c
	CHT (2)	101.17±2.6 b-e	44.53±1.11 c-f	23.11±0.6 bc	39.51±2.20 b	16.03±0.58 bc
	CHT (3)	107.58±1.21 b	47.64±2.22 b-e	25.09±0.99 ab	41.68±1.5 b	15.40±0.26 cd
	BRs (1)	96.67±2.1 ef	46.00±4.85 b-f	23.09±0.46 bc	34.71±0.84 c-f	13.83±0.28 c-j
	BRs (2)	99.50±3.13 c-e	50.84±0.85 b	26.42±1.68 a	41.05±1.57 b	15.47±1.63 cd
	BRs (3)	117.17±0.92 a	59.72±3.34 a	27.26±1.98 a	49.11±0.82 a	19.00±2.57 a
6 days	Control	75.17±2.53 k-m	38.21±3.41 g-j	13.90±1.4 ij	27.8±0.85 ij	10.16±0.97 k-m
	CHT (1)	95.08±1.72 e-g	41.58±1.97 e-h	19.25±0.43 d-f	32.68±0.62 e-h	14.32±0.92 c-h
	CHT (2)	99.83±1.451 c-e	44.8±2.30 b-f	22.81±2.09 bc	35.90±1.70 cd	14.87±1.19 c-f
	CHT (3)	104.83±2.53 b-d	48.81±1.09 bc	22.73±1.02 bc	34.80±1.32 c-f	14.95±2.45 c-f
	BRs (1)	99.67±3.03 c-e	40.53±0.75 f-i	22.62±0.61 bc	31.77±0.75 f-h	13.42±0.65 c-j
	BRs (2)	104.33±2.05 b-d	48.27±0.94 b-d	25.49±1.01 ab	33.65±0.53 c-g	14.56±0.55 c-g
	BRs (3)	107.25±4.73 b	47.74±0.78 b-d	21.30±0.35 cd	35.67±1.55 c-e	18.60±0.38 ab
9 days	Control	74±1.59l m	32.51±2.56 j-l	13.03±0.54 j	21.16±1.44 l	8.91±1.36 lm
	CHT (1)	89.08±1.47 g-i	35.8±2.95 h-k	17.44±0.54 f-h	23.96±1.56 kl	12.29±0.74 e-k
	CHT (2)	97.08±4.12 ef	43.31±1.41 c-g	17.89±0.49 e-g	34.18±0.09 c-g	12.94±0.21 d-k
	CHT (3)	99.17±1.72 de	45.23±0.58 b-f	18.19±0.07 e-g	34.72±0.63 c-f	14.030±1.06 c-i
	BRs (1)	88.83±4.06 g-i	37.54±1.68 g-j	17.04±0.6 f-h	27.63±1.77 j	11.94±0.26 g-k
	BRs (2)	101.58±1.82 b-e	43.5±2.86 c-g	20.68±1.34 c-e	29.94±0.83 h-j	12.99±0.57 d-k
	BRs (3)	106.42±2.81 bc	44.64±1.48 c-f	16.27±1.09 f-i	36.23±0.29 c	15.18±0.40 c-e
12 days	Control	70.33±1.58 m	27.73±1.99 l	9.91±0.18 k	17.80±0.53 m	8.3±1.24 m
	CHT (1)	83.75±3.41 h-j	32.18±1.99 j-l	14.66±0.41 h-j	20.84±0.68 lm	11.18±0.65 i-m
	CHT (2)	85.33±1.6 h-j	34.27±2.02 jk	16.80±1.54 f-i	26.93±1.46 jk	11.20 ±1.32 i-l
	CHT (3)	90.17±3.03 f-h	35.48±1.42 h-k	17.26±0.41 f-h	22.68±0.59 l	11.40±0.82 i-l
	BRs (1)	79.5±0.9 j-l	30.55±0.94 kl	16.32±1.87 f-i	21.35±0.51 l	11.46±1.21 h-l
	BRs (2)	82.12±3.05 i-k	35.77±2.42 h-k	17.53±0.54 f-h	23.59±0.78 l	12.07±0.54 f-k
	BRs (3)	101.75±3.04 b-e	35.13±1.89 i-k	15.19±0.83 g-j	27.18±0.96 j	12.30±0.29 e-k

CHT (1) = chitosan at 50 ppm, CHT (2) = chitosan at 100 ppm, CHT (3) = chitosan 200 ppm, BRs (1) = Brassinolide at 50 ppm, BRs (2) = Brassinolide at 100 ppm, BRs (3) = Brassinolide at 200 ppm. Each value represents the mean ± standard error of three replicates , Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

each irrigation intervals, the plants sprayed with the higher concentrations of two tested bio-stimulators (CHT or BRs) had significant increase ($p<0.05$) in most of growth parameters compared to control plants (pants exposed to water stress and not received any bio-stimulators treatments). The data also exhibited that under the same level of the two bio-stimulators, BRs was generally superior in its effect than CHT and among the different concentrations, the highest dose (200 ppm) was the most effective one in increasing the tested growth parameters. The results are similar to those obtained by previous studies where CHT was tested (Pirbalouti *et al*., 2017; Byczyńska, 2018; El-Khateeb *et al*., 2018; Abdel-Mola and Ayyat, 2020; El-Serafy, 2020; Ashour *et al.*, 2021; Arshad *et al*., 2022), as well as BRs (El-Khateeb *et al*., 2017; Abd-Allah *et al.*, 2018; Latha and Vidya Vardhini, 2018; Mohamed, 2020; Sheng *et al.*, 2022). Moreover, other studies (Hosseinpour *et al.*, 2020; Mohammadi *et al.*, 2020; Omidian, *et al.*, 2022) stated that application of BRs has a positive effect on growth parameters of ornamental plants exposed to drought stress.

Chemical constituents

Pigments content. As shown from data listed in Table 5 irrigation intervals, bio-stimulators treatment and interaction effects had significant influence on pigments content (chlorophyll a, b, carotenoids and

Table 4 - Plant height, fresh and dry weight of shoots and fresh and dry weight of roots of *Azadirachta indica* as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons)

Irrigation intervals	Bio-stimulators	Stem diameter (mm)	Root length (cm)	Number of leaves	Leaf area (cm^2)
3 days	Control	5.64±0.56 c-j	41.25±0.66 f-i	22.00±1.38 d-g	11.79±1.07 e-j
	CHT (1)	6.81±0.17 a-c	42.00±1.84 f-i	24.17±1.08 c-e	13.42±0.88 c-h
	CHT (2)	6.80±0.17 a-c	44.00±2.46 e-g	27.42±1.29 bc	15.54±0.69 a-c
	CHT (3)	6.91±0.02 ab	53.33±1.64 b	31.67±0.22 a	16.76±1.60 ab
	BRs (1)	6.85±0.35 ab	47.67±1.69 c-e	27.95±1.81 b	13.79±0.66 c-f
	BRs (2)	6.92±0.47 ab	50.67±1.74 b-d	33.08±1.54 a	14.88±0.47 b-d
	BRs (3)	7.10±0.49 a	59.25±1.32 a	35.00±2.81 a	18.24±1.12 a
6 days	Control	4.77±0.13 jk	30.75±1.38 l-n	17.33±0.44i-k	10.88±2.04 g-l
	CHT (1)	6.46±0.28 a-f	37.58±0.82 h-k	19.58±1.36 f-j	12.10±0.27 e-j
	CHT (2)	6.52±0.67 a-f	40.17±3.43 g-j	22.33±0.65 d-f	13.58±1.07 c-g
	CHT (3)	6.62±0.32 a-d	45.08±2.09 e-g	24.58±0.30 b-d	14.10±0.40 b-e
	BRs (1)	6.07±0.49 a-i	42.75±0.80 e-h	19.92±1.01 f-i	12.01±1.63 e-j
	BRs (2)	6.11±0.1 a-i	45.92±1.67 d-f	24.42±0.71 cd	13.52±0.51 c-h
	BRs (3)	6.53±0.22 a-e	54.17±3.83 ab	24.08±1.31 c-e	15.48±0.59 bc
9 days	Control	3.78±0.04 k	29.25±1.84 mn	14.67±1.36 kl	8.73±0.86 kl
	CHT (1)	5.33±1.14 f-j	33.83±0.88 k-m	16.33±0.33 jk	10.55±0.47 i-l
	CHT (2)	5.74±0.06 b-j	36.67±1.62 i-k	19.42±1.17 f-j	12.04±1.09 e-j
	CHT (3)	6.15±0.57 a-i	42.42±1.40 e-h	19.67±0.79 f-j	12.34±0.47 d-i
	BRs (1)	5.26±0.17 g-j	35.00±2.36 j-l	19.33±0.96 f-j	10.19±0.86 i-l
	BRs (2)	6.01±0.05 a-i	40.00±3.74 g-j	20.00±0.14 f-i	11.56±0.51 e-j
	BRs (3)	6.45±0.43 a-g	51.67±2.53 bc	20.83±0.60 e-h	13.34±0.13 c-h
12 days	Control	3.61±0.69 k	26.17±2.02 n	12.33±0.44 l	8.29±0.66 l
	CHT (1)	4.98±0.5 ij	31.08±1.08 l-n	15.00±1.23 kl	9.56±0.51 j-l
	CHT (2)	5.55±0.09 d-j	33±0.58 k-m	17.50±1.0 h-k	10.10±0.61 i-l
	CHT (3)	5.25±0.45 h-j	41.00±2.10 f-i	17.58±0.93 h-k	11.34±0.21 f-k
	BRs (1)	5.40±0.21 e-j	34.83±1.74 j-l	18.92±2.32 g-j	9.38±1.05 j-l
	BRs (2)	5.73±0.32 b-j	35.00±1.23 j-l	17.67±1.31 h-k	10.78±2.12 h-l
	BRs (3)	6.39±0.36 a-h	45.92±0.79 d-f	20.17±1.20 f-i	12.72±0.96 d-i

CHT (1) = chitosan at 50 ppm, CHT (2) = chitosan at 100 ppm, CHT (3) = chitosan 200 ppm, BRs (1) = Brassinolide at 50 ppm, BRs (2) = Brassinolide at 100 ppm, BRs (3) = Brassinolide at 200 ppm. Each value represents the mean ± standard error of three replicates , Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

total chlorophyll). Data in Table 6 showed that within each level of the two bio-stimulators (CHT or BRs), chlorophyll a, b, carotenoids and total chlorophyll were reduced gradually (in most cases) in repose to prolonged irrigation intervals. Photosynthetic pigments play a vital role in photosynthetic process. Under drought stress, stomata functioning is changed that affect photosynthesis and CO_2 uptake, thus resulting various chlorophyll contents level through the entire growth period of the plant (Khatana *et al*., 2018). Similar reductions in pigments content as a result of water deficient stress were reported by various studies (Ashour and El-Attar, 2017; Abd-Elmoneim *et al*., 2018; Khatana *et al*., 2018; Sarker and Oba, 2018; El-Shanhorey and Sorour, 2019; Al-Arjani *et al*., 2020; Singh and Singh, 2020; El-Gamal and Khamis, 2021; Papú *et al*., 2021; Sorour, 2021; Shaltout *et al*., 2022).

The data in Table 6 also revealed that within each irrigation intervals, the highest concentrations of CHT or BRs caused significant increase in pigments content compared to control plants. The data also clarified that BRs was better in its effect than CHT particularly the highest concentration (200 ppm) since recorded the highest values of the tested traits. These results are in agreement with findings of prior authors who reported that application of CHT resulted in increase in pigments content (Byczyńska, 2018; El-Khateeb *et al*., 2018; El-Serafy, 2020; Elansary *et al*., 2020; Abdel-Mola and Ayyat, 2020; Gerami *et al*.,

Table 5 - Mean square for the effect of irrigation intervals and bio-stimulators treatments and their interaction on some chemical constituents of *Azadirachta indica*

Traits	Source of Variation				
	Treatment			Error	CV
	Irrigation intervals (A)	bio-stimulators (B)	(A × B)		
Chlorophylls A content (mg/g f.w)	22.90 ***	8.91 ***	0.32 *	0.93	16.99
Chlorophylls B content (mg/g f.w)	2.72 ***	1.35 **	0.21 *	0.39	24.19
Carotenoids content (mg/g f.w)	5.30 ***	5.94 ***	0.21 *	0.64	20.35
Total chlorophyll (mg/g f.w)	39.85 ***	16.72 ***	0.56 *	1.74	15.96
Total carbohydrates (%) in leaves	206.87 ***	156.23 ***	6.81 ***	1.05	4.09
Proline (µ moles/g fresh matter)	25.67 ***	9.65 ***	0.82 ***	0.11	7.60
N% in shoot	1.23 ***	2.59 ***	0.17 ***	0.01	5.03
P% in shoot	0.18 ***	0.46 ***	0.05 ***	0.001	9.21
K% in shoot	0.34 ***	0.85 ***	0.05 ***	0.01	6.29
Total indoles (mg/100 g DW)	1310.44 ***	4091.18 ***	101.34 ***	2.75	2.45
Total Phenols (mg/100 g DW)	514.11 ***	3724.62 ***	116.61 ***	3.44	2.94

*, **, *** significant at P≤0.05, P≤0.01, P≤0.001, respectively.

Table 6 - Pigments content of *Azadirachta indica* as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons)

Irrigation intervals	Bio-stimulators	chlorophyll a (mg/g FW)	chlorophyll b (mg/g FW)	Carotenoids (mg/g FW)	Total chlorophyll (mg/g FW)
3 days	Control	3.88±0.23 h-j	2.05±0.07 d-f	2.95±0.05 i-k	5.93±0.15 h-j
	CHT (1)	5.76±0.44 c-g	2.40±0.49 c-f	4.78±0.59 a-e	8.16±0.89 d-g
	CHT (2)	5.86±0.81 c-f	2.84±0.30 b-d	4.02±0.66 b-j	8.70±1.10 c-g
	CHT (3)	7.22±0.40 bc	2.80±0.25 b-d	4.64±0.10 a-f	10.02±0.62 b-d
	BRs (1)	5.79±0.04 c-g	2.96±0.30 b-d	4.41±0.17 b-g	8.75±0.34 c-g
	BRs (2)	6.73±0.91 cd	3.48±0.69 ab	5.33±0.96 ab	10.22±1.53 b-d
	BRs (3)	9.56±1.08 a	3.99±0.22 a	5.94±0.73 a	13.55±0.90 a
6 days	Control	4.27±0.16 g-j	1.71±0.14 ef	2.98±0.07 h-k	5.99±0.24 h-j
	CHT (1)	5.18±0.48 d-h	2.62±0.20 b-e	4.22±0.28 b-i	7.80±0.32 e-h
	CHT (2)	5.47±0.62 d-g	2.98±0.41 a-d	3.54±0.18 e-k	8.44±0.41 c-g
	CHT (3)	6.39±0.94 c-e	2.86±0.39 b-d	4.36±0.38 b-g	9.25±1.32 b-e
	BRs (1)	5.69±0.13 c-g	2.54±0.15 b-e	4.07±0.69 b-j	8.23±0.15 d-g
	BRs (2)	6.48±0.50 c-e	2.81±0.39 b-d	4.29±0.65 b-h	9.29±0.48 b-e
	BRs (3)	8.54±0.82 ab	2.86±0.29 b-d	5.03±0.39 a-c	11.40±1.08 ab
9 days	Control	3.58±0.28 ij	1.62±0.12 ef	2.87±0.11 jk	5.20±0.36 ij
	CHT (1)	4.49±0.42 f-j	2.52±0.10 b-f	3.73±0.65 c-k	7.01±0.33 f-i
	CHT (2)	5.02±0.33 e-i	2.55±0.29 b-e	3.42±0.45 f-k	7.57±0.05 e-h
	CHT (3)	5.88±0.78 c-f	2.27±0.19 d-f	4.13±0.20 b-j	8.16±0.89 d-g
	BRs (1)	5.08±0.37 e-i	2.45±0.43 c-f	3.95±0.19 c-j	7.54±0.76 e-h
	BRs (2)	6.45±0.29 c-e	2.63±0.21 b-e	3.78±0.24 c-k	9.08±0.34 c-f
	BRs (3)	7.22±0.50 bc	2.82±0.45 b-d	4.88±0.44 a-d	10.05±0.91 b-d
12 days	Control	3.05±0.13 j	1.51±0.04 f	2.47±0.17 k	4.56±0.17 j
	CHT (1)	4.34±0.25 f-j	2.51±0.57 b-f	3.41±0.62 f-k	6.85±0.60 g-i
	CHT (2)	4.48±0.54 f-j	2.36±0.55 c-f	3.09±0.46 g-k	6.84±0.69 g-i
	CHT (3)	5.51±0.72 d-g	2.03±0.22 d-f	3.12±1.06 g-k	7.54±0.92 e-h
	BRs (1)	4.54±0.19 f-j	2.08±0.11 d-f	3.29±0.21 g-k	6.62±0.15 g-j
	BRs (2)	5.45±0.53 d-h	2.86±0.45 b-d	3.67±0.44 d-k	8.31±0.95 d-g
	BRs (3)	7.12±0.69 bc	3.36±0.66 a-c	4.06±0.29 b-j	10.49±1.33 bc

CHT (1) = chitosan at 50 ppm, CHT (2) = chitosan at 100 ppm, CHT (3) = chitosan 200 ppm, BRs (1) = Brassinolide at 50 ppm, BRs (2) = Brassinolide at 100 ppm, BRs (3) = Brassinolide at 200 ppm. Each value represents the mean ± standard error of three replicates , Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

2020; Ashour *et al.*, 2021; Arshad *et al*., 2022; Samany *et al*., 2022; Attaran Dowom *et al*., 2022). While, the valuable enhance in tested components due to BRs treatments are in harmony with another reports (El-Khateeb *et al*., 2017; Abd-Allah *et al.*, 2018; Rezaei *et al.*, 2018; Mohamed, 2020; Pacholczak *et al*., 2021). Furthermore, previous workes (Hemmati *et al.*, 2018; Mohammadi *et al.*, 2020; Mojaradi *et al*., 2020; Zafari *et al*., 2020; Omidian *et al.*, 2022) showed that application of BRs has a favorable effect on photosynthetic pigments of plants subjected to drought stress.

The positive effect of BRs in increase the pigments content may be due to application of BRs increases the photosynthetic rate of plants by increasing the RuBisCo activity and other main enzymes included in the Calvin cycle, BRs also enhance the uptake of CO_2 which increase the stomatal conductance (Vikram *et al.*, 2022).

Total carbohydrates (% of dry matter)

As shown in figure 1 A, the data indicated that within each level of CHT or BRs total carbohydrates were reduced in parallel with increasing irrigation intervals from 3 to 6, 9 or 12 days. The reductions in total carbohydrates percentage due to water deficient stress are in agreement with findings of other researchers (Ashour and El-Attar, 2017; Abd-Elmoneim *et al*., 2018; Sarker and Oba, 2018; El-Shanhorey and Sorour, 2019; Sorour, 2021). The water deficit may elevate the production of reactive oxygen species under drought stress which lead to oxidative stress and harm to chloroplasts structure and chlorophyll loses. Reducing chlorophylls contents and photosynthetic activity could indirectly cause a reduction in carbohydrates content. Moreover, water deficient assists translocation of absisic acid via xylem vessels to the shoot of stressed plants for stomatal closure which may be resulted in reduction of net photosynthesis and carbohydrate accumulation (Baccari *et al*., 2020).

Results in the same figure also showed that within each irrigation intervals, spraying the plants with any concentration of CHT or BRs resulted in significant increase in total carbohydrates compared to control plants. Under the same level of the two tested bio-stimulators, CHT was generally better in its effect than BRs for increasing total carbohydrates. The lowest value (15.99 %) was obtained from plants irrigated with the longest intervals (12 days) and not received any bio-stimulators treatments, whereas the highest mean value (38.64%) was resulted from plants irrigated with the shortest intervals (3 days)

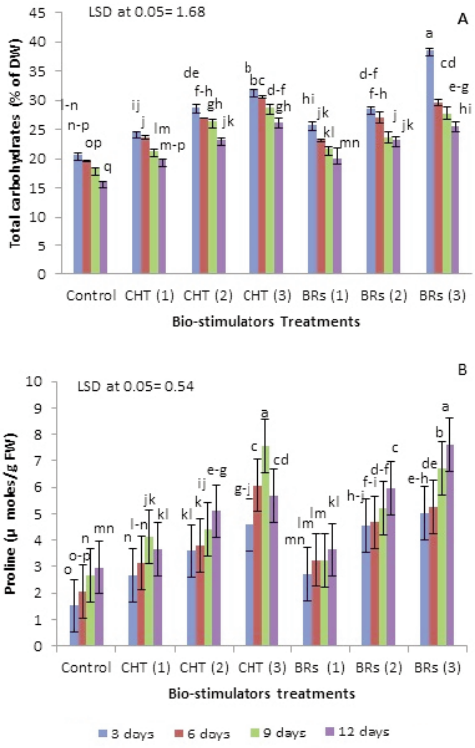

Fig. 1 - Total carbohydrates (% DW) (A), proline content (B) as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons). Column with different letters indicate a significant difference at 5% level. Vertical bars indicate to standard error (SE) of three replicates.

and sprayed with BRs at 200 ppm. The results of increasing total carbohydrates due to CHT treatments are the same as the results of previous researches (Zohreh *et al*., 2017; Shafiei-Masouleh, 2019; Elansary *et al*., 2020; Ashour *et al*., 2021; Salachna and Pietrak, 2021). While, the beneficial increase in total carbohydrates due to BRs treatments are in agreement with earlier studies (Mohamed, 2020; Pacholczak *et al*., 2021). Additionally, previous workers (Hemmati *et al.*, 2018; Mojaradi *et al*., 2020; Cai *et al*., 2021) indicated that application of BRs has a useful effect on carbohydrates accumulation of plants subjected to drought stress.

Proline content

Data in figure 1 B elucidated that in most cases under bio-stimulators treatments, proline content was increased progressively with prolonging irrigation intervals. Proline, an amino acid, plays a highly useful role in plants subjected to different stress conditions. Besides acting as an excellent osmolyte, proline plays three main roles during stress, i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat *et al*., 2012). Similar

results were reported by many studies (Ashour and El-Attar, 2017; El-Shanhorey and Sorour, 2019; Al-Arjani *et al*., 2020; El-Gamal and Khamis, 2021; Papú *et al*., 2021; Sorour, 2021; Samany *et al*., 2022; Shaltout *et al*., 2022).

The data also demonstrated that under each irrigation intervals, spraying any concentration of CHT or BRs resulted in significant higher values of proline content compared to control plants and BRs was superior in its effect than CHT. The highest mean value (7.61 µ moles/g FW) was registered from plants irrigated with the longest intervals (12 days) and sprayed with BRs at 200 ppm, while the lowest value (1.52 µ moles/g FW) was produced from plants irrigated with the shortest intervals (3 days) and not received any bio-stimulators treatments. The results of increasing proline content due to application of CHT treatments has been reported by earlier authors (Zohreh *et al*., 2017; Elansary *et al*., 2020; Ashour *et al*., 2021; Attaran Dowom *et al*., 2022). Whereas, the obvious increases in proline content due to application of BRs on ornamental water stressed plants are in good accordance with those elicited by prior authors (Zafari *et al*., 2020; Hosseinpour *et al.*, 2020; Mohammadi *et al.*, 2020; Omidian *et al.*, 2022). The accumulation of proline due to application of BRs may be due to BRs enhanced gene expression of biosynthetic genes (Sharma *et al*., 2019).

N, P and K (% of dry matter)

As shown in Table 5, N, P and K % were significantly affected by irrigation intervals, bio-stimulators treatment and interaction effects. The data in Table 7 indicated that within each level of the two bio-stimu-

Table 7 - N, P and K% of *Azadirachta indica* as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons)

Irrigation intervals	Bio-stimulators	N%	P%	K%
3 days	Control	1.28±0.03 lm	0.16±0.01 j-m	1.17±0.01 l-n
	CHT (1)	1.62±0.10 g-i	0.28±0.01 ef	1.42±0.01 f-i
	CHT (2)	1.95±0.04 d	0.32±0.01 d	1.51±0.02 ef
	CHT (3)	2.23±0.01 c	0.86±0.02 a	1.51±0.02 ef
	BRs (1)	1.55±0.01 h-j	0.30±0.01 de	1.75±0.05 bc
	BRs (2)	2.13±0.58 c	0.58±0.01 b	1.92±0.05 a
	BRs (3)	2.98±0.01 a	0.90±0.03 a	1.97±0.04 a
6 days	Control	1.31±0.01 kl	0.17±0.01 i-l	1.14±0.05 mn
	CHT (1)	1.59±0.11 g-i	0.28±0.03 ef	1.29±0.01 i-l
	CHT (2)	1.66±0.01 f-h	0.34±0.01 d	1.45±0.02 e-h
	CHT (3)	2.46±0.03 b	0.43±0.04 c	1.50±0.05 e-g
	BRs (1)	1.71±0.04 fg	0.20±0.01 h-j	1.59±0.02 de
	BRs (2)	2.22±0.02 c	0.30±0.01 de	1.68±0.14 cd
	BRs (3)	2.20±0.06 c	0.46±0.02 c	1.89±0.14 ab
9 days	Control	1.16±0.02 m-p	0.13±0.01 mn	1.12±0.01 mn
	CHT (1)	1.44±0.01 jk	0.18±0.01 i-l	1.18±0.01 l-n
	CHT (2)	1.42±0.02 jk	0.18±0.01 i-k	1.30±0.03 i-l
	CHT (3)	1.90±0.10 de	0.23±0.01 gh	1.36±0.07 g-j
	BRs (1)	1.49±0.01 ij	0.19±0.01 h-j	1.45±0.01 e-h
	BRs (2)	1.79±0.01 ef	0.20±0.01 g-i	1.29±0.01 i-l
	BRs (3)	1.94±0.11 d	0.30±0.01 de	1.33±0.01 h-k
12 days	Control	1.06±0.02 op	0.10±0.01 n	1.07±0.01 n
	CHT (1)	1.15±0.02 m-p	0.13±0.01 mn	1.11±0.02 mn
	CHT (2)	1.18±0.02 l-p	0.14±0.01 lm	1.10±0.02 mn
	CHT (3)	1.25±0.12 l-n	0.19±0.01 h-j	1.19±0.04 k-n
	BRs (1)	1.12±0.04 n-p	0.14±0.01 lm	1.23±0.10 j-m
	BRs (2)	1.25±0.02 l-n	0.15±0.01 k-m	1.22±0.01 k-m
	BRs (3)	1.27±0.04 lm	0.24±0.03 fg	1.21±0.02 k-n

CHT (1) = chitosan at 50 ppm, CHT (2) = chitosan at 100 ppm, CHT (3) = chitosan 200 ppm, BRs (1) = Brassinolide at 50 ppm, BRs (2) = Brassinolide at 100 ppm, BRs (3) = Brassinolide at 200 ppm. Each value represents the mean ± standard error of three replicates. Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

lators (CHT or BRs), prolonging irrigation intervals generally decreased three nutrients (N, P and K %). Similar reduction has been obtained by other reports (Ashour and El-Attar, 2017; Abd-Elmoneim *et al*., 2018; El-Shanhorey and Sorour, 2019; Singh and Singh, 2020; El-Gamal and Khamis, 2021).

The unfavorable effect of water deficient on the uptake and accumulation of the three nutrients in plant may be due to water deficient stress caused by extending the irrigation intervals resulted in low soil moisture content which affects the elements solubility and their absorbing efficiency by plants which in turn leading to reduce their accumulation in plant tissues. Additionally, limited transpiration rates and impaired active transport and membrane permeability lead to reduce nutrient uptake by the roots and accumulation in the shoots (Farooq *et al*., 2009).

The data in the same Table disclosed that within each level of irrigation frequency, in most cases three nutrients were significantly higher in the plants sprayed with any concentrations of two tested bio-stimulators (CHT or BRs) than those recorded with control plants. Under the same level of the two tested bio-stimulators, BRs appeared to be more effective than CHT and among BRs concentrations; the highest dose (200 ppm) was the most effective one. The results of increasing N, P or K% due to application of CHT confirmed the reports of previous study (Abd-El-Hady, 2020; Salachna and Pietrak, 2021). While, the increase due to application of BRs are similar to those obtained by prior workers (Mohamed, 2020).

Total indoles and phenols content

Results in figure 2 A showed that with each level of CHT or BRs, increasing irrigation intervals caused steady reduction in content of total indoles**.** Within each irrigation intervals, application of any concentrations of CHT or BRs resulted in significant increase in total indoles compared to control plants**.** Additionally, CHT appeared to be more effective than BRs and the highest concentration (200 ppm) was the most effective one.

Results in figure 2 B indicated that total phenols content showed different trend in response to water deficient, under each level of CHT or BRs, content of total phenols was increased linearly with increasing irrigation intervals from 3 to 6, 9 or 12 days. The present increase in total phenols content as result of water deficient are similar to those obtained by (Sarker and Oba, 2018; El-Gamal and Khamis, 2021; Papú *et al*., 2021).

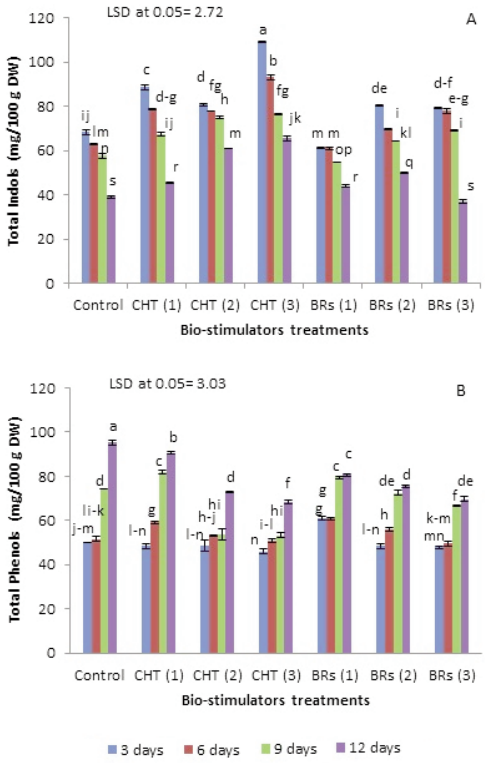

Fig. 2 - Total indoles (A), total phenols (B) as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons). Column with different letters indicate a significant difference at 5% level. Vertical bars indicate to standard error (SE) of three replicates.

The data also revealed that within each irrigation intervals, application of CHT or BRs at the highest concentration (200 ppm) reduced the mean values compared to control. Although previous studies revealed increase in total phenols content due to CHT treatments (Pirbalouti *et al*., 2017; Vosoughi *et al*., 2018; El-Serafy, 2020; Elansary *et al*., 2020; Arshad *et al*., 2022; Attaran Dowom *et al*., 2022) or due to BRs treatments (Amraee *et al*., 2020; Mohammadi *et al*., 2020). However, under the present study total phenols content was reduced in response to application of CHT or BRs which support the results of Ashour *et al*. (2021) who found that application of CHT decreased total phenols content.

4. Conclusions

Water deficient stress had a harmful effect on growth parameters, pigments content, total carbohydrates and nutrient uptake while, increased proline

and total phenols content. Foliar application of chitosan or brassinolide at the higher concentrations (200 ppm) increased growth parameters, pigments content, total carbohydrates, proline content and nutrient uptake. Based on the obtained results it can be concluded that, foliar application of chitosan or brassinolide at 200 ppm can alleviate the adverse effects of water deficient stress on the growth and physiology parameters of *Azadirachta indica*.

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Effect of continuous lighting on the growth and leaf chemical components of *Artemisia princeps* grown hydroponically in a plant factory condition

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Key words: Chlorogenic acid, Japanese mugwort, nutrient solution pH, photoperiod, polyphenol.

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Abstract: Young leaves of *Artemisia princeps* Pamp. (Japanese mugwort), already used as a foodstuff in Japan, can be positioned as a functional health food because of remarkably higher contents of chlorogenic acid and total polyphenol compared to common vegetables. To procure young leaves in demand on a year-round basis by hydroponic production in fully artificial light-type plant factories, we investigated whether 24-h photoperiod, known to enhance some beneficial constituents, could improve the growth and chemical constituents of Japanese mugwort plants grown hydroponically in a plant factory condition. As we previously demonstrated that lowering the nutrient solution concentration increased chlorogenic acid and total polyphenol contents of the leaves without reducing the growth, plants were cultivated with a lower concentration of nutrient solution. The results indicated that it is possible to grow Japanese mugwort hydroponically under 24-h photoperiod in a plant factory condition with a nutrient solution concentration as low as 25% of the standard. In addition, under 24-h photoperiod, plant growth was greatly accelerated and chlorogenic acid as well as total polyphenol were increased, suggesting that 24-h photoperiod is highly beneficial for Japanese mugwort production in a fully artificial light-type plant factory.

1. Introduction

Mount Ibuki, located on the border of Shiga and Gifu prefectures in Japan, has been famous for its medicinal plants since ancient times, and it was written in 'Engishiki' (compiled in 927 A.D.) that Omi (Shiga Prefecture) and Mino (Gifu Prefecture) ranked first and second, respectively, in the number of herbal medicinal items as paying tribute to the imperial court from all over Japan (Oda, 1985). In particular, in the early Edo era (around 1700 A.D.), the area around Mt. Ibuki was a major producer of domestic mugwort, such as *Artemisia princeps* or *Artemisia montana*, and the resulting moxa, called for 'Ibuki-Moxa' was publicized nationwide (Oda, 1998, 1999). The authors focus on the use of such

domestic mugwort.

Gaiyoh (Artemisiae folium) used in Wakan-yaku (traditional herbal drugs) is defined as the dried leaves and branch tips of *A. princeps* or *A. montana*, and it is used as a raw material for moxa and is included in various Chinese herbal preparations as an astringent hemostatic and analgesic (Nunome, 2018; Ministry of Health, Labour and Welfare, 2021). *Artemisia princeps* (Japanese mugwort) is also used as a foodstuff, with its young leaves, picked in early spring, being mixing with rice cakes or dumplings as 'Mochigusa', or used in soaking and tempura (Odachi and Hiyama, 2013; Ando *et al*., 2022). In particular, according to the Functional Components Database (National Agriculture and Food Research Organization, 2020), Japanese mugwort has remarkably higher chlorogenic acid and total polyphenol contents compared with common vegetables, indicating that it can be positioned as a functional health food.

Japanese mugwort is generally procured by harvesting wild plants or through cultivation in open fields (Ando *et al.*, 2022). It is preferable to harvest the young, tender leaves in early spring for use as a food ingredient or functional health food. However, under natural conditions, the number of mature leaves increases with plant growth, and after flowering in autumn, the plant eventually withers and stops growing until the following spring (Ito, 2015), making it difficult to procure young in-demand leaves on a year-round basis, even after harvesting both wild and cultivated plants. To address this problem, we focused on the use of a fully artificial light-type plant factory system. With the multi-shelf cultivation system used in plant factories (Kozai, 2013), it is possible to produce a large number of young plants at a low plant height on a year-round basis, allowing to provide the young leaves desired throughout the year (Kim *et al.*, 2021). In addition, plant factory production has the advantage of being pesticide-free.

However, to date, there is limited knowledge on the hydroponic cultivation of Japanese mugwort; therefore, it is necessary to establish an effective management system for its hydroponic cultivation in fully artificial light-type plant factories. In our previous report (Hata and Kawamura, 2021), we investigated the effects of nutrient solution concentration on the growth and leaf chemical components of Japanese mugwort cultivated hydroponically using 'Ibuki-yomogi' (a line of *A. princeps* indigenous to Shiga Prefecture), to establish an effective hydroponic cultivation method. The results showed that lowering the nutrient solution concentration to 25% of the standard increases the ascorbic acid, chlorogenic acid, and total polyphenol contents of the leaves without reducing plant growth.

Hata *et al*. (2012 a) studied the differences in the growth rate and leaf sesamin content of sesame (*Sesamum indicum*) grown under various photoperiods and found not only a maximum leaf yield, but also a distinctively high sesamin content, under a 24-h photoperiod. Furthermore, Higashiuchi *et al*. (2016) also reported that the active ingredient (asperuloside) level in white flower snake-tongue grass (*Hedyotis diffusa*), a medicinal plant, increases noticeably under a 24-h photoperiod compared with under 14- and 19-h photoperiods. Thus, enhanced leaf yields and accumulations of beneficial components may be achieved using a 24-h photoperiod in the cultivation of Japanese mugwort in a plant factory; however, supporting research is required.

Consequently, in the present study, we used 'Ibuki-yomogi' in our experiments and investigated whether a 24-h photoperiod increased the growth and chemical component contents of Japanese mugwort plants grown hydroponically in a low nutrient solution concentration under plant factory conditions.

2. Materials and Methods

Plant materials and seedling cultivation methods

The strain maintained at the Ibuki Yakuso-no Sato Cultural Center (Maibara-city, Shiga Prefecture, Japan) was used as the experimental material. Inflorescences collected in the fall of 2017 were air-dried and stored in a desiccator for use in the cultivation experiments.

The seedlings were grown in the growth chamber. The photosynthetic photon flux density from the Hf-fluorescent lamp (FHF32EX-N-H, Panasonic Co., Japan) on the surface of a seedling box was 260 µmol m^{-2} s^{-1}. The photoperiod and temperature were set at 12 h and 23°C, respectively.

On the basis of our previous report (Hata and Kawamura, 2021), seeds were spread by rubbing the flower heads with fingers, and then they were placed on root prevention sheets (20701FLD, Unitika Ltd., Japan) laid on Kim Towels (Nippon Paper Group Crecia Co., Ltd., Japan) moistened with tap water. At 1 week after sowing, young seedlings of approxi-

mately 3 mm were transplanted into polyurethane cubes (2.35 × 2.35 × 3 cm, Tomiyamass Co., Japan). Afterwards, the seedlings were grown for 3 weeks by subirrigation with 1/2-strength Enshi formula nutrient solution. This solution consisted of 2 mM of $Ca(NO_3)_2 \cdot 4H_2O$, 4 mM of KNO_3, 0.67 mM of $NH_4H_2PO_4$, 1 mM of $MgSO_4 \cdot 7H_2O$, 1.5 mg L^{-1} Fe, 0.25 mg L^{-1} Mn, 0.25 mg L^{-1} B, 0.025 mg L^{-1} Zn, 0.01 mg L^{-1} Cu, and 0.005 mg L^{-1} Mo.

Hydroponic methods

Hydroponic cultivation was conducted in a walk-in type plant growth room (internal dimensions: 4.1 m long, 4.1 m wide, and 2.1 m high) at the Experimental Agricultural Facility of The University of Shiga Prefecture. During the cultivation period, the temperature and CO_2 concentrations were set at 23°C and 400 ppm, respectively, while the relative humidity was not set at a constant level. The photosynthetic effective photon flux density on the surface of the growing container at a distance of 42 cm vertically from the Hf-fluorescent lamps (FHF32EX-N-H, Panasonic Co., Japan) was 130 µmol m^{-2} s^{-1}.

The seedlings were planted at 4 weeks after sowing and grown hydroponically for 4 weeks under a 12-h or 24-h photoperiod. A 2.5-cm thick Styrofoam board with two 2.5-cm diameter holes (11 cm between plants) was floated as a planting board on 6.0 L of nutrient solution in each container (NF Box #11 Blue, inner dimensions: 15.3 × 27.8 × 16.5 cm, capacity: 7.0 L, JEJ Astage Co., Ltd., Japan), and two seedlings were planted per board. The nutrient solution used was 1/4-strength Enshi formula, which was continuously aerated at 0.4 L min^{-1} with an air pump. The initial pH of the nutrient solution was adjusted to 6.0 with H_2SO_4 before use, but it was not adjusted during the cultivation period. The nutrient solution was renewed after 2 and 3 weeks of hydroponic cultivation. The pH of the nutrient solution was measured with a digital pH meter (pH-208, Sato Shoji Co., Ltd., Japan) before nutrient solution replacement and at harvest (4 weeks after the start of hydroponic cultivation).

Growth evaluation and preparation of dry matter samples

In total, 20 plants were grown in 10 growing containers under each photoperiod. The plants were harvested at 4 weeks after the start of hydroponic cultivation, and the fresh weights of leaves, stems, and roots were measured, as were the main stem lengths and numbers of branches, for all the plants. The stems and roots were dried in an oven at 60°C, after which the constant dry weights were recorded and used for calculating dry matter content. Approximately 10-15 g of leaves randomly taken from the whole leaves was similarly dried at 60°C to form a dried sample for the inorganic component analysis as well as the dry matter content calculation. The rest of the leaves were freeze-dried for other component analyses and stored in a -80°C freezer.

Chemical composition analysis

The 60°C-dried and freeze-dried samples were thoroughly ground independently with a mortar and pestle. In each photoperiodic treatment, 10 samples were analyzed for each component, with one sample being a mixture of equal amounts of the two individuals growing in one container. Each analysis described below was conducted similarly in accordance with our previously reported methods (Hata and Kawamura, 2021).

Determination of inorganic components

For each sample, 100 mg of the powdered sample was decomposed using the wet method in a nitric acid and hydrogen peroxide mixture in a 100-mL beaker. After decomposition, the solution in the beaker was volumetrically diluted with 1 M nitric acid and passed through a 0.45-µm syringe filter (Surplux PTFE-H (hydrophilic) 25 mm, LMS Co., Ltd., Japan). The P, K, Ca, Mg, Na, Fe, Mn, and Zn concentrations were measured using (SII SPS3100, Hitachi High-Tech Science Co., Ltd., Japan). Multi-element standard IV and single-element standard (P) for ICP (Merck Millipore Ltd., Germany) were used as calibration standards, and the content of each inorganic component in the leaves was calculated from the intensity value of each sample.

Determination of ascorbic acid

For each sample, 50 mg of the powder, weighed in a 2-mL microcentrifuge tube, was extracted using ultrasonic waves for 30 min in distilled water. After extraction, the ascorbic acid content in centrifuged supernatant liquid was measured using a reflectometer (RQ Flex 10, Merck Millipore Ltd., Germany) to calculate the corresponding content in the leaves.

Determination of chlorogenic acid

For each sample, 50 mg of the powder, weighed in a 2-mL microcentrifuge tube, was extracted at

40°C for 30 min with shaking at 2,000 rpm in 80% (v/v) ethanol solution. After the extraction, the supernatant was collected by centrifugation at 12,500 rpm for 5 min, and the extract was collected again from the extraction residue. The collected extract mixture was passed through a 0.45-µm syringe filter (GL Chromato-Disk 4N, GL Sciences Inc., Japan) before being used for the chlorogenic acid concentration analysis with the UPLC-FLD method. In brief, samples were analyzed with the ACQUITY UPLC system (Waters Co., USA) using a Waters ACQUITY UPLC HSS T3 Column (100 mm × 2.1 mm, 1.8 µm). Detection was performed using a Waters 470 Scanning Fluorescence Detector set at an excitation wavelength of 371 nm and an emission wavelength of 443 nm. The mobile phases were 0.2% (v/v) formic acid (solvent A) and 100% acetonitrile (solvent B). The gradient elution program, with a mixture of solvents A and B, was as follows: 90-80% A for 0-1 min (curve no. 7), 80-55% A for 1-5 min (curve no. 7), 55-35% A for 5-6 min (curve no. 9), and 35-90% A for 6-7 min (curve no. 9). The flow rate was 0.3 mL min^{-1}. The column oven was set at 40°C, and 3 µL of each sample was loaded. The amount of chlorogenic acid in a sample was quantified from the peak area of the authentic standard compound (chlorogenic acid hemihydrate dissolved in 80% ethanol) to calculate the content in the leaves. Solvents of HPLC grade, and all other chemicals, were purchased from Nacalai Tesque, Inc., Japan.

Determination of total polyphenol

A 10-fold dilution of the extract solution for the chlorogenic acid analysis with 80% (v/v) ethanol was prepared and analyzed in accordance with the Folin–Ciocalteu method. First, 0.3 mL of the sample solution and 0.3 mL of distilled water were mixed in a 2-mL microcentrifuge tube, and then, 0.6 mL of a solution of phenol reagent (Nacalai Tesque, Inc., Japan) diluted two-fold with distilled water was added and left for 3 min after mixing. Next, 0.6 mL of 10% (w/v) sodium carbonate solution was added, mixed, and allowed to react for 60 min. Within 30 min of the reaction finishing, the absorbance at a wavelength of 750 nm was measured using a spectrophotometer. Chlorogenic acid hemihydrate dissolved in 80% ethanol was used as the calibration standard, and the total polyphenol content in the leaves was calculated as chlorogenic acid equivalents from the absorbance values (750 nm) of each sample.

Data analyses

For growth data, average values for each growing container were compared, whereas the data for the nutrient solution pH levels and chemical components were compared among the obtained values per growing container. Significant differences between two photoperiods were analyzed using Student's t-test (n = 10).

3. Results

Change in nutrient solution pH

After 2 weeks of hydroponic cultivation, the pH of the nutrient solution rose to 6.5 under 12-h photoperiod, while it rose to 7.7 in the 24-h photoperiod (Fig. 1). Even after returning to the initial pH of 6.0 by replacing the nutrient solution, the pH rose to only 6.5 under 12-h photoperiod but to 7.7 under 24-h photoperiod in the following week. Even when the nutrient solution was renewed again after 3 weeks of hydroponic cultivation, the pH rose to 6.6 under 12-h photoperiod but to 7.7 under 24-h photoperiod, at the end of cultivation one week later.

Plant growth

Flower buds did not form under either the 12- or 24-h photoperiod until the end of cultivation at 8 weeks after sowing. From 2 weeks after the start of

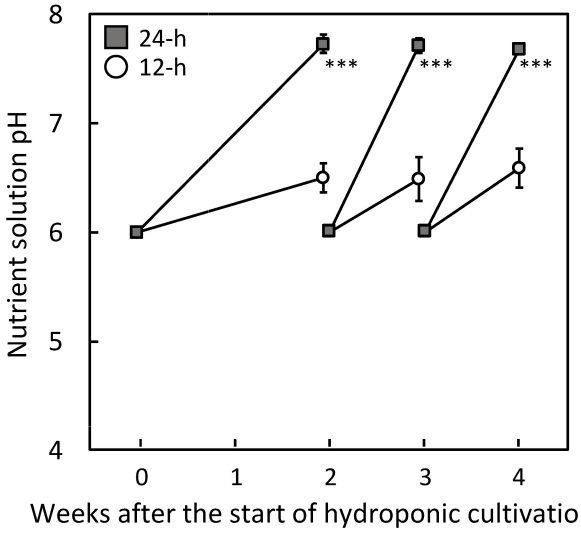

Fig. 1 - Changes in nutrient solution pH during the hydroponic cultivation of Japanese mugwort plants grown under 12-h (open circles) and 24-h (closed squares) photoperiods. Values represent means ± SEs (n = 10). Statistical significances between the two photoperiods were determined using Student's t-test. ***, $p < 0.001$.

hydroponic cultivation, plant growth was more vigorous under the 24-h photoperiod than under the 12-h photoperiod (Fig. 2). Generally, darker leaf colors and greater anthocyanin accumulations in the main stems were observed under the 24-h photoperiod (Fig. 3), and 2 of 20 plants showed lower leaf senescence (Fig. 4). There was no visual difference in the number of trichomes on leaves and stems between the two photoperiodic treatments (Fig. 3, 5).

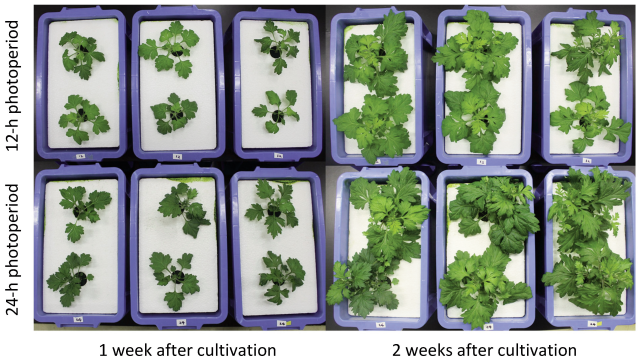

Fig. 2 - Differences in early developmental stages of Japanese mugwort plants grown under 12-h and 24-h photoperiods.

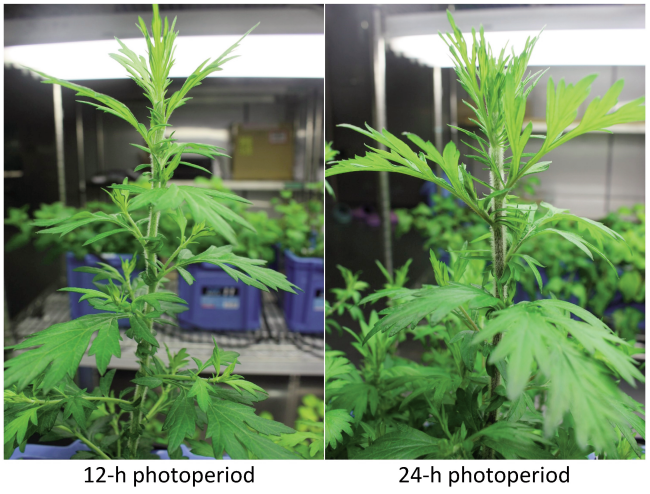

Fig. 3 - Differences in main stem colors of Japanese mugwort plants grown under 12-h and 24-h photoperiods for 4 weeks.

The fresh weights of leaves, stems, and roots at harvest under the 24-h photoperiod were 22.8, 11.1, and 14.6 g, respectively, which were almost twice as high as those under the 12-h photoperiod (Table 1). The dry weights of leaves, stems, and roots showed the same trends as fresh weights, and the dry matter ratio of leaves to stems was also significantly greater under the 24-h photoperiod. All the traits related to stem elongation, such as number of branches, main

Fig. 4 - Appearance of lower-leaf browning in the Japanese mugwort plant grown under a 24-h photoperiod for 4 weeks.

Fig. 5 - Appearances of trichomes on the abaxial leaf surfaces of Japanese mugwort plants grown under 12-h and 24-h photoperiods for 4 weeks.

stem length, number of main stem nodes, and average internode length, were significantly higher under the 24-h photoperiod compared with under the 12-h

Table 1 - Effects of photoperiod on the biomass production of Japanese mugwort plants

Photoperiod (h)	Leaves			Main stem + branches			Root		
	FW (g)	DW (g)	DMR (%)	FW (g)	DW (g)	DMR (%)	FW (g)	DW (g)	DMR (%)
12	13.132	1.4	11	3.7	0.4	10	8.0	0.6	8
24	22.81	3.0	13	11	1.4	12	15	1.0	7
Significance [z]	***	***	***	***	***	***	***	***	NS

FW= Fresh weight; DW= Dry weight; DMR= Dry matter ratio.
[z] Statistical significances between the means of two photoperiods were determined using Student's t-test (n = 10). NS= not significant; ***, p<0.001.

photoperiod (Table 2).

Inorganic component contents

On a dry weight basis, the K and Zn contents were significantly lower at the 1% significance level under the 24-h photoperiod compared with under the 12-h photoperiod (Table 3).

Furthermore, the Fe and Mn contents were also significantly lower under the 24-h photoperiod at the 0.1% significance level. The P, Ca, Mg, and Na contents were not significantly different between the two photoperiods at the 5% significance level.

On a fresh weight basis, the Ca and Mg contents were significantly higher under the 24-h photoperiod at the 0.1% and 1% significance levels, respectively. However, the Mn content was significantly lower under the 24-h photoperiod at the 5% significance level. The P, K, Na, Fe, and Zn contents were not significantly different between the two photoperiods at the 5% significance level.

Ascorbic acid content

On a dry weight basis, the ascorbic acid content tended to be higher under the 24-h photoperiod compared with under the 12-h photoperiod, but there was no significant difference at the 5% significance level between the two photoperiods (Fig. 6). On a fresh weight basis, the ascorbic acid content was 1.8-times higher under the 24-h photoperiod, which was significant at the 5% level.

Chlorogenic acid and total polyphenol contents

The chlorogenic acid content was 2.5-times higher on a dry weight basis and 3.1-times higher on a fresh weight basis under the 24-h photoperiod than under the 12-h photoperiod, and these differences were significant at the 5% and 1% levels, respectively (Fig. 6). Similarly, the total polyphenol content was 1.5- and 1.8-times higher on dry and fresh weight bases, respectively, under the 24-h photoperiod, and these differences were significant at the 1% and 0.1% levels, respectively. There was a positive correlation between chlorogenic acid and total polyphenol contents, with a correlation coefficient of 0.48 on a dry weight basis, whereas the correlation coefficient was 0.64 on a fresh weight basis, indicating a stronger correlation (Fig. 7).

Table 2 - Effects of photoperiod on the stem development of Japanese mugwort plants

Photoperiod (h)	No. of branches	Main stem		
		Length (cm)	No. of nodes	Mean of internode length (cm)
12	15.0	20.65	23.25	0.9
24	22.0	33.10	28.45	1.1
Significance [z]	***	**	***	**

[z] Statistical significances between the means of two photoperiods were determined using Student's t-test (n = 10). **, p < 0.01; ***, p<0.001.

Table 3 - Effects of photoperiod on the mineral contents of Japanese mugwort leaves

Photoperiod (h)	Mineral content on a dry weight basis							
	P (mg g DW^{-1})	K (mg g DW^{-1})	Ca (mg g DW^{-1})	Mg (mg g DW^{-1})	Na (mg g DW^{-1})	Fe (µg g DW^{-1})	Mn (µg g DW^{-1})	Zn (µg g DW^{-1})
12	13.2	48.7	11.5	3.2	0.3	152.2	325.8	60.7
24	10.8	39.1	11.8	3.1	0.3	126.2	211.7	44.3
Significance[z]	NS	**	NS	NS	NS	***	***	**

Photoperiod (h)	Mineral content on a fresh weight basis (mg g FW^{-1})							
	P	K	Ca	Mg	Na	Fe	Mn	Zn
12	138.0	511.9	121.2	33.4	3.1	1.6	3.5	0.6
24	140.9	510.3	154.3	41.0	3.5	1.7	2.8	0.6
Significance[z]	NS	NS	***	**	NS	NS	*	NS

Statistical significances between the means of two photoperiods were determined using Student's t-test (n = 10). NS= not significant; * p < 0.05; ** p < 0.01; *** p < 0.001.

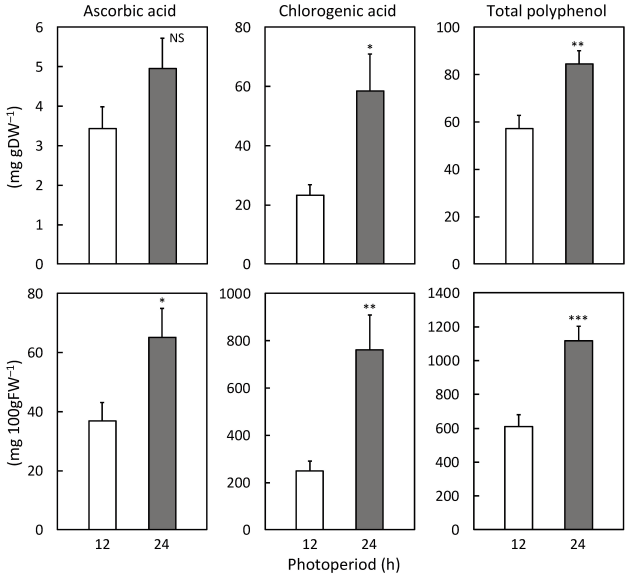

Fig. 6 - Effects of photoperiod on ascorbic acid, chlorogenic acid, and total polyphenol contents in Japanese mugwort leaves. Values represent means ± SEs (n = 10). Statistical significances between the two photoperiods were determined using Student's t-test. NS, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

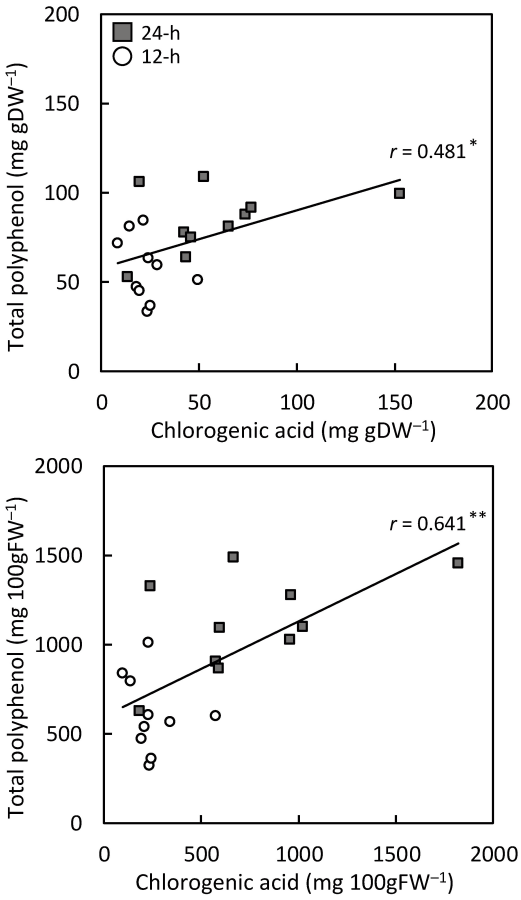

Fig. 7 - Correlations between chlorogenic acid and total polyphenol contents in the leaves of Japanese mugwort plants grown under 12-h (open circles) and 24-h (closed squares) photoperiods. * and ** indicate significant correlations as determined by Pearson's test at p < 0.05 and p < 0.01, respectively (n = 20).

4. Discussion and Conclusions

Change in nutrient solution pH

We reported previously (Hata and Kawamura, 2021) that when growing 'Ibuki-yomogi' plants hydroponically in a greenhouse for 4 weeks at differ-

ent nutrient solution concentrations, the nutrient solution pH increases as the nutrient solution concentration decreases and that after lowering the latter to 25% of the standard, the nutrient solution pH reached 8.1. Similarly, in the present study, the nutrient solution pH increased, even when the nutrient solutions' initial pH was adjusted to 6.0, indicating that the nutrient solutions' pH tended to increase even under artificial light sources, regardless of the photoperiod, by lowering the concentration to 25% of the standard.

When anion uptake is dominant, the rhizosphere pH increases as a result of OH^- or HCO_3^- release from the roots to maintain the cellular charge balance, and when cation uptake is dominant, H^+ is similarly released from the roots and the rhizosphere pH decreases (Hinsinger *et al*., 2003; Fageria, 2012). In particular, because NO_3^- and NH_4^+ account for approximately 70% of the cations and anions absorbed by plants, the form of nitrogen application has a significant effect on rhizosphere pH (nutrient solution pH). Furthermore, Zheng *et al*. (2004, 2010) reported that the medium pH increases when the applied nutrient solution concentration is lowered from 100% to 25% in the pot cultivation of rose and gerbera, indicating that the rhizosphere pH (nutrient solution pH) may not decrease, but rather increase, owing to the lack of NH_4^+ at a low nutrient solution concentration. Because the total nitrogen content in the Enshi formula nutrient solution used in the present study consisted of 92.5% NO_3^- and 7.5% NH_4^+, the plants absorbed less NH_4^+ as the nutrient solution concentration decreased, which may have caused the nutrient solution pH to increase, rather than decrease, during the growing period.

Masuda *et al*. (2001) reported that when pepper plants are cultivated hydroponically with fluorescent lamps under a 24-h photoperiod, the pH of the recirculating nutrient solution rapidly increases immediately after planting. In the present study, similarly, the nutrient solution pH at 2 weeks after planting or 1 week after nutrient solution renewal increased more under the 24-h photoperiod compared with the 12-h photoperiod. On the other hand, Hata and Xu (2020 a) reported that when leaf lettuce is grown hydroponically using a nutrient solution containing NH_4^+ as the nitrogen source, the degree of decrease in nutrient solution pH is greater under the 24-h photoperiod than under the 12-h photoperiod, suggesting that a faster the growth rate, the pH is more likely to decrease. Thus, the faster the growth rate, the greater the change in the pH of the nutrient solution in proportion to the amount of nitrogen absorbed. Consequently, greater growth rates of the mugwort plants under the 24-h photoperiod than under the 12-h photoperiod makes the pH of the nutrient solution more likely to increase, when grown using a low-concentration of nutrient solution.

Plant growths

For plants that are capable of cultivation under longer photoperiods, the production cost per plant in a fully artificial light-type plant factory decreases as the photoperiod increases, and a 24-h photoperiod is desirable (Takatsuji, 2012). Plants capable of longer photoperiods are highly tolerant of the continuous light injury that occurs under a 24-h photoperiod, and their growth is greatly accelerated. The occurrence of a marked level of continuous light injury in Asteraceae plants has not been reported to date, and maximum plant growth rates have been reported under 24-h photoperiods in lettuce and garland chrysanthemum (Hata *et al*., 2011 a, b). This was also the case for the Japanese mugwort plants used in the present study. Continuous light-induced chlorosis did not occur in newly developed leaves, the leaf color darkened under the 24-h photoperiod, and the dry weights of leaves, stems, and roots at the end of cultivation were nearly two-fold greater under the 24-h photoperiod than under the 12-h photoperiod. Thus, like lettuce and garland chrysanthemum, Japanese mugwort, which is a member of the Asteraceae family, is not susceptible to continuous light injury. Thus, a 24-h photoperiod could be used to increase the leaf yield and productivity of Japanese mugwort in a fully artificial light-type plant factory.

Stem elongation in plants is inhibited by greater red to far-red light ratios (R/FRs), whereas lower R/FRs may promote plant stem elongation owing to greater internode elongation (Demotes-Mainard *et al*., 2016; Ballaré and Pierik, 2017). White fluorescent light has a higher R/FR ratio than sunlight (6.5–9.6 and 1.0, respectively) (Hamamoto and Yamazaki, 2013), and internode elongation is likely to be suppressed in a plant factory environment that uses such fluorescent lighting. In fact, in our previous report using the same 'Ibuki-yomogi' seeds and hydroponic cultivation method, the average internode length of individuals grown in a glasshouse was 2.3 cm (Hata and Kawamura, 2021), whereas the average internode length of individuals grown in an artificial growth room, as in the present study, was 0.9-1.1 cm. The

internode shortening under fluorescent light is advantageous for producing young leaves of Japanese mugwort plants because of the low plant heights in the multi-shelf cultivation system used in plant factories. In addition, the number of branches was significantly higher under the 24-h photoperiod than under the 12-h photoperiod, suggesting that cultivation under the former is advantageous for increasing the number of harvested stems.

Inorganic component contents

The carbon content increases, whereas other essential inorganic element contents generally decrease, in many plant species when grown at greater than atmospheric CO_2 concentrations (Loladze, 2014; Soares *et al*., 2019). The factors responsible for the decrease in these inorganic elements include (1) a decreased transpiration rate leading to a lowered absorption, and (2) an increased carbon content which results in a reduced element relative content (dilution by carbohydrates). Although there have been limited studies on the effects of a 24-h photoperiod on the inorganic component contents in plants, Hata and Xu (2020 b) found that when leaf lettuce is grown under a 24-h photoperiod, the leaf carbon content increases more compared with under a 12-h photoperiod, whereas many inorganic component contents decrease, suggesting that reactions similar to those under high CO_2 conditions occur under a 24-h photoperiod. The K, Fe, Mn, and Zn contents per dry weight of Japanese mugwort in the present study were also significantly lower under the 24-h photoperiod compared with the 12-h photoperiod, suggesting that there may be a number of plant species in which the inorganic component contents tend to decrease under a 24-h photoperiod. As in leaf lettuce (Hata and Xu, 2020 a, b), no clear nutrient deficiency symptoms associated with decreased inorganic component contents were observed in Japanese mugwort in the present study under a 24-h photoperiod, but lower-leaf browning was observed in some individuals, suggesting that the potassium concentration in the culture medium requires optimization.

Ascorbic acid content

Ascorbic acid in plants is synthesized through the D-Man/L-Gal pathway, in which D-fructose, a photosynthetic product, is used as a metabolic intermediate to synthesize D-mannose and L-galactose (Venkatesh and Park, 2014). In leaf lettuce, the ascorbic acid content per fresh weight increases 1.3-fold when grown under a 24-h photoperiod compared with under a 16-h photoperiod owing to an increase in the activity of L-galactono-1,4-lactone dehydrogenase, an enzyme that converts L-galactono-1,4-lactone, an ascorbic acid precursor, to ascorbic acid (Zha *et al*., 2019). In the present study, the ascorbic acid content per fresh weight was 1.8-times higher under the 24-h photoperiod than under the 12-h photoperiod, which was consistent with previous results. This is suggested that a 24-h photoperiod may be used effectively in the production of crops having enhanced ascorbic acid contents, unless the target plants develop continuous light injuries. The addition of ascorbic acid suppresses the degradation of polyphenols, such as chlorogenic acid, during apple juice processing (Kolniak-Ostek *et al*., 2013), indicating that the increased ascorbic acid content in Japanese mugwort leaves may contribute to the increased stability of polyphenols, such as chlorogenic acid, during utilization.

Chlorogenic acid and total polyphenol contents

Hata and Xu (2020 b) reported that the chlorogenic acid and total polyphenol contents per dry weight increased by 1.5 to 4.2 times and 1.1 to 1.2 times, respectively, in leaf lettuce grown under a 24-h photoperiod compared with under a 12-h photoperiod. Furthermore, the differences between the two photoperiods widened in the chlorogenic acid and total polyphenol contents per fresh weight because the dry matter ratio increased more under the 24-h photoperiod than under the 12-h photoperiod. In the present study, the chlorogenic acid content was 2.5- and 3.1-times higher per dry and fresh weights, respectively, and the total polyphenol content was 1.5- and 1.8-times higher per dry and fresh weights in Japanese mugwort under a 24-h photoperiod compared with a 12-h photoperiod. These results were similar to those previously reported for leaf lettuce (Hata and Xu, 2020 b), and they suggest that a 24-h photoperiod may be effectively used for the production of crops with high polyphenol contents, such as chlorogenic acid, unless target plants develop continuous light injuries.

We reported previously (Hata and Kawamura, 2021) that when 'Ibuki-yomogi' plants are grown hydroponically in a glasshouse, the chlorogenic acid and total polyphenol contents of the leaves increase

as the nutrient solution concentration decreases to 25% of the standard. There were positive correlations (r = 0.45) between chlorogenic acid and total polyphenol contents, both per dry weight and per fresh weight bases. In the present study, positive correlations between chlorogenic acid and total polyphenol contents (r = 0.48 for content per dry weight and r = 0.64 for content per fresh weight) were also observed, suggesting that the increase in the former largely contributed to the increase the latter under a 24-h photoperiod. In addition, high anthocyanin pigment accumulations are often observed in some plant species under a 24-h photoperiod (Hata *et al*., 2012 b), and here, we observed some individuals accumulating anthocyanin pigments in the main stems under the 24-h photoperiod. This suggests that anthocyanin synthesis is also enhanced in Japanese mugwort under a 24-h photoperiod and that increases in some flavonoid compounds may also contribute to the increase in the total polyphenol content.

These results indicate that it is possible to grow Japanese mugwort hydroponically under a 24-h photoperiod and plant factory conditions in a nutrient solution having a concentration as low as 25% of the standard. In addition, under the 24-h photoperiod, plant growth was greatly accelerated and chlorogenic acid, a useful secondary metabolite, as well as ascorbic acid, contents increased, suggesting that a 24-h photoperiod is highly beneficial for Japanese mugwort production in a fully artificial light-type plant factory. However, because the pH of the nutrient solution fluctuated drastically during the cultivation period, it is necessary to investigate separately the composition of the nutrient solution suitable for a 24-h cultivation photoperiod.

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Effect of different nitrogen forms and bio-treatments on the growth and seed yield of downy safflower (*Carthamus lanatus*)

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Key words: Arbuscular mycorrhiza fungi, Downy safflower, nitrogen fertilization, *Trichoderma viride*, vermicompost.

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Abstract: A field experiment was carried out to investigate the effect of different nitrogen forms and some biotreatments (*Trichoderma viride*, vermicompost and arbuscular mycorrhiza fungi) alone or in combination on vegetative growth, seed yield and some chemical traits of downy safflower (*Carthamus lanatus* L.). Nitrogen was supplied as ammonium sulfate, ammonium nitrate and urea at the rates (5, 3 and 2 g/plant, respectively). Bio treatments included *Trichoderma viride*, vermicompost and arbuscular mycorrhiza fungi. The results showed that all nitrogen forms significantly increased the plant growth and yield, pigments content, and total carbohydrates in leaves and seeds, as well as N, P and K%, total phenols and oil content in seeds. All bio treatments significantly increased the tested parameters compared to control. The integration of ammonium sulfate with *T. viride* was the most effective treatment since determined the highest increases of the tested traits. Results showed that for enhancing downy safflower plant growth, and nutritional values of seed, the combined treatment of *T. viride* at 5 ml/plant and ammonium sulfate at 5 g/plant is recommended.

1. Introduction

Carthamus lanatus L. (also called downy safflower, woolly distaff thistle or saffron thistle) is an erect spiny biennial plant native of the Mediterranean region. It is closely related to safflower, which is in the same genus. Downy safflower is reported to be sudorific (sweat inducing), fever-reducing and anthelmintic (Hellwig, 2004; DiTomaso *et al*., 2017; Adel El-Gazzar *et al*., 2019), Previous studies revealed its importance due to different components of diverse chemical nature such as flavonoids, sesquiterpenes glycosides, lipids, aromatic acids, sterols, triterpenes , volatiles alkaloids, tannins and saponins (Abu El-Khair, 2020).

Plant nutrition is one of the most essential factors which increase plant production. Nitrogen (N) is the most recognized in plant as it is present in the structure of the protein molecule and plays a vital role in syn-

thesis of plant compositions via the action of various enzymes activities and protein synthesis (Taiz and Zeiger, 2002). Nitrogen has an important role in plant metabolism that impacts quantitative and qualitative plant production by enhancing the growth and stimulating the essential processes which leads to increase the active substances. Ammonium sulfate (AS), ammonium nitrate (AN) and urea are the main forms of inorganic N fertilizers and are extensively utilized in modern agriculture. AS application was better than AN and urea for increasing vegetative growth yield parameters, chlorophyll content, NPK % in seeds and seed oil % of sunflower and jojoba (El Mantawy, 2017; El Sayed, 2020; Hegab *et al*., 2021).

Trichoderma is a genus of saprotrophic fungi and a widespread component of the soil rhizosphere; it has been reported to enhance plant growth and to control many of plant diseases (Colla *et al*., 2014). One of the well-known stimulatory effects of *Trichoderma* on plants is the ability to dissolve phosphate through acidification, chelation or redox activity to improving the utilization by plants (Mansour *et al*., 2021). The benefits of *Trichoderma* species in stimulating plant growth can be realized via various mechanisms including boost nutrient uptake, solubilization, sequestration of inorganic nutrients and enhancement of root hair development (Harman, 2006; Lorito *et al*., 2010). *Trichoderma* spp. promote plant hormone synthesis that improve root growth and root hair formation which lead to more efficient use of nitrogen, phosphorus, potassium and micronutrient (Mastouri *et al*., 2010). Moreover, *Trichoderma* is able to produce metabolites with hormonal activities such as indole-3-acetic acid (Contreras-Cornejo *et al*., 2011). The positive impact of *T. viride* inoculation on physiological and biochemical features of plants has been reported by many authors. The fungus is able to improve growth and yield parameter (Ghoneem *et al*., 2019), promote photosynthetic pigments (Kumar *et al*., 2015; Ghoneem *et al*., 2019), enhance nutrient status in leaves and roots (Metwally, 2020), increase essential oil and total phenol content (Shaikh *et al*., 2019; Hassanin *et al*., 2020; Sanei and Razavi, 2018; Ghoneem *et al*., 2019) and promote peroxidase activity.

Vermicompost is an organic product that is obtained from biodegradation and stabilization of organic waste via the interaction between earthworms and microorganisms, lead to break up organic matter residues into fine particles (Ndegwa and Thompson, 2001; Campitelli and Ceppi, 2008). It has a favorable effect on the physical and chemical structure of soil as well as plant growth (Bachmana and Metzger, 2008). Additionally, it induces and boosts the absorption of nutrients by plants and favors a biological control of bacterial and fungal plant pathogens (Rivera and Wright, 2009). It has high microbial and enzymatic activity and contains large amounts of plant growth regulators like auxins, gibberellins cytokinins, macronutrients and micronutrients (Atiyeh *et al*., 2002). The favorable effect of vermicompost application on the growth and yield of many plants has been reported by previous studies (Adamipour *et al*., 2019; Levinsh, 2020; Abd El-Hamed *et al*., 2021).

Arbuscular mycorrhizal fungi (AMF) are soil fungi which are prevalent in most agricultural ecosystems to associate with more than 80% of plant species (Wang and Qiu, 2006). Previous studies have shown that plant inoculation with AMF improves growth, seeds yield, promotes photosynthetic pigments and carbohydrates content, enhances accumulation of macro- and micronutrients in leaves (Amiri *et al*., 2017; Gashgaril *et al.,* 2020; Mohamed, 2020), as well as increases the nutritional values of seeds like proteins and oil percentage (Ashour *et al*., 2021).

Although the beneficial roles of nitrogen and bio treatments on medicinal and aromatic crops and their valuable effect on improving growth and production, there are no sufficient available data about their effectiveness on the growth and yield of downy safflower plants. Therefore, this research is aimed to evaluate the influence of different nitrogen forms (ammonium sulfate, ammonium nitrate and urea) and some biotreatments (*T. viride*, vermicompost or arbuscular mycorrhiza fungi) on vegetative growth, seed yield and some chemical parameters of downy safflower (*Carthamus lanatus*) plant.

2. Materials and Methods

The field experiment was conducted at the Experimental area of the Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza Governorate during the two successive seasons of 2019/2020 and 2020/2021. The latitude, longitude and altitude of the experimental site was 0°01'92.70" N, 31°20'68.08" E and 22 m above sea level, respectively.

Experimental procedure

Seeds of *Carthamus lanatus* plants were acquired

from experimental farm of Faculty of Pharmacy, Cairo University. On 1st November (of the two consecutive years), seeds were sown in a seedling trays (50 x 90 cm diameter) at saran house with 42% shading, 28/18°C (day/night) temperature, 14 h light conditions, and 30-35% relative humidity. After 30 days from seeds sowing, uniform seedlings, with an average height of 18-20 cm, were transplanted in the experimental open field in plots (3×3 m), with a distance of 50 cm among rows, 70 cm between plants. Some physical and chemical properties of the experimental soil (average value of the two seasons) were determined according to Jackson (1973), and the results are presented in Table 1.

Nitrogen fertilization included ammonium sulfate (21 %N and 23-24%S) at 5 g/plant, ammonium nitrate (33 %) at 3 g/plant and urea (46% N) at 2 g/plant. Nitrogen forms were applied as two separate doses. The first addition was before transplanting and the second was before flowering.

Plants treated with nitrogen forms were also inoculated with *T. viride*, vermicompost and arbuscular mycorrhiza fungi (Amf), the control plants were not treated. *T. viride*, were obtained from Pest Rearing Department, Central Agricultural Pesticides Laboratory, Agricultural Research Centre, Dokki, Giza, Egypt. 10^9cfu/ml conidial suspension of *T. viride* was diluted in 5 liters of water so as to prepare solution strength of $2X10^5$cfu/ml. For each seedling, 100 ml of solution was used which accounted $2X10^7$cfu of Trichoderma per seedlings. 100 ml of the solution was used to drench the soil per seedlings (Mastouri *et al*., 2010; Chirino-Valle *et al*., 2016).

Table 1 - Physical and chemical properties of experimental soil (mean of two seasons)

Soil characteristics	Data
Physicical characteristics	
Soil Texture	Clay
Clay (%)	43.30
Coarse sand (%)	4.20
Fine sand (%)	21.70
Silt (%)	30.80
Field capacity (V %)	67.85
Chemical characteristics	
Macro-nutrients (%)	
N	94.19
P	21.29
K	59.64
Organic matter (%)	1.76
$CaCO_3$ (%)	1.54
Electrical conductivity (dS/m)	1.54
Cation exchange capacity (meq/100 g)	40.22
pH	7.27

$CaCO_3$= calcium carbonate, pH= soil acidity.

Vermicompost was acquired from Central Laboratory for Agricultural Climate (CLAC), Agriculture Research Center, Ministry of Agriculture, Giza, Egypt. It was applied at 5 g/seedling. Chemical analyses of vermicompost used in this work (average value of the two seasons) are shown in Table 2.

Amf inoculum contained roots, hyphae, spores colonized by *Glomus mosseae* NRC31 and *Glomus fasciculatum* NRC15 obtained from Agricultural Microbiology Department, National Research Center, Dokki, Giza, Egypt. Inoculum material consisted of 275 spores g^{-1} (the infectivity 10^4 propagola). AMF inoculation treatments were carried out by injecting 5 g/seedling of the inoculum.

The three bio treatments were applied as two doses, the first addition was after 3 weeks from transplanting (21th December in both seasons, respectively) and the second was after 2 months from transplanting at branching start (21th February in two seasons, respectively). Irrigation, manual weeding, pest and diseases control were done when needed.

The layout of the experiment was factorial 4x4 in randomized complete blocks design with 16 treatments. The first factor was 4 nitrogen forms (including the control). The second factor was 4 biotreatments (including the control) with 3 replicates, each replicate consisting of 32 plants (2 plants from each treatment).

Vegetative growth and yield parameters measurement

Vegetative growth parameters were registered after 120 days from transplanting (On 1st April). Two

Table 2 - Chemical analysis of vermicompost used in this work (mean of two seasons)

Properties	pH	EC (dS/m)	Organic matter (%)	N (%)	P (%)	K (%)	Fe ppm	Zn ppm	Mn ppm
Vermicompost	8.41	6.6	42.9	1.65	1.14	1.69	166	109	96

samples of plants were taken and used to measure growth parameters including plant height (cm), number of branches/plant, stem diameter (cm, at 5 cm above the soil surface), fresh and dry weights of leaves, stems and roots as well as leaf area (cm^2). At the harvesting stage (on 1st to15th May) yield parameter were measured: number of flower heads/plant, weight of flower heads/plant, weight of seeds/plant and weight of 100 seeds (gr).

The seed content of total carbohydrates, (N, P and K), total phenols and oil were also determined.

Chemical analysis

The chemical analysis were performed at the end of each season (on 1st to15th May).

Chlorophyll and carotenoid contents. Chlorophyll pigments including Chl a, Chl b and carotenoid contents (mg g^{-1}) were determined according to Lichtenthaler and Buschmann (2005), leaves extracted by suspending them in 5 ml of 95% aqueous acetone at 60° C then the total volume completed to 10 ml with 95% aqueous aceton. The aqueous acetone supernatant was then taken for spectrophotometric measurement. A blank of acetone was taken at wavelengths of 663, 645 and 452.5 nm respectively, and data were then calculated using the following equations:

Chlorophyll a (mg g^{-1}) = 0.0127 A663 – 0.00269A645
Chlorophyll b (mg g^{-1})= 0.0029A663 – 0.00468A645
Carotenoids (mg g^{-1}) = 4.2 E 452.5 – 0.0264

Total carbohydrates. Total carbohydrates content in leaves and seeds (percentage of dry matter) was determined in dried samples according to Dubois *et al*. (1956). A known weight (0.1 g) of the dried samples was completely hydrolyzed with 10 ml sulphuric acid (67%) in a test tube on a boiling water bath for one hour. The solution was decolorized and the filtrate was diluted to 100 ml with distilled water. A known volume (1 ml) of the extract was taken in a test tube, to which 1 ml phenol solution (5%) was added, followed by 5 ml of concentrated sulphuric acid. The optical density of the resulting color was measured at 490 µm, using a spectrophotometer, against a blank reagent. The standard curve of glucose was used to calculate the total carbohydrates concentration in the extract.

N, P and K content of seeds. Half gram of dried seeds samples was digested using tertiary acid mixture ($HClO_4$ + HNO_3 + $H2SO_4$) and the extract was analyzed to determine concentrations of N, P and K (as percentage of dry seeds) according to Estefan *et al*. (2013). Nitrogen concentration was determined by using the micro-Kjeldahl method. Phosphorus was determined calorimetrically by using the chlorostannous molybdophosphoric blue colour method in sulphuric acid. Potassium was determined by using the flame photometer apparatus (CORNING M 410, Germany).

Total phenolics content. Total phelolics content was determined in the seeds extract by using the Folin Ciocalteau's reagent colorimetric method and results are expressed as milligram of gallic acid equivalent per gram of seeds dry weight extract (mg GAE/g DW) (John *et al*., 2014). Briefly, 1 mL of seed extract was mixed with 2.5 mL of 10% (w/v) Folin-Ciocalteu reagent. After 5 min, 2.0 mL of Na_2CO_3 (75%) was subsequently added to the mixture and incubated at 50°C for 10 min with intermittent agitation. Afterwards, the sample was cooled and the absorbance was measured utilizing a UV Spectrophotometer (Shimazu, UV-1800) at 765 nm against a blank without extract. The outcome data were expressed as mg/g of gallic acid equivalents in milligrams per gram (mg GAE/g) of dry extract.

Seed oil (%). The oil content of seeds was determined according to AOAC (1995) using soxhlet apparatus using petroleum ether as a solvent. The clean air dried seeds were separately crushed in a Willey mill, then extracted in Soxhlet apparatus, samples of 10 g of seeds were moved into Soxhlet apparatus in 100 ml of N-hexane and the extraction period extended to 6 hours (30-36 syphon cycle approx.). The N-hexane extract was dried over anhydrous sodium sulfate, then filtered and the oil was obtained by distillation under vacuum. oil % was calculated according to the equation:

oil % = extracted oil weight (g)/seeds sample weight (g)/× 100.

Statistical analysis

Results of the two field trials performed in two different growth seasons were combined in order to obtain an average value for each parameter. The means of all results were subjected to Two-Ways analysis of variance (ANOVA) in randomized complete blocks design. Means of data were compared by using Duncan's multiple range tests at P = 5% (Snedecor and Cochran, 1989).

3. Results and Discussion

Vegetative growth parameters

The data in Table 3, 4 and figure 1 revealed that

Table 3 - Mean square for the effect of nitrogen forms and biotreatments and their interaction on vegetative growth, yield parameters of *Carthamus lanatus*

Traits	Source of variation				
	Treatment			Error	CV
	Biotreatments (A)	Nitrogen sources (B)	(A × B)		
Plant height (cm)	4102.63 ***	3164.686 ***	133.843 ***	2.236	0.926
No. of branches/plant	123.894 ***	140.852 ***	18.727 ***	0.785	6.276
Stem diameter (cm)	0.779 ***	0.888 ***	0.058 ***	0.011	8.215
Fresh weight of leaves (g/plant)	3539.035 ***	2397.09 ***	484.993 ***	3.234	1.495
Dry weight of leaves (g/plant)	345.389 ***	253.726 ***	47.235 ***	1.86	3.728
Fresh weight of stems (g/plant)	56027.069 ***	16413.722 ***	1476.819 ***	9.154	1.383
Dry weight of stems (g/plant)	3390.971 ***	989.016 ***	74.53 ***	0.902	1.799
Fresh weight of roots (g/plant)	1451.436 ***	1788.839 ***	476.746 ***	5.688	4.887
Dry weight of roots (g/plant)	117.628 ***	128.286 ***	25.247 ***	0.611	6.305
Leaf area (cm^2)	870.237 ***	1304.444 ***	115.687 ***	0.734	2.09
No. of flower heads/ plant	453.436 ***	382.616 ***	46.209 ***	1.077	6.178
weight of flower heads/ plant	388.412 ***	606.905 ***	45.35 ***	1.783	3.706
weight of seeds/ plant	964.929 ***	1057.951 ***	102.45 ***	0.736	3.534
weight of 100 seeds/ plant	0.549 ***	0.799 ***	0.072 ***	0.002	1.993

*, **, *** significant at P≤0.05, P≤0.01, P≤0.001 respectively, (n=3).

Table 4 - Plant height, No. of branches/plant, Stem diameter, Fresh and dry weights of leavesof *Carthamus lanatus* as affected by the interaction between nitrogen forms and biotreatments (mean of two seasons)

Biotreatments (A)	Nitrogen forms (B)	Plant height (cm)	No. of branches/plant	Stem diameter (cm)	Fresh weight of leaves (g/plant)	Dry weight of leaves (g/plant)
Control	Control	124.33±0.60 k	7.00±0.58 g	0.70±0.05 e	86.50±2.18 i	26.07±0.81 h
	AS	140.83±1.17 gh	11.33±0.33e	1.15±0.06 c	101.83±0.67 g	30.97±0.48 fg
	AN	130.83±0.44 j	9.33±0.44 f	0.82±0.06 dc	96.83±1.17 h	28.20±0.9 h
	N-urea	139.67±1.59 hi	11.50±0.29 e	1.17±0.03 c	107.50±0.76 f	34.03±0.12 e
T. viride	Control	148.67±0.88 f	11.00±0.29 e	0.93±0.07 d	109.17±1.01 f	34.07±0.62 e
	AS	189.00±0.76 a	23.17±0.17 a	1.87±0.03 a	178.33±1.42 a	54.57±1.4 a
	AN	175.50±1.50 d	15.50±0.29 c	1.53±0.09 b	136.17±2.6 c	41.42±0.86 c
	N-urea	179.50±0.29 c	17.83±0.44 b	1.53±0.06 b	136.67±1.86 c	41.63±0.78 bc
Vermicompost	Control	137.83±1.17 i	9.17±0.33 f	1.30±0.06 c	110.17±0.44 f	30.63±1.12 g
	AS	184.17±0.60 b	13.33±0.73 d	1.62±0.09 b	142.67±1.09 b	43.65±0.71 b
	AN	175.83±1.09 d	15.33±0.93 c	1.52±0.01 b	107.5±1.61 f	32.98±0.61 ef
	N-urea	179.67±0.88 c	16.50±0.87 bc	1.65±0.01 b	128.17±0.83 d	38.37±0.59 d
Amf	Control	142.67±1.17 g	11.17±0.33 e	0.82±0.03 de	108.33±0.88 f	32.70±0.73 e-g
	AS	187.00±0.76 a	21.83±0.93 a	1.52±0.07 b	121.17±0.67 e	36.48±0.81 d
	AN	169.50±1.04 e	15.17±0.44 c	1.15±0.01 c	118.67±0.44 e	38.02±0.46 d
	N-urea	179.50±0.76 c	15.33±0.33 c	1.57±0.02 b	134.67±0.83 c	41.55±0.42 bc

Amf= Arbuscular mycorrhiza fungi, AS= ammonium sulfate, AN= ammonium nitrate. Data represent the mean value ±S.E. the mean of three replicates. Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

under the same level of N forms, application of *T. viride*, vermicompost or Amf treatments resulted in significant increase of tested vegetative growth parameters (*viz*., plant height, number of branches/plant, stem diameter, fresh and dry weights of leaves, stems and roots and leaf area) compared to control. Among the tested treatments, application of *T. viride* appeared to be the most effective treatment since

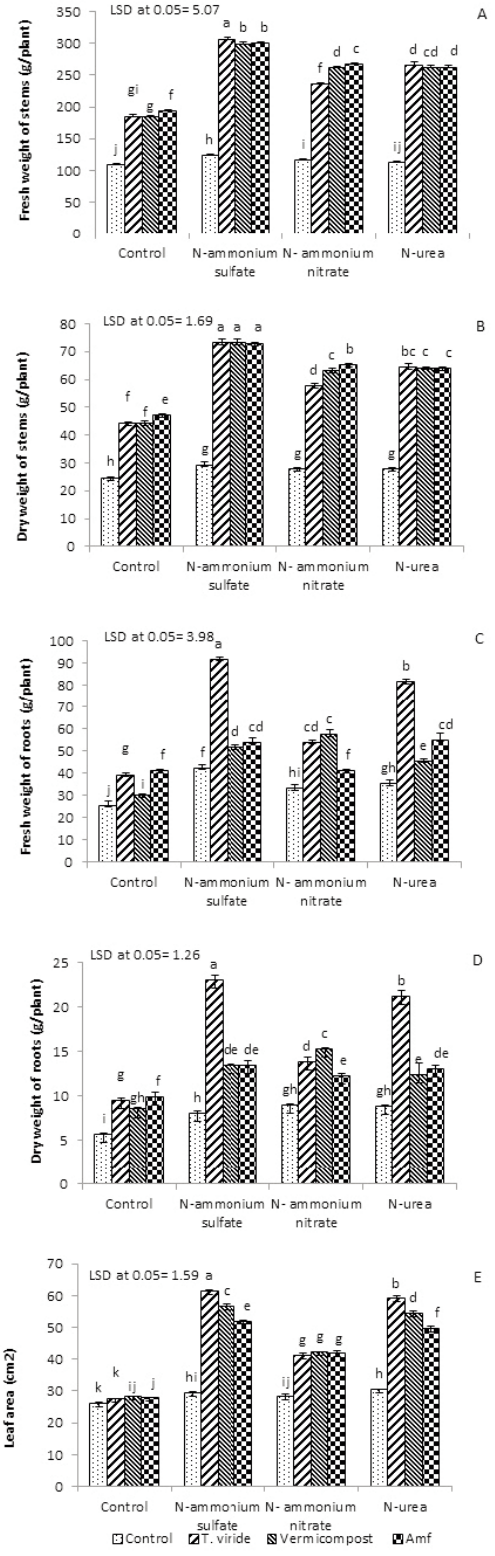

Fig. 1 - Fresh weight of stems (g/plant) (A), dry weight of stems (g/plant) (B), fresh weight of roots (C), dry weight of roots (D), leaf area (E) of *Carthamus lanatus* as affected by the interaction between nitrogen forms and bio treatments (mean of two seasons). Column with different letters indicate a significant difference at 5% level. Data represent the mean values ± SE the mean of three replicates.

registered the highest values. These results are in line with those findings of prior authors (Lakshman and Ghodke, 2018; Shaikh and Mokat, 2018; Ghoneem *et al*., 2019; Guo *et al*., 2020; Hassanin *et al*., 2020; El-Dabaa *et al*., 2021), who reported increases in vegetative growth parameters due to *T. viride* inoculation.

The useful effect of *T. viride* on vegetative growth parameters may be related to participation of such microorganisms in biotransformation of cellulose, increasing cell reproduction, nitrogen mineralization and phosphorus solubilization. They also increase the volume of roots which in turn, increases absorption of water and nutrients, consequently increasing both growth and yield of the crops (Nepali *et al*., 2020).

The data in Table 4 and figure 1 also revealed that under the same rate of bio treatment (*T. viride*, vermicompost or Amf) treating the plants with different nitrogen forms resulted in significant increase in vegetative growth parameters compared to control and among the tested nitrogen forms, with the application of ammonium sulfate leading to superior growth compared with ammonium nitrate or urea. The increases in vegetative growth parameters due to ammonium sulfate treatments are in agreement with the findings of several studies on different plants including *Cynara cardunculus* (Sarhan *et al*., 2014), *Urtica pilulifera* (Wahba *et al*., 2014), *Nigella sativa* (Khalid and Shedeed, 2015), Sunflower (El Mantawy, 2017; El Sayed, 2020), *Thymus vulgaris* (Basal *et al*., 2019) and Jojoba (Hegab *et al*., 2021).

The positive effect of ammonium sulfate may be attributed to the role played by the acidic component that decrease the values of soil pH and thus simplify the uptake of nutrients by the plant roots (Fouda, 2017).

Yield parameters

Results of figure 2 indicated that within each level of N forms, in most cases, application *T. viride*, vermicompost or Amf treatments caused a significant increase in yield parameters (namely, No. of flower heads/plant, weight of flower heads/plant, weight of seeds/plant, weight of 100 seeds) compared to control. *T. viride* treatment appeared to be the most effective one since recorded the highest values. Increases in yield parameters due to *T. viride* treatments are matched well with those of previous studies on different crops including *Coriandrum sativum* (Khan and Parveen, 2018), *Triticum aestivum* (Mahato *et al*., 2018), *Cuminum cyminum* (Ghoneem *et al*., 2019).

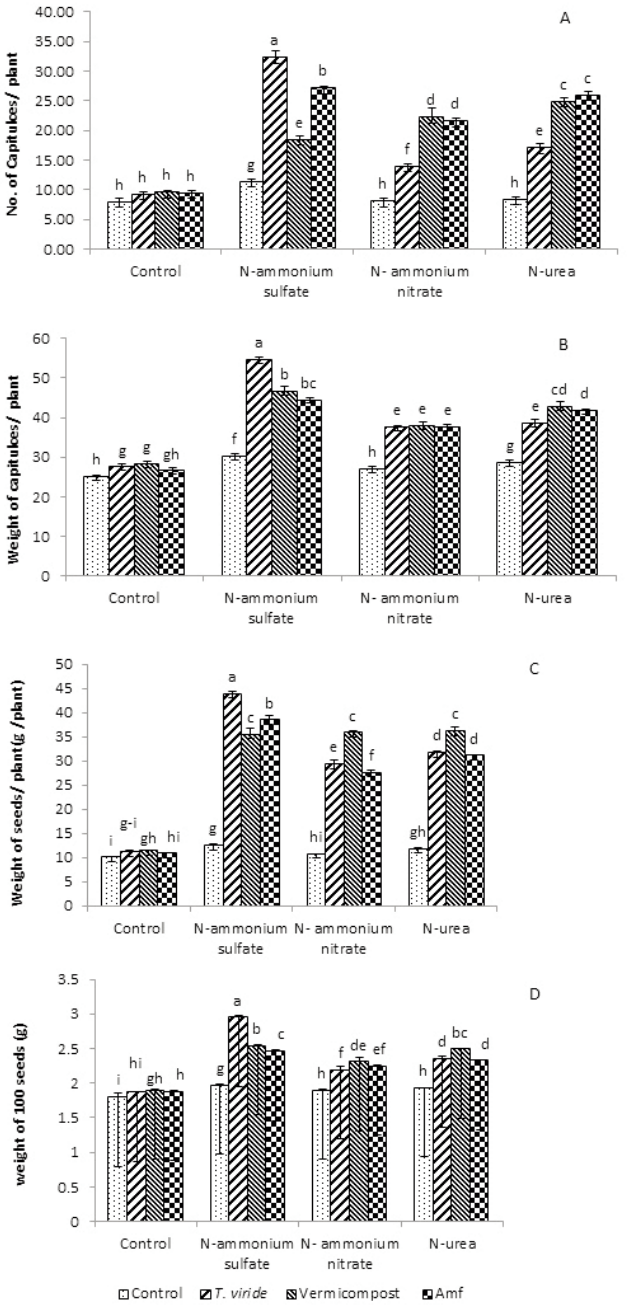

Fig. 2 - Evolution of total phenolic content in season 2018 (mg gallic acid/100 g of fresh fruit) of four selected farms (131, 272, 351, 432) and their average trend. Bars represent standard error of the mean (±SEM). Values followed by the same letter in every sampling point are not significantly different from each other. Mean separation by LSD test (P≤0.05).

Data in figure 2 also exhibited that within each rate of *T. viride*, vermicompost or Amf, the plants treated with nitrogen forms had significantly higher values of yield parameters, in most cases, than those of control. Among the tested nitrogen sources, the most effective one was ammonium sulfate, for which the highest mean value was found. The increases in yield parameters due to ammonium sulfate treatments are the same as the results of Sarhan *et al*., 2014 on *Cynara cardunculus*, Wahba *et al*. (2014) on *Urtica pilulifera*, Khalid and Shedeed (2015) on *Nigella sativa*, El Mantawy (2017); El Sayed (2020) on *Helianthus annuus*, Prinsloo and Plooy (2017) on *Sutherlandia frutescens*, Hegab *et al*. (2021) on *Simmondsia chinensis*.

Contents of pigments and total carbohydrates in leaves

As shown in Table 5 and 6 within each rate of N forms, in most cases, application of *T. viride*, vermicompost or Amf rates resulted in significant increase in the mean values of pigments content (chlorophyll a, b and carotenoids) and total carbohydrates in leaves compared to control. The highest mean values were found for the plants treated with *T. viride*. Such increase in pigments content or total carbohydrates in leaves due to *T. viride* inoculation is in accordance with those obtained by previous reports on *Salvia officinalis* (Kumar *et al*., 2015), *Cuminum cyminum* (Ghoneem *et al*., 2019), *Allium cepa* (Metwally, 2020).

Chlorophyll is used by plants for light-trapping and energy transduction during the anabolic process of photosynthesis. A higher content of photosynthetic pigments can be correlated to the augmentation in carbohydrates content of leaves.

Results of Table 6 also pointed out that within biotreatments (*T. viride*, vermicompost or Amf), in most cases, the mean values of pigments content and total carbohydrates in leaves of the plants treated with various nitrogen forms were significantly higher than the control. Nitrogen in the form of ammonium sulfate was superior to the other two nitrogen sources for enhancing the value of the parameters considered. The results are analogy with that recorded by earlier research (Sarhan *et al*., 2014; Wahba *et al*., 2014; El Mantawy, 2017), they reported increase in pigments content or total carbohydrates in leaves due to application of ammonium sulfate.

The superior effect of ammonium sulfate in increasing pigments contents may be related to sulfur element that is a constituent of succinyl Co-A which involved in chlorophyll synthesis in leaves and its activation at cellular level enhances photosynthesis that eventually boost vegetative growth.

Table 5 - Mean Square for the effect of nitrogen forms and bio treatments and their interaction on some chemical constituents of *Carthamus lanatus*

Traits	Source of variation				
	Treatment			Error	CV
	bio treatments (A)	Nitrogen sources (B)	(A × B)		
Chlorophylls A content (mg/g f.w)	14.545 ***	9.932 ***	0.801 ***	0.002	2.18
Chlorophylls B content (mg/g f.w)	8.327 ***	71.103 ***	3.727 ***	0.112	2.932
Carotenoids content (mg/g f.w)	0.288 *	0.268 *	0.148 **	0.073	7.57
Total carbohydrates [%] in leaves	35.699 ***	55.094 ***	2.029 *	0.771	4.30
Total carbohydrates [%] in seeds	22.491 ***	47.094 ***	2.901 *	1.188	4.68
N% in seeds	1.682 **	1.761 **	0.102 *	0.158	17.71
P% in seeds	0.036 ***	0.029 ***	0.004 **	0.002	9.91
K% in seeds	0.078 ***	0.161 ***	0.01 *	0.004	4.58
Total phenols [%] in seeds	0.568 ***	0.795 ***	0.068 ***	0.003	1.79
Oil [%] in seeds	29.47 ***	48.638 **	0.423 *	1.498	4.63

*, **, *** significant at P≤0.05, P≤0.01, P≤0.001 respectively, (n=3).

Table 6 - Pigments, total carbohydrates in leaves as affected by the interaction between nitrogen forms and bio treatments (mean of two seasons)

Bio treatments (A)	Nitrogen forms (B)	Chlorophylls A content (mg/g f.w.)	Chlorophylls B content (mg/g f.w.)	Carotenoids content (mg/g f.w.)	Total carbohydrates [%] in leaves
Control	Control	3.68±0.03 k	7.04±0.12 i	2.64±0.57 b	15.44±0.59 g
	AS	5.06±0.01 i	13.74±0.07 c	3.55±0.01 a	19.88±1.15 d-f
	AN	4.09±0.11 j	11.06±0.33 f	3.58±0.01 a	16.22±0.58 g
	N-urea	5.88±0.04 g	11.27±0.1 ef	3.58±0.01 a	20.18±0.52 c-e
T. viride	Control	5.88±0.04 g	7.28±0.05 i	3.60±0.03 a	18.90±0.58 ef
	AS	8.24±0.09 a	15.26±0.14 a	3.88±0.06 a	24.37±1.15 a
	AN	6.11±0.09 f	12.13±0.34 d	3.63±0.02 a	21.32±0.57 b-e
	N-urea	6.74±0.02 d	12.26±0.21 d	3.65±0.02 a	23.02±1.73 ab
Vermicompost	Control	6.62±0.07 de	8.95±0.02 h	3.57±0.08 a	17.28±1.15 fg
	AS	7.8±0.14 b	13.78±0.04 bc	3.59±0.11 a	22.58±1.21 a-c
	AN	5.92±0.11 fg	11.79±0.02 de	3.63±0.09 a	21.21±0.64 bc-e
	N-urea	7.83±0.10 b	14.31±0.06 b	3.57±0.07 a	22.42±0.59 a-d
Amf	Control	5.50±0.02 h	8.78±0.35 h	3.55±0.06 a	18.90±0.58 ef
	AS	7.30±0.05 c	11.11±0.05 f	3.64±0.13 a	21.55±0.55 bcd
	AN	6.45±0.12 e	10.11±0.31 g	3.70±0.04 a	20.67±0.57 b-e
	N-urea	7.95±0.03 b	13.37±0.13 c	3.62±0.03 a	22.83±1.17 ab

Amf= Arbuscular mycorrhiza fungi, AS= ammonium sulfate, AN= ammonium nitrate. Data represent the mean value ±S.E. the mean of three replicates. Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

Moreover, it is known the function of sulfur in the synthesis of proteins, oils, vitamins, and flavored compounds in plants since, it is a constituent of the three amino acids methionine (21% S), cysteine (26% S) and cystine (27% S), that are the building blocks of protein (El Mantawy, 2017).

Contents of total carbohydrates, N, P and K in seeds

It is clear from data reported in figure 3 that within each rate of N forms, in most cases, application of *T. viride*, vermicompost or Amf caused significant increase in total carbohydrates, macronutrients (N, P and K %) in seeds compared to control. Among the

tested treatment *T. viride* appeared to be the most effective one. In this regard, Kumar *et al*. (2015) on *Salvia officinalis* stated that the plants inoculated with *T. viride* had higher phosphorus content in shoot and root as compared with control. Also, Metwally (2020) on *Allium cepa* declared that the plants inoculated with *T. viride* improved total carbohydrates and N, P or K% in plant organs.

Data in figure 3 also exhibited that within *T. viride*, vermicompost or Amf treatmens, the plants treated with nitrogen forms had significantly higher values of total carbohydrates, N, P and K in their seeds than the control. Among the tested nitrogen forms ammonium sulfate was superior in its effect than the other two nitrogen sources. Such results confirmed the reports of prior works (Wahba *et al*., 2014; Khalid and Shedeed, 2015; El Sayed, 2020; Hegab *et al*., 2021) who showed increase in total carbohydrates or N, P and K% in seeds as result of ammonium sulfate application.

The increased content of total carbohydrate in seeds may be related to the increase in chlorophyll content of plants, corresponding to improved photosynthesis efficiency (Khalid and Shedeed, 2015).

Total phenols in seeds

Data in figure 4 A displayed that within each rate of N forms, in most cases, application of *T. viride*, vermicompost or Amf treatments caused a significant increase in total phenols in seeds compared to control. The highest mean values were found to be associated with the plants treated with vermicompost. Similar results were reported by Abd El-Hamed *et al*. (2021) who found that vermicompost caused increase in total phenols in leaves of *Dracocephalum moldavica*.

Within each treatment of *T. viride*, vermicompost or Amf, the plants fertilized with different rates of nitrogen forms had significantly higher values of total phenols in seeds than those of control. Nitrogen as ammonium sulfate was superior to the other nitrogen forms in augmentation total phenols in seeds. These results confirmed the reports of earlier researches (Munene *et al*., 2017; Petropoulos *et al*., 2018; Prinsi *et al*., 2020; Machado *et al*., 2022) that reported increase in total phenols in plant organs due to ammonium sulfate treatments.

Seed oil (%)

It is evident from data figure 4 (B) that within each rate of N forms application of *T. viride*, vermicompost

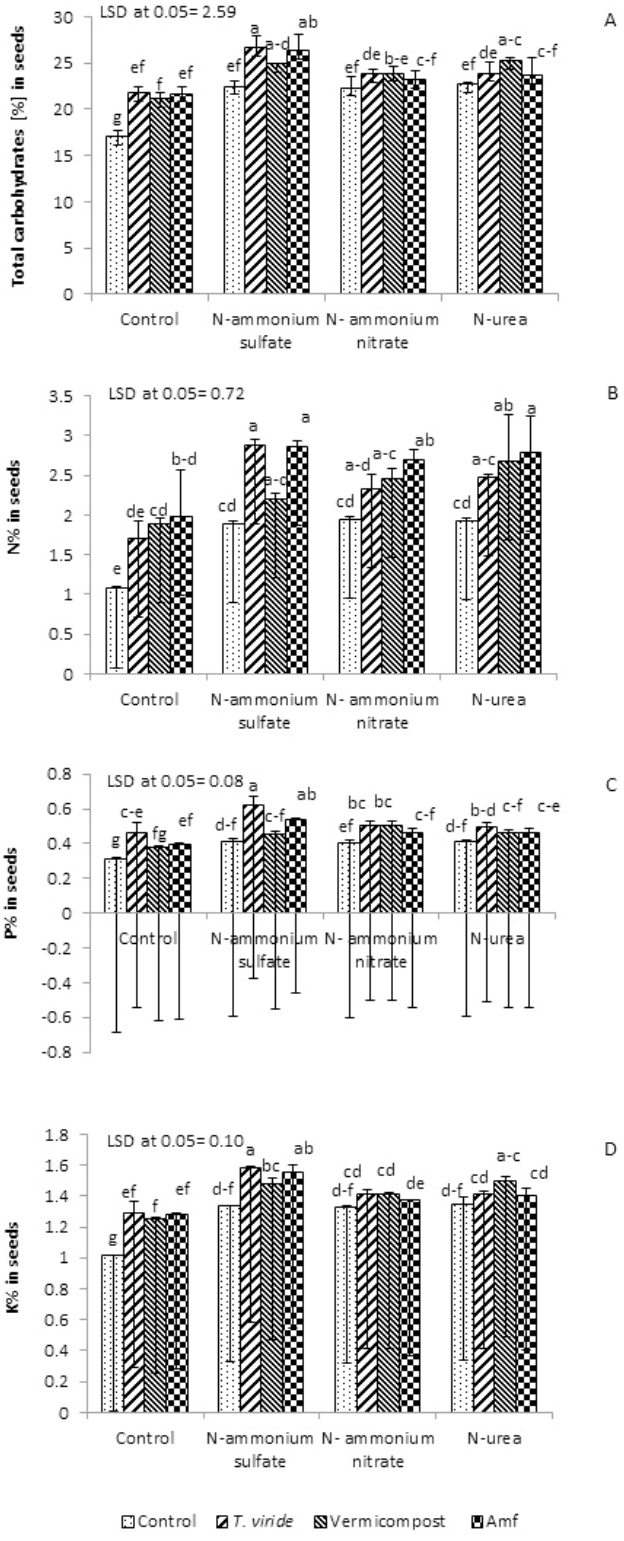

Fig. 3 - Evolution of superficial SS index of four selected farms (131, 272, 351, 432) and their average trend during storage in 2018. Bars represent standard error of the mean (±SEM). Values followed by the same letter in every sampling point after harvest are not significantly different from each other. Mean separation by LSD test (P≤0.05).

or Amf rates caused a significant increase in oil percentage in seeds compared to control. Among the tested treatments, *T. viride* appeared to be the most effective one since recorded the highest values. The positive effect of *T. viride* treatments for enhancing oil % in seeds is similar to those obtained by previous reports (Shaikh and Mokat, 2018; Guo *et al*., 2020; Hassanin *et al*., 2020).

Improved the quantity of oil percentage may be due to *T. viride* regulating the genes that encode the enzymes involved in essential oil metabolism through a potential MAPK-mediated signaling pathways (Guo *et al*., 2020).

Results in figure 4 also indicate that, within each treatment of *T. viride*, vermicompost or Amf, oil percentage in seeds of plants treated with different nitrogen forms were significantly higher than those of control. Ammonium sulfate was slightly better in its effect than the other two nitrogen forms. These

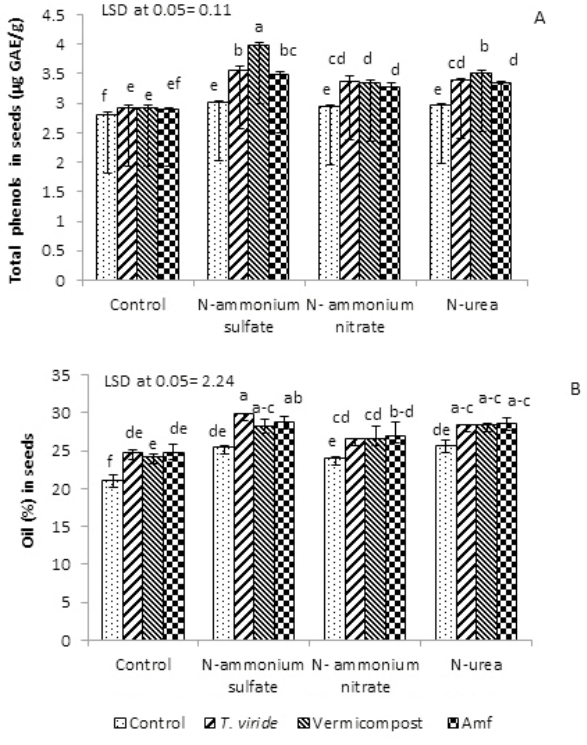

Fig. 4 - Evolution of antioxidant capacity (mg ascorbic acid/100 g of fresh fruit) and SS index after 4 months of cold storage (T2) in seasons 2019 of four farmers (131, 272, 351, 432) and their average trend. Bars represent standard error of the mean (±SEM). Values followed by the same letter between four producers are not significantly different from each other considering DPPH values at T0 and T1 or SS index during storage. Mean separation by LSD test (P ≤0.05).

results are in agreement with those reported by previous researches on different plants including *Cynara cardunculus* (Sarhan *et al*., 2014), *Helianthus annuus* (El Mantawy, 2017) and *Simmondsia chinensis* (Hegab *et al*., 2021), they indicated that ammonium sulfate treatments caused increase in oil percentage in seeds.

The increments in oil content due to ammonium sulfate application may be attributed to its promoting role in the formation of amino acids methionine (21% S) and cysteine (27% S); synthesis of proteins and oil content of seeds. Also, sulfur is an important element for oil crops which a constituent of acetyl Co-A, which converted into malonyl Co-A to synthesis of fatty acid (El Mantawy, 2017).

4. Conclusions

Summing up the results, it can be concluded that for enhancing growth, nutritional values of seeds, the interacted treatment of *T. viride* inoculation at 5ml /plant and ammonium sulfate at 5 g/plant is recommended for downy safflower plants.

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Improving fruit set and yield of tissue cultured date palm cv. Berhi by using a combined pollination technique

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Key words: Abnormal fruit set, metaxenia, parthenocarpic fruits, *Phoenix dactylifera* L., pollen source.

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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: Tissue-cultured (TC) date palms produce no fruit or low yield due to abnormal fruit setting. To improve the yield of TC 'Berhi' palms, trees were pollinated using five pollen sources (Gantar, Ghannami, Mazafati, Zahedi, and Jarvis). The experiment was carried out in three replications for two successive years in a randomized complete blocks design. The fruit set, the fruit and seed physical traits at the Khalal stage, bunch weight at the Tamar and Khalal stages, ripeness of Tamar bunch, and the fruit quality at both Khalal and Tamar stages were measured and monitored. Year factor significantly affected the fruit set and the fruit and seed characteristics. Pollen sources affected fruit set and some seed characteristics significantly. Zahedi+Jarvis pollen treatment that induced 50% normal fruit set and the highest ratio of pulp to seed was found superior. It was also a top treatment in Khalal's bunch weight (3.11 Kg). Zahedi+Gantar treatment was realized superior in Tamar's bunch weight (6.00 Kg). Ghannami, Jarvis+Ghannami, and Zahedi+Jarvis treatments produced Khalal's fruits with higher quality indices but Zahedi+Jarvis treatment was superior in fruit quality at the Tamar stage. Overall, the combined application of Zahedi and Jarvis pollens yielded the most desired outcomes.

1. Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most important commercial fruit crops of the arid and semi-arid subtropical regions of the world. The global total annual production reaches about 9.1 million tons. Iran with a production of about 1.3 million tons in 2020 is the world's third-largest date producer (FAO, 2020). Although many cultivars of dates grown in the country are popular in the domestic market, there has been a shift in recent years toward cultivars that are in high demand on both

the local and international markets. Among such cultivars, 'Berhi' is considered a superior, elite cultivar that has earned high popularity in export markets. The international high demand for this cultivar is because of the rare characteristic of its fruits. Unlike most date cultivars, fruits from 'Berhi' are best marketed at the Khalal stage for their tannin-free, sweet and crispy flesh. However, 'Berhi' fruits are also consumed at Rutab and Tamar stages (Zaid and de Wet, 2002 a) though with lower frequency.

'Berhi' is comparatively a high-yielding cultivar (average of 200 Kg per palm) (Zaid and de Wet, 2002 a) ensuring higher income for the growers, which in turn is driving demand for its propagules to expand its plantations (Mohammadi *et al.,* 2017). The current demands for date palm trees cannot be satisfied with traditional propagation by offshoots (Ali-Dinar *et al.,* 2021), particularly for the cultivars whose offshoot production is low like 'Berhi' (Zaid and de Wet, 2002 a). Alternatively, micropropagation is an efficient method of propagating date palm clones on a large scale (Al-Khayri and Naik, 2017; Ali-Dinar *et al.,* 2021). Nevertheless, despite the numerous benefits of micropropagation, it has also resulted in various abnormalities in the growth and yield of TC (tissue culture)-derived palms.

The most economically damaging disorder, which occurs in high frequency, is floral fertilization failure resulting in abnormal fruit set with a great impact on productivity and crop yield, and predominantly leading to a very low proportion of normal fruitlets that ultimately affects the overall economics of the plantation (Al-Dinar *et al.,* 2021). The abnormal fruits, usually appearing in the form of triple parthenocarpic fruitlets, are of no commercial value. This abnormal fruiting has been reported in date palms in the past years frequently (Cohen *et al.*, 2004; Al-Kaabi *et al.,* 2007; Mohammadi *et al.,* 2017). TC-derived trees of 'Berhi' display a high prevalence of this phenotype, which has made it the most common unstable cultivar in this ground (Cohen *et al.,* 2004; Al-Wasel 2005; Mohammadi *et al.,* 2017; Al-Dinar *et al.,* 2021). The main cause of failure in normal fruit setting is failed fertilization originating from ineffective pollination of the female flowers. Although several reports have reviewed/evaluated the growth and yield of TC-derived date trees (Al-Wasel, 2000; Hajian, 2007; Kavand *et al.,* 2015) few attempts have been undertaken to overcome this shortcoming in the field-established TC-derived date trees, especially 'Berhi' (Mohammadi *et al.,* 2017; Al-Najm *et al.,* 2021). Pollination intervention can be an influential strategy to improve the fruit set and yield of such off-type palms (Mohammadi *et al.,* 2017). However, so far no optimized pollination procedure has been released. Thus, more research is required in this context.

Date palm is a dioecious species and artificial (hand) pollination by man is inevitably done to gain economic yield. There are several reports on the role of pollen source in the fruiting of non-tissue cultured date cultivars (Omar and El-Abd, 2014; Soliman *et al.,* 2020), but similar research on TC-derived date trees are few (Rezazadeh *et al.,* 2013; Mohammadi *et al.,* 2017).

Metaxenic effect reflected as changes in the physical and chemical characteristics of fruits under the direct influence of pollen source has been reported in many date cultivars previously (Nixon, 1934; Shafique *et al.*, 2011; Rezazadeh *et al.*, 2013; Mohammadi *et al.*, 2017; Al-Najm *et al.*, 2021). Exploiting this effect to improve fruit quality is of interest. Overall, despite several works carried out during the past several years, no clear-cut solution has been found for resolving abnormal fruiting and low yield in many TC-derived 'Berhi' trees yet.

The present study was performed to avoid abnormal fruit-setting and improve fruit yield and quality of TC-derived 'Berhi' trees through optimizing pollen source and pollination treatment.

2. Materials and Methods

Plant materials

Field pollination experiments were carried out at Date Palm and Tropical Fruits Research Station in Dashtestan (29°23´ N; 51°5´ E; altitude 50 m), Bushehr, Iran. Fourteen-year-old TC-derived trees of 'Berhi', uniform in growth, were selected as female parents.

Pollen collection and pollination

Pollen grains were collected from five clonally male selections preserved in the date palm germplasm collection at the research station, four locally superior selections *viz.* 'Gantar', 'Ghannami', 'Mazafati', and 'Zahedi (Zahidi)', and one internationally recognized elite clone "Jarvis". Pollen collection and storage were done in February 2018 and 2019 according to the procedure described by Mohammadi *et al*. (2017). To assess the viability of pollen grains, two tests was applied: acetocarmine

staining test and *in vitro* germination test (Fig. 1) few days ahead of hand pollination. The pollens used for viability tests had been stored in a refrigerator at 4°C for 4-5 weeks.

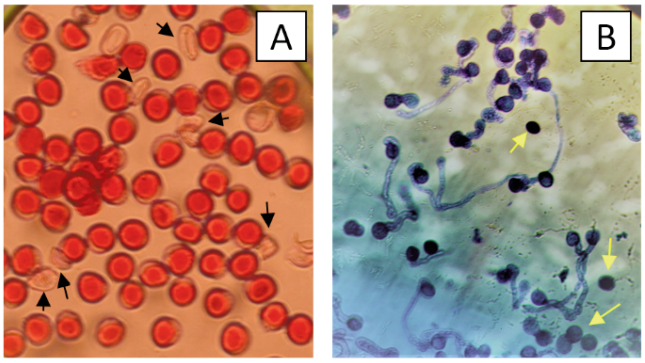

Fig. 1 - (A) Differential stainability of viable and non-viable pollen grains. Red-stained grains are viable and non-stained grains are non-viable. Arrows show abnormal pollen grains, which all are non-viable. (B) Germinated pollen grains with pollen tubes of various lengths. Arrows show non-germinated pollen grains.

Manual pollination was performed on the day of natural spathe craking in early April in the first year and in late March in the second year. Pollens of 'Gantar', 'Ghannami', and 'Mazafati' were used both individually and in combination with each of the Zahedi and Jarvis pollens. A pollination treatment consisting of a combined application of 'Zahedi' and 'Jarvis' pollens was also included. The pollination operation was done according to the traditional method used by the local date growers. In this method, a traditional pollination device, made of cotton fabric and the dried midrib of date palm leaf and filled with fresh pollen, is tapped mildly on two sides of each selected receptive female inflorescence releasing pollen grains gently through the fabric onto the flowers (Fig. 2). The experimental inflorescences were selected on candidate female palms based on uniformity in length (approx. 80 cm) and the number of spikelets per inflorescence (14±2). For the pollination treatments that contained two different pollen sources, an equal quantity of each source was weighed, mixed well with each other, and poured into the pollination device. For each pollination treatment, a separate pollination device filled with the corresponding pollen source(s) was used. To prevent possible contamination of the flowers by foreign pollens, the pollinated inflorescences were then immediately covered with white cotton bags. The bags were removed three weeks later. All targeted inflorescence were tagged properly corresponding to the pollen treatment and the block number.

Fig. 2 - Pollinating female inflorescences through mild tapping of a traditional pollination device, releasing pollen grains gently through the cotton fabric onto the receptive florets.

Calculating fruit set traits

Five weeks after pollination, percentages of normal fruit set, parthenocarpic fruit set, and fruit drop were calculated for each tagged inflorescence. Five strands on each inflorescence were randomly selected. Numbers of normal fruits, parthenocarpic fruits, and flower scars as the representative of dropped flowers and fruitlets were counted separately, recorded, and each one divided by the total number of flowers in the chosen strands, then multiplied by 100.

Measuring bunch weight and bunch ripeness

In the first year of the study, each tagged bunch was harvested separately on September 18 and shaken off to collect the whole fruits. The weight of total fruits of each bunch was considered as the bunch weight, and the percentage of bunch ripeness was calculated based on the weight ratio of the bunch Tamar fruits to total fruits of the same bunch (including Tamar, Rutab, and Khalal fruits). In the second year, the weight of the whole Khalal fruits of each tagged bunch was considered as the bunch weight at the Khalal stage.

Measuring fruit and seed physical characteristics

During both experimental years, fruit sampling was done in mid-August at the Khalal stage. Fifteen single normal fruits were picked randomly from each tagged bunch, and the weight, length, diameter, and volume of the fruits and seeds were measured. In addition, pulp weight, the ratios of pulp weight to seed weight, and fruit length to fruit diameter were also calculated.

Measuring fruit phytochemical quality

Fruit phytochemical quality was measured twice; the first year at the Tamar stage and the second year

at the Khalal stage. Fifteen normal fruits were harvested from each tagged bunch. Three replicated homogenized samples containing the pulp of five fruits were prepared and analyzed. To measure total soluble solids (TSS), fruit juice was extracted according to Aurand *et al*. (1987) and TSS was read using a digital refractometer (Atago PAL-3, Japan). The pH was measured directly in the extracted juice by a digital pH-meter (AZ 8686, Taiwan). The contents of reducing and total sugars were determined using the procedure described by Lane and Eynon (1923). Titratable acidity (TA) was quantified by titrating a known volume of juice with 0.01 N NaOH, using phenolphthalein as the indicator (AOAC, 2016) and expressed as the percent of acetic acid. The percentage of dry matter and moisture content were determined using 10 g of fruit pulp in a Petri dish dried in an oven at 72°C for 48 h.

Experimental design and Statistical analysis

The field experiment was conducted in a randomized complete blocks design with ten pollen treatments and three replications (blocks). Each block consisted of ten female inflorescences each of them pollinated with one of the ten pollen treatments. Inflorescences in each block were assigned to three female palms. Overall, nine female palms hosted 30 inflorescences uniform in length, and the number of spikelets were assigned for the experimental blocks. The current study was performed over two successive years. To determine the significance level of effect of pollen treatments on fruit-set parameters, and fruit and seed physical characteristics recorded for two years, a combined analysis was performed by GLM procedures of the SAS software package (SAS Institute, Cary, NC, USA). The mean comparison was also performed using Duncan's multiple range tests (DMRT) based on the averages of the two years data at a 5% probability level. For fruit qualitative traits, bunch weight at Khalal and Tamar stages and bunch ripeness at the Tamar stage, a one-way ANOVA was conducted for each year. The mean comparison was also executed for the fruit qualitative traits using DMRT at a 5% probability level.

3. Results

Pollen viability

The results have been illustrated in figure 3. Mean comparison for pollen viability by the staining test showed that the values had a ranging from a maximum of 98% for Zahedi+Mazafati pollens to a minimum of 79.3% for Ghannami pollen (Fig. 3). Apart from Ghannami pollen and Jarvis+Ghannami pollen source, there was not a significant difference among almost all other sources, which had pollen viability above 90%. Pollen viability assessed by the germination test ranged from 24.3 to 74%. The highest score was for Jarvis+Mazafati pollens though not significantly different from that of Zahedi+Gantar pollen source (69.7%) (Fig. 3). The lowest percentages of germinated pollens were observed with Ghannami male (24.3%) and Zahedi+Jarvis source (32.3%). The results revealed that combining Zahedi pollen with each of Gantar, Ghannami, and Mazafati pollens showed that the percentage of germinated pollens was increased significantly in cases of Gantar and Ghannami pollens but not for Mazafati pollen showing a reverse result. The results showed high differences among pollen sources in the ability of *in vitro* germination. Such significant differences can be due to genetic variation and storage conditions. Since the fresh pollen used in

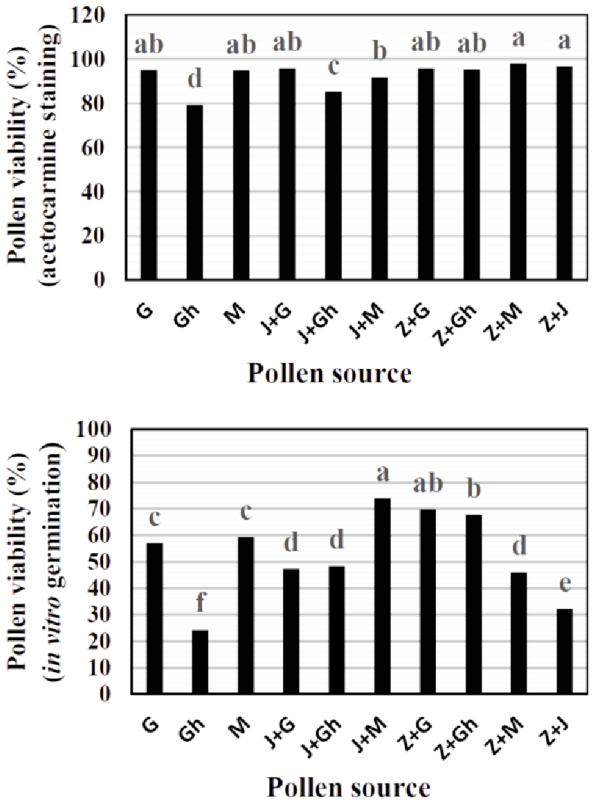

Fig. 3 - Mean comparison of pollen viability for 10 pollen sources tested by staining with acetocarmine and by *in vitro* germination. Means were calculated based on the two years data. Columns having same letter(s) do not show a significant difference with each other using Duncan's multiple range test at P≤0.01. G= Gantar, Gh= Ghannami, M= Mazafati, J= Jarvis, Z= Zahedi.

this study was kept under the refrigerator temperature for a few weeks before the date of hand pollination, the most important variable that differentiated these pollen sources based on their germination ability can be assumed as the genetic differences.

Fruit set

The results (Table 1) revealed that all the experimental variables including year, pollen source, and the interaction effect affected fruit set traits significantly at P≤0.01. Results of the mean comparison test conducted for the main factor (pollen source) have been illustrated in figure 4. The highest values of normal fruit set were achieved with the pollen treatments including Mazafati, Zahedi+Jarvis, and Zahedi+Gantar (53.1%, 49.2%, and 48.9%, respectively) while the lowest parthenocarpic fruit set was with the same pollen sources (18.1%, 10.3%, and 17.7%, respectively).

Mazafati pollen also caused the lowest fruit drop (28.8%) (Fig. 4). Gantar pollen and Jarvis+Gantar pollen treatment yielded the lowest percentage of normal fruits (28.9% and 34.6%, respectively). Gantar pollen also caused the highest percentage of parthenocarpy (36.7%) and a moderate fruit drop (34.4%). Meanwhile, the combined application of Jarvis and Gantar pollens acquired the first rank in fruit drop (43.5%). The interesting point was that the Zahedi+Jarvis pollen treatment caused the lowest parthenocarpy (10.3%) and the second-best normal fruit set (49.2%) which was statistically the same as the highest normal fruit set obtained with Mazafati (53.1%).

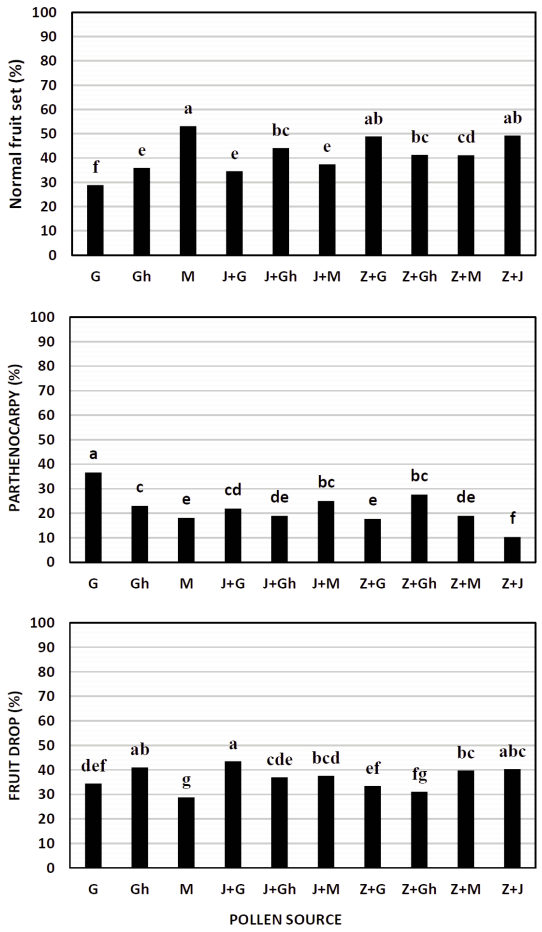

Fig. 4 - Mean comparison of fruit set traits for the ten pollen sources used. Means were calculated based on the two years data. Columns having the same letter(s) do not show a significant difference from each other using Duncan's multiple range test at P≤0.01 (G= Gantar, Gh= Ghannami, M= Mazafati, J= Jarvis, Z= Zahedi).

Table 1 - Combined analysis of variance for fruit set traits and some characteristics of Khalal fruit of TC-derived 'Berhi' date palms pollinated with 10 pollen sources

Fruiting characteristics	Sources of variation (Mean square)					CV%
	Year (df= 1)	Error (year) (df=4)	Pollen source (df=9)	Year × PS (df=9)	Error 2 (df= 36)	
Normal fruit set %	814.82 **	18.485	341.22 **	630.15 **	17.764	10.16
Parthenocarpy %	7863.40 **	20.90	297.24 **	717.87 **	6.712	11.86
Fruit drop %	3602.82**	15.66	132.31 **	129.91 **	8.321	7.86
Fruit weight (FW)	218.80 **	1.401	1.37 NS	3.25 **	0.865	10.47
Fruit length (FL)	49.5 **	1.58	2.02 NS	4.92 **	1.49	4.28
Fruit diameter (FD)	217.74 **	1.77	0.99 NS	3.56 *	1.42	5.22
Fruit volume (FV)	265.44 **	0.80	1.30 NS	3.68 **	1.04	11.71
Fruit volume (FV)	120.98 **	1.573	1.302 NS	19.73 **	0.957	11.22
Seed weight (SW)	0.308 **	0.004	0.016 **	0.024 **	0.003	7.58
Seed length (SL)	0.14 NS	3.135	1.389 **	1.308 **	0.402	3.21
Seed diameter (SD)	2.86 **	0.41	0.435 NS	0.918 **	0.250	5.46
Seed volume (SV)	3.10 **	0.076	0.165 NS	0.185 NS	0.113	23.28
Pulp weight (PW)	206.23 **	1.20	1.001 NS	2.82 **	0.817	11.1
PW/SW ratio	129.71 **	0.563	2.201 *	0.389 NS	1.018	9.45
FL/FD ratio	0.273 **	0.003	0.0028 NS	0.0007 NS	0.002	3.87

NS= not significant; ** significant at P≤0.01; * significant at P≤0.05.

Ghannami pollen that recorded the second-highest fruit drop also caused one of the lowest percentages of normal fruit set among all treatments (35.9%) with moderate parthenocarpy (23%). Overall, we concluded that the Zahedi+Jarvis pollen treatment followed by the Mazafati pollen respectively as the best and the second-best pollen treatments for the improvement of normal fruit set in TC-derived 'Berhi' dates.

Bunch weight and bunch ripeness percentage

Investigating bunch characteristics showed that pollen source affected (P≤0.01) bunch weight at Tamar and Khalal stages as well as the percentage of bunch ripeness. Means comparison for these traits has been depicted in figure 5. The Jarvis+Ghannami treatment caused the highest percentage of bunch ripeness with an average of 92.98%. The lowest bunch ripeness was recorded with Gantar and Jarvis+Gantar sources indicating a significant delaying effect of Gantar pollen on fruit maturation and bunch ripeness compared to other pollen sources.

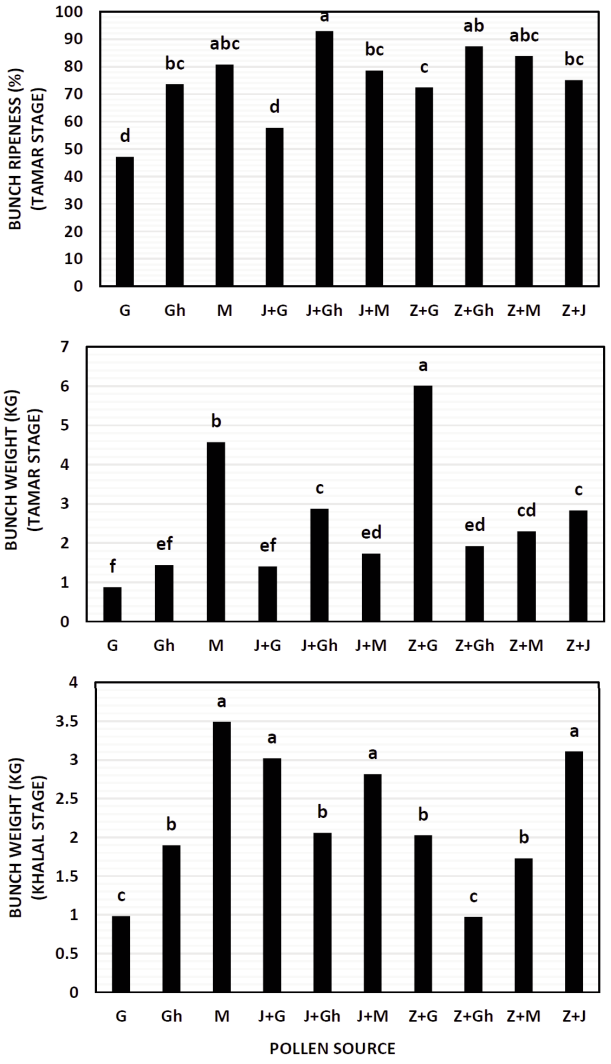

Fig. 5 - Means comparison of bunch ripeness and bunch weight (at Tamar and Khalal stages) for the ten pollen sources used. Columns having the same letter(s) do not show a significant difference from each other using Duncan's multiple range test at P≤0.05 (G= Gantar, Gh= Ghannami, M= Mazafati, J= Jarvis, Z= Zahedi).

The Zahedi+Gantar treatment yielded the Tamar bunches with an average of 6 Kg which were heavier than those of all other treatments (P≤0.05), followed by the Mazafati pollen that yielded the bunches carrying averagely 4.58 Kg fruits (Fig. 5). Gantar pollen appeared as the poorest source since it produced bunches with averagely 0.88 Kg of fruits. Measurement of bunch weight at the Khalal stage (in the second year) showed four pollen treatments including Mazafati (3.49 Kg), Zahedi+Jarvis (3.11 Kg), Jarvis+Gantar (3.02 Kg), and Jarvis+Mazafati (2.82 Kg) were the top-yielding treatments. The poorest pollen sources in Khalal's bunch weight were Gantar (0.98 Kg) and Zahedi+Ghannami (0.97 Kg).

Fruit and seed characteristics

Combined ANOVA showed that the year factor affected (P≤0.01) all the Khalal fruit and seed characteristics except seed length (Table 1). Pollen sources could not affect fruit physical characteristics significantly (Tables 1 and 2), but seed weight and length were affected (P≤0.01) (Table 1). In addition, the pollen source influenced the ratio of pulp weight to seed weight at P≤ 0.05 (Table 1). The means for the fruit and seed characteristics of the normal fruits at the Khalal stage have been presented in Table 2. As the table shows, though pollen sources have influenced these characteristics and some sources have improved them, the differences among the pollen sources have not been significant (P≤0.01) in anyone.

As illustrated in figure 6, the mean comparison for seed weight showed that the lightest seed was produced with Zahedi+Ghannami pollen (0.66 g) though not significantly different from that of Zahedi+Jarvis and Gantar sources. The smallest seeds were obtained with Zahedi+Ghannami pollen (18.7 mm long) and the longest ones with Jarvis+Mazafati source (20.3 mm). The highest weight ratio of fruit pulp to seed (11.94) was obtained in the fruits produced through pollination with the Zahedi+Jarvis pollen treatment.

Fruit phytochemical characteristics

ANOVA revealed that all measured qualitative traits of the Tamar fruits were affected by pollen sources (P≤0.01). In Khalal fruits, however, pollen sources affected TSS, reducing sugars and total sugars at P≤0.01 and titratable acidity, pH, dry matter,

Table 2 - Mean comparison of fruit and seed characteristics for the ten pollen sources used. Means were calculated based on the data from the two year

Pollen sources	Fruiting characteristics							
	Fruit weight (g)	Fruit length (mm)	Fruit diameter (mm)	Fruit length/ fruit diameter	Fruit volume (cm^2)	Seed diameter (mm)	Seed volume (cm^3)	Pulp weight (g)
G	8.40	28.4	22.4	1.28	8.20	9.12	0.61	7.67
Gh	9.17	28.4	23.0	1.23	8.77	9.35	0.88	8.38
M	9.58	28.8	22.9	1.27	9.25	9.33	0.68	8.78
J+G	8.92	28.4	23.2	1.23	9.25	8.92	0.81	8.16
J+Gh	8.65	28.3	22.6	1.26	8.37	9.37	0.84	7.86
J+M	9.28	29.2	22.9	1.28	8.95	9.43	0.82	8.45
Z+G	9.03	29.3	23.1	1.27	8.72	9.10	0.83	8.30
Z+Gh	7.88	27.3	22.0	1.25	7.80	8.62	0.68	7.37
Z+M	8.90	28.6	23.4	1.22	8.98	9.48	0.70	8.12
Z+J	9.01	29.1	23.0	1.27 a	8.88	9.07	0.77	8.32

G= Gantar, Gh= Ghannami, M= Mazafati, J= Jarvis, Z= Zahedi
Means in each column are statistically the same at P≤ 0.01 by DMRT.

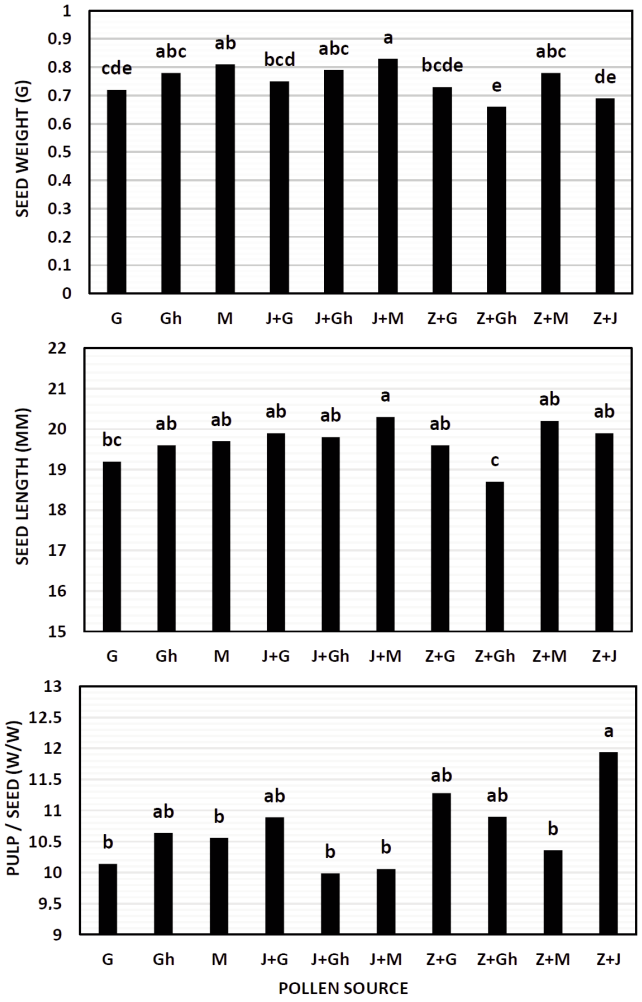

Fig. 6 - Mean comparison of seed weight, seed length, and pulp weight/seed weight ratio for the ten pollen sources used at the Khalal stage. Means were calculated based on the two years data. Columns having the same letter(s) do not show a significant difference from each other using Duncan's multiple range test at P≤0.01 (G= Gantar, Gh= Ghannami, M= Mazafati, J= Jarvis, Z= Zahedi).

and moisture content at P≤0.05.

In Tamar fruits, TSS ranged from 52 to 64 percent so the highest value was obtained by Ghannami pollen and the lowest with Zahedi+Jarvis pollen source (Table 3). Fruit juice pH was in the range of 6.58 to 6.87. The lowest pH belonged to the fruits set by Ghannami pollen (6.58) the same source that earned the highest TSS. The highest titratable acidity (0.028%) was recorded with the Zahedi+Jarvis pollen treatment. Total sugars ranged from 38.86 to 53.74 percent, the highest contents obtained by Zahedi+Mazafati and Jarvis+Ghannami pollen treatments (>53%), and the lowest remained for Zahedi+Ghannami pollen source (38.68%). The content of reducing sugars was significantly the highest with the Jarvis+Ghannami pollen treatment (46.5%). Application of Zahedi pollen in combination with either Ghannami, Mazafati, or Jarvis pollens caused maximum pulp dry matter (~88%) and minimum moisture content (11-12%). This is while the lowest dry matter (76.9%) and the highest moisture content (23.1%) were recorded in the fruits produced by Zahedi+Gantar pollen treatment.

In Khalal fruits, TSS ranged from a maximum of 34.03% induced by Jarvis+Gantar pollen to a minimum of 21.77% made by Gantar pollen (Table 4). Fruit juice pH was not different among most of the pollen sources used (P≤0.05). Titratable acidity showed a narrow range so the lowest value (0.47%) was for the Zahedi+Jarvis source and the highest one (0.58%) for the Zahedi+Ghannami pollen treatment. While the highest amount of total sugars was recorded with Zahedi+Mazafati pollens as well as with Jarvis+Ghannami pollen treatment (53.74% and 53.55%, respectively), the maximum amount of

Table 3 - Mean comparison for some fruit qualitative characteristics of TC-derived 'Berhi' date palms at Tamar stage pollinated by 10 pollen treatments

Pollen sources	Fruit quality characteristics						
	TSS (%)	pH	Titratable acidity (%)	Total sugars (%)	Reducing sugars (%)	Dry matter (%)	Moisture (%)
G	59.3 cde	6.85 a	0.012 cd	45.47 cd	36.42 bc	85.6 abc	14.4 cde
Gh	64.0 a	6.58 c	0.014 cd	47.18 bc	35.63 c	81.9 cd	18.1 bc
M	61.5 abc	6.63 bc	0.016 c	42.73 de	38.81 b	80.6 de	19.4 ab
J+G	54.9 f	6.70 abc	0.011 d	41.11 ef	31.11 d	83.8 bcd	16.2 bcd
J+Gh	62.5 ab	6.60 bc	0.013 cd	53.55 a	46.50 a	86.9 ab	13.1 de
J+M	56.9 def	6.86 a	0.021 b	44.85 cd	35.62 c	86.3 abc	13.7 cde
Z+G	59.0 cde	6.77 abc	0.013 cd	44.32 cd	37.15 bc	76.9 e	23.1 a
Z+Gh	56.5 ef	6.85 a	0.022 b	38.86 f	32.84 d	88.1 ab	11.9 de
Z+M	60.0 bcd	6.87 a	0.022 b	53.74 a	38.11 bc	88.7 a	11.3 e
Z+J	52.0 g	6.79 ab	0.028 a	49.59 b	36.83 bc	88.1 ab	11.9 de

G= Gantar, Gh= Ghannami, M= Mazafati, J= Jarvis, Z= Zahedi
Means in each column are statistically the same at P≤0.01 by DMRT.

Table 4 - Mean comparison for some fruit qualitative characteristics of TC-derived 'Berhi' date palms at Khalal stage pollinated by 10 pollen sources

Pollen sources	Fruit quality characteristics						
	TSS (%)	pH	Titratable acidity (%)	Total sugars (%)	Reducing sugars (%)	Dry matter (%)	Moisture (%)
G	21.77 e	5.74 c	0.55 ab	20.63 c	15.00 d	22.7 abc	77.3 abc
Gh	30.17 bc	5.83 abc	0.57 a	29.55 a	18.19 abc	20.3 c	79.7 a
M	27.57 bc	5.90 abc	0.49 bc	26.23 b	17.93 abc	26.4 a	73.6 c
J+G	34.03 a	6.02 ab	0.48 c	26.51 b	16.46 bcd	23.2 abc	76.8 abc
J+Gh	30.57 b	5.82 abc	0.55 ab	28.05 ab	18.61 ab	20.5 c	79.5 a
J+M	29.33 bc	5.89 abc	0.51 abc	28.69 ab	18.89 a	21.8 bc	78.2 ab
Z+G	26.87 cd	5.80 bc	0.51 abc	26.61 ab	18.20 abc	19.1 c	80.9 a
Z+Gh	28.33 bc	5.99 ab	0.58 a	27.65 ab	18.25 abc	21.0 bc	79.0 ab
Z+M	23.9 de	5.86 abc	0.56 a	22.74 c	16.13 cd	21.6 bc	78.4 ab
Z+J	30.87 b	6.04 a	0.47 c	28.38 ab	17.93 abc	25.3 ab	74.7 bc

G= Gantar, Gh= Ghannami, M= Mazafati, J= Jarvis, Z= Zahedi
Means in each column are statistically the same at P≤0.01 by DMRT.

reducing sugars (46.50%) was obtained only with Jarvis+Ghannami source. Mazafati pollen caused the highest pulp dry matter (26.4%) and the lowest pulp moisture (73.6%). The highest fruit moisture was made by Zahedi+Gantar pollen (80.9%).

4. Discussion and Conclusions

In the present study, different pollen sources revealed various potentials for the improvement of normal fruit set, fruit quantitative and qualitative characteristics, and the bunch yield. The results approved that certain pollen sources were more effective in reducing the problems of abnormal fruit setting. The pollen sources also influenced fruit physical characteristics and bunch yield variably, some with promising performance in tree yield. The effect of pollen source on the fruit set and fruit traits was variable between the two years of the study suggesting a significant interaction with climatic factors. Screening pollen sources for improved fruit yield and

quality has been done earlier for some horticultural crops (Fattahi *et al.*, 2014; Kuroki *et al.*, 2017) including date palm (Omar and El-Abd, 2014; Mohammadi *et al.*, 2017; Soliman *et al.*, 2020).

Fruit set characteristics

The results unveiled that the Mazafati pollen was the only pollen source that could set normal fruits averagely beyond 50%. Other tops were Zahedi+Jarvis and Zahedi+Gantar sources both with about 49% normal fruit set. Previously, Mohammadi *et al.* (2017) announced Zahedi pollen as the most promising pollen source with an average of 50.1% normal fruit set for low fruiting TC-derived 'Berhi' date palms. Our results are following theirs confirming the desirable potential of Zahedi pollen in improving normal fruit set in such trees. However, in our work, the percentage of survived parthenocarpic fruitlets was less than 20% in all three superior pollen treatments. The Zahedi+Jarvis pollen treatment caused reduced parthenocarpic fruitlets to 10% whereas Mohammadi *et al*. (2017) reported 32% parthenocarpy for their top pollen treatment (Zahedi pollen). This outstanding reduction in the percentage of parthenocarpy was achieved by performing combined pollination consisting of two elite pollen sources simultaneously *i.e.* Zahedi and Jarvis.

Comparing the singular and combined applications of pollen sources discloses that singular pollination with the Gantar source yielded the lowest percentage of normal fruit set but it behaved variably when it was applied in combination with each of the two prominent pollen sources; Zahedi and Jarvis. These two male sources had been known as superior sources from our previous work (Mohammadi *et al.*, 2017). The combined application of Gantar pollen with Jarvis pollen improved the normal fruit set slightly while its combined application with Zahedi pollen achieved the third rank, not significantly different from the first and the second ranks of pollen treatments in this parameter. Regarding Ghannami pollen, its application in combination with Jarvis source as well as with Zahedi source improved the normal fruit set compared to its singular application. In the case of Mazafati pollen, the opposite results were observed compared to the Gantar and Ghannami source's behaviors. Singular application of Mazafati pollen performed better in normal fruit set than its combined application with each of the Jarvis and Zahedi pollens.

Having looked from another point of view, those pollen treatments that recorded the highest figures for normal fruit set *i.e.* Mazafati, Zahedi+Jarvis, and Zahedi+Gantar, though caused a lower percentage of parthenocarpy compared to their corresponding fruit drop percentages but acted differently in the values of these two traits. This means that a more favorable fruit set was obtained with Zahedi+Jarvis pollen treatment, which reduced the surviving parthenocarpic fruits to a minimum rate at the cost of a high rate of fruit drop. This is while the other two superior pollen sources (Mazafati and Zahedi+Gantar) reduced fruit drop more, instead, they added proportionally to the rate of parthenocarpy. Since the dropped organs have mainly consisted of the unfertilized flowers and the parthenocarpic fruitlets, a higher percentage of fruit drop accompanied by a lower rate of surviving parthenocarpic fruits allows the normal fruits on the bunch to receive more metabolites. This situation is more beneficial as it would result in improvement of the fruit's physical and phytochemical characteristics, ultimately leading to an improved bunch weight with more marketable fruits. Therefore, the Zahedi+Jarvis pollen source was concluded as the best pollination treatment for the improved fruit set. Various factors have been suggested to cause variation in date fruit set such as pollen viability, growth of the pollen tube, or fertilization (Zaid and de Wet, 2002 b), differences in compatibility barriers (Al-Obeed and Abdul-Rahman, 2002), and compatibility levels between pollen variety and female tree (Mohammadi *et al.*, 2017).

It has been shown that growth-promoting phytohormones play a significant role in pollen germination and pollen tube growth (Kojima, 2005), the processes that are initially critical for successful ovule fertilization leading to a normal fruit set. The hormonal content of pollen grain can play a key role in this context. In date palms, parthenocarpic fruit growth is mostly triggered by hormonal imbalance in certain tissues. Auxins (IAA), gibberellins (GA_3), and abscisic acid (ABA) have been considered the major hormones in parthenocarpic fruits (Al-Dinar *et al.*, 2021). Pollens from various male sources possess different hormonal profiles. This variability can be attributed to the genetic makeup of pollen-producing mother plants and the environmental conditions in which the male palms grow and produce pollens. Shahsavar and Shahhosseini (2021) found the hormonal content of the pollen grains from different male sources variable. They reported the highest amounts of cytokinin (zeatin) in Jarvis pollen, gibberellin in pollens of Zahedi and Jarvis males, and

auxins in Fard and Zahedi pollen sources. Interestingly, in our study, one of the highest figures of normal fruit set was achieved with the combined application of pollens of Zahedi and Jarvis males, the two sources that contained the highest amounts of growth-promoting phytohormones in the report of Shahsavar and Shahhosseini (2021). It is well known that though *in vivo* germination of pollen grain on the stigma is critical for the process of normal fruit set, it must be followed by healthy directional growth of a pollen tube in the style to deliver the male gametes to the embryo sac (Salomón-Torres *et al.*, 2021).

Bunch characteristics

The results indicated that by choosing a more compatible pollen source, the bunch weight (both Khalal and Tamar stage) could be improved. For Tamar bunch weight, Zahedi+Gantar pollen treatment was seen as the top source. On the other side, this treatment showed about 72% ripened fruits per bunch at the time of bunch harvest whereas four other treatments showed bunch ripeness of over 80%, the highest belonging to the Jarvis+Ghannami treatment. Therefore, according to our results, for the production of maximum Tamar fruits in TC-derived 'Berhi' palms, pollination by Zahedi+Gantar pollen source is recommended but the harvest time should be postponed probably for two more weeks to get maximum Tamar fruits. For Khalal bunch weight, Mazafati, Zahedi+Jarvis, and Jarvis+Gantar sources produced the weightiest bunches (3-3.5 Kg). Through these pollen treatments Khalal bunch weight was doubled and even tripled compared to the other pollen sources like Gantar pollen. Khalal bunch weight in offshoot-originated Berhi palms has been reported up to about 20 Kg (Abd-Elhaleem *et al.*, 2020). This indicates that more research is needed to optimize the pollination issue for tissue-cultured Berhi palms.

Fruit and seed characteristics

Pollen sources used in this study manifested xenic as well as metaxenic effects. The Zahedi+Jarvis pollen treatment gained the most superior place in the ratio of pulp to seed weight. This source also produced fruits with acceptable dimensions, causing one of the lowest seed weights. Previously, Mohammadi *et al*. (2017) obtained the highest value for the weight ratio of pulp to seed by Jarvis pollen in TC-derived 'Berhi' trees. Several reports have confirmed the effect of different pollinizers on the fruit characteristics of dates (Helail and El-Kholey, 2000; Awad and Al-Qurashi, 2012; Rezazadeh *et al.*, 2013).

It seems variation in the performance of pollen sources in fruit and seed characteristics can be attributed to the differences in content and composition of the phytohormones that are synthesized within the embryo. As the genome of each pollen source is different from the other sources, the genetic make-up of the embryo produced by each pollen source would be different compared to the others. This distinction in seed genetics would cause the synthesis of a unique hormonal profile by the embryo that consequently will regulate seed and fruit characteristics (Shafique *et al.*, 2011). In a study on the Hayany date cultivar, it was reported that different pollen sources change the amounts of various phytohormones such as auxin and gibberellins at various stages of fruit development. It was also indicated that larger fruits were obtained by the pollen sources that had higher gibberellin content comparatively (El-Hamady *et al.*, 2010). Shahsavar and Shahhosseini (2021) emphasized that pollen source has a key role in fruit growth and development with a special focus on the hormonal content of each pollen source.

Overall, considering the highest values in the normal fruit set and pulp-to-seed ratio, the Zahedi+Jarvis source was realized as the best pollination treatment for the production of Khalal fruits. We suggest Zahedi male as an elite pollen source to be included in future works aiming to improve the yield of low-fruiting TC-derived 'Berhi' date palms. Earlier, improvement in the yield of the 'Berhi' cultivar was reported by the use of Saki and Maktoumy pollen sources (Al-Obeed and Abdul-Rahman, 2002) and several Iranian local male selections (Rezazadeh *et al.*, 2013). The exploitation of the potential of pollen source in other fruit and nut species such as hazelnut (Fattahi *et al.*, 2014) and Japanese pear (Kuroki *et al.*, 2017) has been reported earlier.

Fruit quality

Pollen source affected fruit quality significantly both at Khalal and at Tamar stages. These results are compatible with those reported earlier in date (Awad and Al-Qurashi, 2012; Shafique *et al.*, 2011) and in fruit species such as mandarin (Wallace and Lee, 1999), and fig (Gaaliche *et al.*, 2011) confirming the critical role of pollen source in fruit quality. However, there are also reports that state the influence of pollen sources on some chemical traits of date fruits is low (Awad and Al-Qurashi, 2012; Rezazadeh *et al.*, 2013). This variation in the findings may indicate that other factor(s) such as crop load, local weather con-

ditions, soil fertility, irrigation, etc. can also influence the fruit quality.

In the Tamar stage, we observed the highest TSS in the fruits set by the Ghannami pollen, the pollen source that caused one of the lowest bunch weights. This indicates a reverse relationship between crop load and TSS. Such a relationship was more apparent when we realized that the Zahedi+Jarvis pollen source that induced one of the top rates in normal fruit set and bunch weight (both at Tamar and Khalal stage), possessed the lowest TSS value and the highest amount of TA. The results also indicated that the Zahedi pollen tends to accumulate more dry matter in Tamar fruits when it is used in combination with the other pollen sources but the Gantar source. Overall, Zahedi+Jarvis pollen treatment was concluded as the most preferable pollen treatment for the quality of fruit at the Tamar stage. It brought moderate sweetness (concerning amounts of TSS, titratable acidity, total and reducing sugars) and drier pulp tissue. Concerning the quality of Khalal fruits, Ghannami and Jarvis+Ghannami pollen sources appeared as the best pollen treatments. They caused the highest amounts in TSS, TA, total sugars, reducing sugars, and the moisture content, which are important in making the Khalal fruits sweeter, tastier, crispier, and more palatable to the consumer. Zahedi+Jarvis pollen treatment can also be considered as another candidate treatment though the fruits set by this treatment were slightly less crispy.

The results of the present study proved that the fruit set and yield are affected differently in different years probably due to variations in environmental conditions. However, based on the average means of the two experimental years, the combined application of Zahedi and Jarvis pollens yielded the most desired results. This pollen treatment obtained about 50% normal fruit set with significantly the lowest parthenocarpy (10%). It also brought about the highest ratio of fruit pulp to seed weight. This pollen treatment was seen as the top pollen source in Khalal's bunch weight. Although the Zahedi+Gantar pollen source was superior in Tamar's bunch weight, the presence of Zahedi pollen in this latter pollen treatment indicates the potential of the Zahedi source over other pollen sources used in the study. Regarding fruit quality, Ghannami, Jarvis+Ghannami, and Zahedi+Jarvis were the pollen treatments that produced Khalal fruits with higher quality indices. However, for the Tamar fruit quality, only Zahedi+Jarvis pollen treatment was chosen as it induced the fruits with moderate sweetness and drier pulp. Overall, considering all fruiting and fruit quality indices, the Zahedi+Jarvis pollen treatment can be drawn as the most desired pollination treatment for low-fruiting TC-derived 'Berhi' date palms. Our results revealed that pollination with elite pollen sources in combination could emerge as a more successful pollination strategy over the singular application of different pollen sources. In addition, the results suggest that relevant future pollination studies utilize the Zahedi pollen source as a potent male source in combination with other elite pollen sources.

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Prediction of chamomile essential oil yield (*Matricaria chamomilla* L.) by physicochemical characteristics of soil

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Abstract: The purpose of this study was to predict the percentage and yield of chamomile essential oils using the artificial neural network system based on some soil physicochemical properties. Several habitats of chamomile cultivation were investigated and 100 soil samples were shipped to the greenhouse. The maximum and minimum of pH, EC, K, OM (organic matter), CCE (calcium carbonate equivalent), and clay in soils were 8.75-7.94, 1.6-1.0, 381-135, 2.30-0.22, 69-16, and 55.6-32.0, respectively. Growth indices, essential oil percentage, and yield were measured. Artificial neural network modeling was carried out to predict the essential oil concentration and yield using three groups of soil properties as a predictor: 1- nitrogen (N), phosphorus (P), potassium (K), and clay; 2- pH, EC, organic matter (OM) and clay; 3- CCE, clay, silt, sand, N, P, K, OM, pH, and EC. So, three pedotransfer functions (PTFs) were developed using the multi-layer perceptron (MPL) with Levenberg-Marquardt training algorithm for estimating chamomile essential oil content. Results evaluation of the accuracy and reliability of showed that, the third PTF (PTF3) which developed by all independent variables had the highest accuracy and reliability. Results also showed that, it is possible to predict the concentration and yield of chamomile essential oil based on soil physicochemical properties. This issue is important in terms of land suitability, identify areas susceptible to chamomile cultivation and planning for essential oil yields.

1. Introduction

Optimum nutrition is a major condition for improving the quality and quantity of the crops, and it is affected by the soil environment (Barker and Pilbeam, 2007; Hargreaves *et al.,* 2008; Ashoorzadeh *et al.,* 2016; Ajili *et al.,* 2018; Tofighi Alikhani, 2021). Peng *et al.* (2012) considered the role of soil characteristics to be highly effective in crops yield. Obviously, the production of organic materials in leaves without the presence of mineral

elements in the process of photosynthesis is not possible. Each of the macronutrients plays a special role in the metabolism of plant growth. Bernier *et al.* (1981) concluded that flowering of plants was under the control of nutritional status, and in this regard, the balance between the elements that plant takes from air and soil is very important. Plant mineral compounds are one of the factors affecting the quality of crops (Prasad and Spiers, 1991).

Considering the importance of developing the cultivation of medicinal plants and using their products as natural ingredients compatible with human health, it is necessary to use different cultivation and nutrition methods that increase the essential oil and effective compounds of medicinal plants (Ajili *et al.,* 2019; Savitikadi *et al.,* 2020). Chamomile (*Matricaria chamomilla* L.) is an annual plant, aromatic, 20-40 cm high that grows wildly on fields and side roads (Omid Beigi, 2004). The plant now has a large dispersal in Europe, Western Asia, North Africa, North and South America, and Australia. The extensive cultivation of this plant is carried out in countries such as Hungary, Germany, Egypt, Czech Republic, Slovakia, and India (Omid Beigi, 2004). In Iran, different species of the genus *Matricaria* grow in different parts of the country. Chamomile flowers are used to treat stomach, flatulence, and skin lesions. In most western countries, they are used as appetizers and digestible foods. The active ingredient of chamomile has been mentioned for many medicinal properties such as sedative, antispasmodic, stimulating white blood cells and strengthening the body's defense system, and antibacterial gram-positive and anti-allergic (Salamon, 1992). Accordingly, chamomile is used in many countries as dry flowers and essential oils in the pharmaceutical, food, cosmetic and sanitary industries. In recent years, it has also become one of the most popular pharmaceutical plants in the world (Gheedi Jashni *et al.,* 2015). Upadhyay and Patra (2011) investigated the effects of calcium and magnesium on chamomile growth and yield and stated that the effect of magnesium on the growth and essential oil of chamomile was higher than that of calcium.

One of the main issues in producing agricultural and garden crops is a lack of ability to forecast production/yield using accessible and easily measured indicators (Mohammadi Torkashvand *et al.,* 2020). Various factors affect the yield and essential oil content of the plant, including nutrition and physicochemical properties of the soil (El-Gohary *et al.,* 2015; Belal *et al.,* 2016; Radkowski and Radkowska, 2018; Mohammadi Torkashvand *et al.,* 2020). For example, it is hypothesized that one can estimate the yield of a product based on the concentration of nutrients in a leaf (Lahiji *et al.,* 2018; Mohammadi Torkashvand *et al.,* 2020), fruit (Mohammadi Torkashvand *et al.,* 2019) or soil characteristics (Rahmani Khalili *et al.,* 2020; Tashakori *et al.,* 2020). In this case, it will be possible to plan fertilization or choosing the soil susceptible and suitable for planting, or the farmer has an estimate of his income and, accordingly, plans its costs for the future programs.

There are various predictive methods for estimating several natural variables, among which more transfer functions are used. Different regression methods have been widely used to derive transitional functions (Vereecen *et al.,* 1992; Sepaskhah *et al.,* 2000; Marashi *et al.,* 2017; Eslami *et al.,* 2019). These methods consider the relationship between the input data and the data to be predicted to be predefined. Since the soil and plant are natural and heterogeneous systems, it is difficult to establish a connection between their properties. Therefore, in these systems, artificial neural networks (ANN) operate more efficiently than regression methods. Numerous studies have been carried out to estimate soil variables through artificial neural networks (Zhou *et al.,* 2008; Bocco *et al.,* 2010; Gago *et al.,* 2010; Parvizi *et al.,* 2010; Mokhtari Karchegani *et al.,* 2011; Besalatpour *et al.,* 2013; Dai *et al.,* 2014; Moghimi *et al.,* 2014; Aitkenhead *et al.,* 2015; Marashi *et al.,* 2017; Khanbabakhani *et al.,* 2019; Marashi *et al.,* 2019). Also, some studies have been conducted to predict crop yield by remote sensing, stochastic, artificial neural network (ANN) and simulation models (Bannayan and Crout, 1999; O'Neal *et al.,* 2002; Bartoszek, 2014; Farjam *et al.,* 2014; Domínguez *et al.,* 2015; Emamgholizadeh *et al.,* 2015; Dias and Sentelhas, 2017; Mohammadi Torkashvand *et al.,* 2017; Niedbała, 2019; Mohammadi Torkashvand *et al.,* 2019) based on weather, soil and growth characteristics as input data. Mohammadi Torkashvand *et al.* (2017) estimated the storage life of kiwifruit based on chemical characteristics of fruits, including the amount of nutrients by analyzing the neural network (NN), identifying it as a superior method in comparison to multiple regression. A similar study was carried out to predict kiwifruit yield based on leaf nutrients by ANN (Mohammadi Torkashvand *et al.,* 2020). Tashakori *et al.* (2020) evaluated the efficiency of artificial neural network (ANN), multiple lin-

ear regression (MLR), and adaptive neuro-fuzzy inference system (ANFIS) in terms of saffron yield estimation by soil properties in some lands of Golestan province, Iran. According to the results, ANN showed the highest accuracy (R^2= 0.58-0.89) in estimating saffron yield as compared to MLR (R^2= 0.41-0.47) and ANFIS (R^2= 0.41-0.69) models.

Poorghadir *et al.* (2021) concluded that the yield and percentage of essence influenced by the soil properties and nutrition.

The purpose of this study was to investigate the importance of soil characteristics on the concentration and amount of chamomile essential oils and their estimation with respect to some important physicochemical properties of the soil, and investigation of feasibility of using artificial neural networks for estimating the concentration and amount of chamomile essential oils.

2. Materials and Methods

Soil experiments

Several habitats or areas of Chamomile cultivation were surveyed in Kermanshah and Hamadan provinces, West of Iran. From 20 areas, 100 soil samples (five of each area) were taken from soil depths of 0-30 cm and transferred to IAU, Science and Research Branch, Tehran, Iran. The environmental characteristics of the sampling areas, in particular the topographic and climatic characteristics, were similar. Soil samples were shipped to the laboratory and air-dried and clods were broken down in small particles with a plastic hammer; then they were passed through a sieve of 2 millimeters (Klute, 1986). Afterward, 0.5 kg of each soil was used for laboratory analysis and the rest was used for greenhouse experiments. The soil samples were analyzed for phosphorus, nitrogen and potassium nutrients, pH, Electrical Conductivity (EC), texture, and organic matter. Soil pH and EC were measured in saturated soil extract. Soil texture was determined by hydrometric method and the amount of calcium carbonate equivalent (CCE) was measured using titration method (Paye *et al.,* 1948). The Kjeldahl method was used to measure nitrogen (Goos, 1995). Soil samples were extracted by Soltanpour and Schwab method (1977) and the concentration of available potassium and phosphorus were measured by flame emission and spectrophotometry methods (Emami, 1996). Organic matter was measured by Walkley and Black (1934) method. Some statistical data of soils are seen in Table 1.

Greenhouse experiment

In a completely randomized design, 100 different soil samples were sprayed in a plot (box) with dimensions of 30 to 35 cm and 25 cm in depth, and 20 seeds were planted in each plot. After germination and early growth of the plant, in the quadruple stage, the number of plants was reduced to 10 in each plot. During the growing season, field operations included irrigation, weed control and pest control for the plots were done alike for all the boxes.

After full flowering, the flowers were harvested at a maximum of five centimeters of length. The flowers were immediately dried at 60°C with an electronic dryer. In addition to the dry flower yield per plot in kg ha^{-1}, the concentration of essential oil for each soil was obtained. The essential oil content of the samples was determined by the Kelevenger apparatus by the water distillation method and expressed as g/100 g of dry flowers. The essential oil yield was expressed

Table 1 - Statistics of data set for estimation of essential oil percentage and essential oil yield

Soil properties	pH	EC (dS/m)	N (%)	P (mg/kg)	K (mg/kg)	OM (%)	CCE** (%)	Sand (%)	Silt (%)	Clay (%)	Essential oil (%)	Essential oil yield (kg/ha)
Max	8.75	1.60	1.30	81.00	381.00	2.30	69.00	39.40	42.40	55.60	1.57	7.37
Min	7.94	1.00	0.25	8.00	135.00	0.22	16.00	14.50	21.00	32.00	0.01	0.28
Average	8.13	1.28	0.73	22.10	226.10	1.43	33.40	24.11	30.57	45.32	0.69	3.71
Median	8.06	1.25	0.78	16.50	244.00	1.46	28.50	23.20	30.25	46.75	0.76	4.13
Standard	0.22	0.15	0.37	18.93	66.28	0.50	13.83	5.69	4.80	6.16	0.51	2.61
Kurtosis	1.72	-0.61	-1.72	5.37	-0.70	0.34	-0.06	1.32	-0.30	-0.95	-1.23	-1.72
Skewness	1.67	0.15	0.02	2.61	0.28	-0.69	0.92	1.02	0.26	-0.38	0.26	-0.12

OM= Organic Matter; CCE= Calcium carbonate equivalent.

as kg ha^{-1} in dry flower yield.

Artificial neural network

One of the best known rules is multilayer perceptron (MLP) learning rule. MLP is a feed forward network in which information flow from input side and pass through the hidden layers to the output layer to produce outputs. In this research, MLP rule and Levenberg-Marquart back propagation algorithm was used for training the artificial neural networks. The Tangent axon function was used as an activation function, which is a nonlinear function. Pourhaghi *et al.* (2013) also used the Tangent axon functions for predicting the input flows by ANN. NeuroSolutions 5.05 (NeuroDimension, Inc., Gainesville, FL, USA) software was used to design the artificial neural network.

The data used in training, validation and test were 60, 20 and 20% of the total data respectively. The training data are used for network education and training. Evaluation data are not used in network training, but this data are used to compare different models and to determine the most suitable network and PTFs.

The analysis of the neural network with three subseries of variables as input variables was performed to estimate or predict the essential oil percentage and essential oil yield:

1 - In first step, total nitrogen, available phosphorus and potassium and clay content of soils, which are the most important factor in soil fertility, were selected as predictors and PTF1 was developed.

2 - In the second step, pH, EC, organic matter and clay, which are found in the most common and important measurements in standard soil analyses, were selected as predictors for estimation desired variables, and PTF2 was developed.

3 - In third step all of the measured soil properties (N, P, K, OM, CCE, pH, EC, Sand, Silt and Clay) were included as predictor for developing PTF3.

Therefore, three PTFs (PTF1, PTF2 and PTF3) were developed using ANNs and their efficiency was compared with each other to find the best and most suitable PTF.

The number of input layer nods was chosen as the number of input parameters for each desired issue. The number of hidden layers determines the complexity of the grid, and the reason for this complexity is that as the number of hidden layers increases, the number of connections between the nerve layers increases, which leads to network complexity. The number of output layer neurons is equal to the number of output parameters of the desired problem.

After training the network with the data of the training and validation series, the precision and accuracy of the generated models were evaluated using the test series data.

In order to evaluate network accuracy, the coefficient of determination (R^2) and square error squared (RMSE) were used.

$$R^2 = 1 - \frac{\sum_{1}^{N}(y_i - \hat{y}_i)}{\sum_{1}^{N}(y_i - \bar{y}_i)^2} \quad (1)$$

$$RMSE = \sqrt{\frac{\sum_{1}^{N}(y_i - \hat{y}_i)^2}{N}} \quad (2)$$

In which: y_i, $\bar{y}_i$, $\hat{y}$, respectively, the measured dependent variable, its mean and the estimated dependent variable, and N is the number of observations. Other criteria used to evaluate the precision of transition functions were the Geometric Mean Error Ratio (GMER) and Geometric Standard Deviation of error ratio (GSDER):

$$GMER = \exp\left(\frac{1}{N}\sum_{1}^{N}\ln\left(\frac{\hat{Y}_i}{Y_i}\right)\right) \quad (3)$$

$$GSDER = \exp\left(\frac{1}{N-1}\sum_{i=1}^{N}\left[\ln\left(\frac{\hat{Y}_i}{Y_i}\right) - \ln(GMER)\right]^2\right)^{1/2} \quad (4)$$

Geometric mean of error ratio (GMER) represents the degree of conformance between measured and estimated values. If the GMER is equal to one, it represents a complete fit between measured and predicted values. If the GMER is greater than one, it indicates that the predicted values are greater than the measured values, and the GMER less than one is an indication of lower estimated values than the measured values. Geometric standard deviation of error ratio (GSDER) is a measure of data diffusion. If it is close to one, it shows less diffusion and the difference between one and the other represents the deviation of most estimates from the measured data.

3. Results and Discussion

Correlation between variables

Table 2 shows the correlation between the variables studied and the percentage and yield of the essential oils. The results presented in this table could be important to find input data series to the neural network. In our study, the percentage and yield of essential oil showed a positive and significant correlation with organic matter, N, P and K contents and clay. Jat and Ahaheat (2006) showed that the use of bio-fertilizers containing nitrogen, phosphorus and potassium increased the growth and amount of essential oil of the fennel plant. Phosphorus plays an important role in seed, flowers germination, vegetative growth, the acceleration of ripening and the completion of metabolic processes of fruits (Bennett, 1993; Malakouti and Shahabi, 2000; Malakouti *et al.,* 2008). It is also involved in controlling enzymatic reactions and regulating metabolic pathways (Rejali, 2005; Miransari *et al.,* 2007). Potassium affects the amount and quality of herbal essential oils due to its effect on metabolic pathways and enzymatic activity (Pacheco *et al.,* 2008). In the case of potassium deficiency, the quality of some products, especially fruits and vegetables, decreases (Egilla *et al.,* 2005; Mohiti *et al*., 2011). Potassium deficiency during plant growth leads to a decrease in photosynthetic rate and chlorophyll content (Gerardeaux *et al.,* 2010), activation of enzymes, and reduced growth and yield (Kanai *et al.,* 2007). Potassium interacts with almost all essential elements.

Furthermore, a synergistic role of K with either N or P has been already noted (Barker and Pilbeam, 2007). Nurzynska-Wierdak (2013), Cecílio Filho *et al.* (2015) and Chrysargyris *et al.* (2017 a) evaluated the impact of different potassium levels (275, 300, 325, 350 and 375 mg/L) on the morphological and biochemical characteristics of spearmint (*Mentha spicata* L.). The results showed that the potassium in 325 mg/L treatment could be appropriate for spearmint cultivation and production for essential oil uses. In the same study, Chrysargyris *et al.* (2017 b) found that the lavender grown in 300 mg L^{-1} of K was appropriate for the essential oil uses/production while the 325 mg L^{-1} of K were more appropriate for lavender cultivation for fresh and dry matter uses.

Due to the significant correlation of essential oils with organic matter, N, P and K contents and clay (Table 2), it was determined three series of data as input data of ANN that is observed in Table 3. Table 3 shows the number of hidden layers, and the number of nodes in the hidden layers in the three input series.

Estimation (prediction) of essential oil

Figure 1 shows the distribution of actual values (measured) and the estimation or prediction of the percentage of essential oil of chamomile and their conformity in three series of input data. R^2 was between the measured values and the estimated essential oils as observed in Table 4. As seen in the first transfer function (PTF1), R^2 was 82.71% in the test data and 78.56% in the training data series. The GMER value indicated that almost the network in the test data was not under or over-estimating, and its

Table 2 - Correlation between the input variables of the neural network and the amount of essential oil (g/100 g of dry flowers) and essential oil yield

Variable	pH	EC	N	P	K	OM	CCE	Sand	Silt	Clay	Essential oil	Essential oil yield
pH	1											
EC	0.511**	1										
N	-0.101	0.152	1									
P	0.806**	0.546**	-0.382*	1								
K	-0.390*	-0.419**	0.099	-0.323*	1							
OM	0.414**	0.675**	0.362*	0.443**	-0.046	1						
CCE	-0.267	0.015	0.001	-0.287	0.044	-0.544**	1					
Sand	-0.032	-0.339*	-0.439**	-0.029	0.268	-0.577**	0.648**	1				
Silt	-0.202	0.219	0.269	-0.069	-0.201	0.409**	-0.258	-0.408**	1			
Clay	0.188	0.178	0.243	0.084	-0.12	0.276	-0.465**	-0.711**	-0.353*	1		
Essential oil	0.092	-0.024	0.391*	0.278	0.434**	0.355*	-0.181	0.155	0.153	-0.277	1	
Essential oil yield	0.235	0.163	0.401*	0.423**	0.382*	0.449**	-0.101	0.226	0.246	-0.421**	0.919**	1

Table 3 - Input data for constructing a neural network in three different transfer functions and the characteristics of neural networks made

Transition function	Model inputs	No. of hidden layers	No. of hidden layer nodes 1	No. of hidden layer nodes 2	No. of hidden layer nodes 3	Type of transfer function	Type of target function
PTF1	N, P, K and clay	3	4	2	1	Tangent axon	Levenberg-Marquardt algorithm
PTF2	pH, EC, Organic matter	3	4	4	2	Tangent axon	Levenberg-Marquardt algorithm
PTF3	All variables	1	10	-	-	Tangent axon	Levenberg-Marquardt algorithm

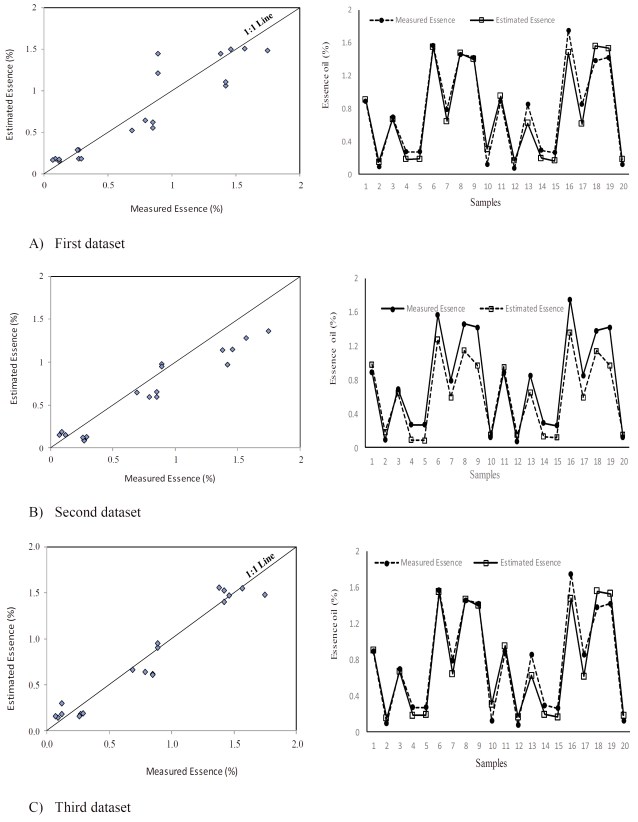

Fig. 1 - Measured values (actual) and estimated concentration of essential oils in diagram 1: 1 test data and their conformance.

values were roughly one, but for the training data series the model overestimated. The R^2 value in the training data of the second series was 18.06% more than the first transfer function. In the test data, the R^2 value increased by 6.83%. The GMER value indicated that the model underestimated, and this is especially evident in the test data series.

According to the results of Table 4, when each of the nine variables was considered as inputs to the network, the distribution of estimated and measured values (Fig. 1) was reduced around the 1:1 axis and R^2 in training data is up to 96.62% and the test rose to 94.78%. Another important aspect is the more accurate estimation of the percentage of chamomile essential oil in both the training and test data, so that the GMER value in both data series was near one, indicating an accurate estimate and a lack or low estimate. The value of GSDER also showed that the lowest non-conformance of predicted essential oils with the measured values in the data of the training and test series in the third transfer function was observed. Based on these results, increasing variables from 4 (PTF1 model) to 9 (PTF3 model) decreased error and increased R^2 of ANN-model. Mohammadi Torkashvand *et al*. (2020) employed an artificial neural network (ANN) to evaluate the kiwi yield of Hayward cultivar based on the concentration

Table 4 - Determination coefficient (R2), error (RMSE), GMER and GSDER in two sets of training and test data in predicting essential oil concentration (g/100 g)

Transition function	Data series	R^2	RMSE	GMER	GSDER
PTF1	training	0.7856	0.226	1.27	2.37
	test	0.8271	0.201	1.04	1.24
PTF2	training	0.9662	0.072	0.91	2.27
	test	0.8954	0.237	0.83	1.57
PTF3	training	0.9562	0.023	0.98	1.22
	test	0.9478	0.086	1.02	1.32

of nitrogen, potassium, calcium, and magnesium in leaves. They concluded that the maximum R^2 and the lowest root mean square error were obtained when all nutrients and related ratios were considered as input variables. Mohammadi Torkashvand *et al.* (2017) tested and compared the performance of an artificial neural network in predicting the firmness of six-month stored kiwifruit with different input datasets. Reversely, they showed that the best answer was obtained using ANN with a RMSE of 0.539 and a correlation coefficient of 0.850 (R^2=0.724) when the nitrogen and calcium (N/Ca ratio) were input data (two variables). Prediction of 6-month fruit firmness using nutrient concentrations and their rations (8 variables) datasets resulted in the lowest R-value and the highest error (Mohammadi Torkashvand *et al.,* 2017).

Figure 2 shows the dispersion of the measured yield values and the estimation of essential oil in the test series in chart 1:1 and in the 20 samples. According to the results of Table 5, the value of R^2 in the test data series in the first transfer function was 91.25%, but less than 80% in the training data. The other thing is the high amount of dispersion, non-conformance of the estimated and measured and over-estimation data in training. Therefore, in the test series, the accuracy has increased, and in addition to reducing the dispersion and increasing the conformance, the prediction accuracy has significantly increased, because GMER was approximately one. Forecasting models of plant yield are prognostic tools that can be an important element in precision agriculture (Shearer *et al.,* 2000; Dias and Sentelhas, 2017; Prasad *et al.,* 2017; Mohammadi Torkashvand *et al.,* 2017, 2020) and the principal factor in decision-making systems (Park *et al.,* 2005). Akbar *et al.* (2018) used a model based on artificial neural network to predict essential oil yield in turmeric (*Curcuma longa* L.). The data of essential oil, soil and environmental factors were collected from 131 turmeric germplasms in 8 agro-climatic regions of Odisha. Results showed that multilayer-feed-forward neural networks was the most reasonable model to use with R^2 value of 0.88. Niazian *et al.* (2018) calculated a root mean square error (RMSE) of 0.192 and R^2 of 0.901 in predicting essential oil content of Ajowan by using the artificial neural network when the number of rays, pedicels, and flowers per umbellet, and a number of umbellets in an umbel, inputted variables. Bahmani *et al.* (2018) utilized an artificial neural network modeling to predict kinetics of essential oils extraction from tarragon (*Artemisia dracunculus* L.) using ultrasound pre-treatment with Clevenger. Based on results, the best prediction performance was belonged to 3-7-1 ANN architecture (0.0008 normalized mean squared error and R^2 were respectively 0.0008 and 0.99) which means that it is possible to predict the extraction yield of essential oils with an acceptable precision. Tashakori *et al.* (2020) research showed that a model with organic matter, phosphorus, potassium, and calcium carbonate as in dependent variables, was the best model (R^2 = 0.87) in estimating saffron yield.

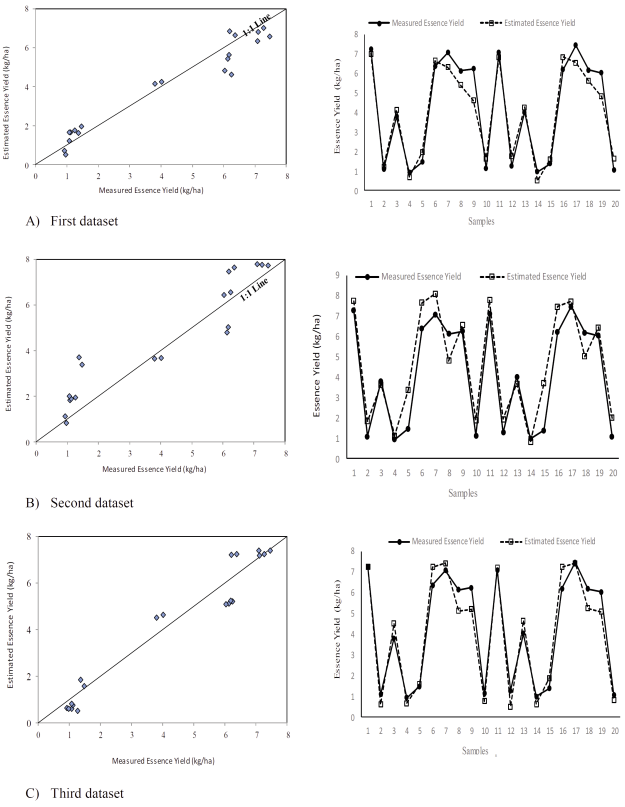

Fig. 2 - Measured (actual) and estimated essential oil yield in diagram 1: 1 test data and their conformance.

If the focus is on the test data, in the second transfer function, the accuracy of the model (R^2 = 83.23%) was less than the other two functions and its accuracy is much lower than the other two, so that the deviation of the measured and estimated essential oil yield data was 1.45 (GSDER) and the model had an over-estimation of 26% (GMER=1.26). The important point is that, like the content of essential oil (g/100 g dry matter), the highest accuracy and precision of the neural network model was obtained in predicting the essential oil yield in the third trans-

fer function, in which all soil variables (nine variables) were considered as input variables of the network. In the test data series, the lowest error (RMSE = 0.088) and the most consistent measured and estimated data on the essential oil yield were obtained in the third transfer function, although the fitted model had a lower estimation than the first function. Of course, it should be noted that in view of the great difference between the first and third functions in training data, the third function had a higher credibility in general.

4. Conclusions

In general, according to the results, the third transfer function (9 variables as input variables of the network) was the most accurate for estimation of the essential oil concentration and for the estimation of essential oil yields. They had the highest R^2 and the lowest RMSE values. Also, the estimated values of these functions were the most consistent with the observed values and the least deviation from the 1:1 line. As the R^2, RMSE, GMER and GSDER values of the proposed model for estimating essential oil percent for test series data were 94.78, 0.86, 1.02 and 1.32, respectively, and to evaluate the essential oil yield in the test series data respectively, equal to 91.51, 0.608, 0.92 and 1.20 respectively. Therefore, the results showed that with high accuracy and precision, it is possible to predict the concentration and yield of chamomile essential oil based on soil physicochemical properties. This issue is important in terms of land suitability, making possible to identify areas susceptible to chamomile cultivation and to plan for essential oil yields. It is suggested to model the prediction of the percentage and yield of chamomile essential oil with an artificial neural network with other characteristics of the soil alone or in combination with these characteristics and compare the results. It is also suggested that other models, such as neuro fuzzy should be evaluated for estimating the concentration and essential oil of chamomile as well as other medicinal plant species.

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Impact of exogenous pre and postharvest salicylic acid applications on MD2 pineapple quality

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Abstract: Salicylic acid (SA) is a natural plant compound that has been proven to enhance the quality of fruits; therefore, its impact on pineapple should be further studied, especially in the most marketable hybrids. This study aimed to evaluate the effect of SA treatments on MD2 pineapple quality. The experiment consisted of two parts with applications pre and postharvest following the next treatments, control: No use of SA, and 5, 7 and 9 mM of SA. The total soluble solids, total acidity, ascorbic acid content, respiration rate, together with the severity and incidence of internal browning and flesh translucency were determined, after 40 days of cold storage. The treatment using 9 mM of SA in pre and postharvest delivered the best results, having the most elevated ascorbic acid (526.75 mg kg^{-1}) and total acidity (0.8%), the lowest severity and incidence outcomes of internal browning and flesh translucency (0% in both cases), with the most reduced respiration rate values during postharvest. In conclusion, SA treatments with concentrations of 9 mM applied in pre and postharvest on MD2 pineapple can improve its quality after 40 days of cold storage.

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

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The authors declare no competing interests.

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1. Introduction

Pineapple is a fruit characterized by its rich source of sugars, organic acids, fibers, minerals, vitamins, flavonoids, and carotenoids (De Ancos *et al.*, 2017). These are essential food properties for healthy human nutrition. Nowadays, low acid hybrids like MD2 are the most exported by the industry worldwide (Hossain, 2016; Cano-Reinoso *et al.*, 2022 a). This hybrid is known for its bright-gold colour, sweeter taste, high ascorbic acid (AsA) content, and uniform size (Bin Thalip *et al.*, 2015; Cano-Reinoso *et al.*, 2022 a). Nevertheless, MD2 is susceptible to physiological disorders like flesh translucency and internal browning, which are major problems that negatively impact its quality (Chen and Paull, 2017; Paull and Chen, 2018).

Currently, the use of natural compounds to deal with these disorders has become a trend (Lu *et al.*, 2011; Goñi *et al.*, 2017). Because of the reduced negative impact these compounds cause to the environment and human health, multiple studies have been implemented by producers and growers (Ponce *et al.*, 2011; Goñi *et al.*, 2017). In this context, salicylic acid (SA) has been investigated as a potential treatment to decrease physiological disorders due to its positive impact on fruit metabolism (Hayat *et al.*, 2010; Goñi *et al.*, 2017). This secondary metabolite has been proved to enhance ion uptake and transport, disease resistance, ripening delay, and control postharvest quality and shelflife of horticultural products (Asghari and Aghdam, 2010; Goñi *et al.*, 2017). For instance, SA has demonstrated outstanding results in reducing the fruit softening rates and the degrading of the sugar and acid content during postharvest (Asghari and Aghdam, 2010; Goñi *et al.*, 2017). Also, SA can mitigate the cell wall degrading and polygalacturonase (PG) enzyme activity, phenomena highly associated with translucency and internal browning in pineapple (Goñi *et al.*, 2017; Paull and Chen, 2018; Cano-Reinoso *et al.*, 2021).

Previous studies in pineapple fruit demonstrated that postharvest SA treatments (in solutions with concentrations between 5 and 9 mM), reduced the internal browning severity and translucency occurrence without affecting the total soluble solids (TSS) and total acidity (TA) negatively (Lu *et al.*, 2010, 2011; Cano-Reinoso *et al.*, 2022 b). Besides, it was determined that in pineapple SA could cause a positive effect on the antioxidant content with a reduction of the peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) enzyme activities, concomitantly with a low respiration rate (Lu *et al.*, 2010, 2011; Cano-Reinoso *et al.*, 2022 b).

However, despite the previous information described currently, there are no sufficient studies on pineapple fruit that document the impact of SA, especially complementing postharvest with preharvest applications. For example, concerning the preharvest stage only two experiments have reported the use of SA, obtaining contradictory results, and mostly implementing just low concentrations (around 2 mM), like in Lu *et al.* (2011) and Cano-Reinoso *et al.* (2022 b). On top of that, some of these former researches have mainly focused on acid hybrids. Therefore, this issue open questions concerning the determination of the exact impact of SA on low acid hybrids like MD2, primordially with a complementary administration, including pre and postharvest. As a result, this study aims to evaluate the impact of exogenous pre and postharvest SA applications on MD2 pineapple quality.

2. Materials and Methods

Preparation of the experiment

This research was implemented in pineapple fields in Lampung, a place located in south Sumatra Indonesia, from January to March 2020, employing the MD2 pineapple hybrid. In order to fulfil the objective of the research, the experiment was divided into two parts. The first part regarding preharvest applications of SA, while the second part concerned the postharvest administration of this natural compound.

Preharvest treatments implementation

Regarding the first part of the experiment, a completed randomized block design was used with four replications, having 20 fruits each of the replications. The fruits were harvested between 144-147 days after flowering, when it has been determined that MD2 can expose the most optimal quality characteristics for commercial consumption (Bin Thalip *et al.*, 2015; Ding and Syazwani, 2016). Besides, for this part of the research, four rows in each block were prepared with a width and length of 0.4 and 3.75 m, respectively. Pineapple plants were organized in two lines of ten plants with a separation of 0.25 m. For this stage the treatments implemented were; control: No use of SA, 5, 7 and 9 mM of SA.

Furthermore, the administration of SA was done using sprayings with their respective solutions until the fruits were wet to runoff. SA solutions were mixed with 1% (v/v) of ethanol and 0.01% (v/v) of tween 20 (emulsifier) before their application. The sprayings were performed at eight, six, four and two weeks before harvest at night, in the fruit shell and crown. Previous experiment of Cano-Reinoso *et al.* (2022 b) demonstrated that two applications prior to harvest (six and three weeks) can cause a positive effect on pineapple quality. Therefore, this research aimed to increase the frequency of application preharvest in order to enhance the influence on the fruit quality. Moreover, the fruit shell and crown were selected as adequate spots to be used based on the findings about mineral mobility in pineapple plants

described by Vásquez-Jiménez and Bartholomew (2018) and Murai *et al.* (2021). They demonstrated that through these plant structures there was mineral assimilation when foliar fertilizations were carried out after flowering.

The soil, where the pineapple plant was cultivated, was fertilized previously with 200 kg ha^{-1} Diammonium Phosphate, 1000 kg ha^{-1} of K_2SO_4 and 200 kg ha^{-1} Kieserite crystal. Three months after plating 700 kg ha^{-1} of Urea were administered by sprayings; also, 700 kg ha^{-1} of $(NH_4)_2SO_4$, 1000 kg ha^{-1} of K2SO4, 170 kg ha^{-1} of MgSO4, 60 kg ha^{-1} FeSO4, 60 kg ha^{-1} ZnSO4, were applied in intervals of 30 days. Besides, borax was sprayed in doses of 30 kg ha^{-1} at flower induction. Climatological conditions were determined where the plants were cultivated with a weather station (LSI Lastem; equipped with a CR6 data logger from Campbell Scientific; Italy). An average of 70.60% of relative humidity (RH), 22.43°C, 10.18 w m^{-1} of solar radiation, and rainfall of 353.10 mm were detected. The physical and mineral composition of the soil of the first part of the experiment is presented in Table 1.

Postharvest treatments implementation

Concerning the second part of the experiment, randomly ten fruits per replication were selected at harvest, organized according to their respective treatments and replications inside a cold storage during 40 days (8°C and 90% RH), and analyzed in intervals of eight days. The treatments implemented in this stage of the research were; control: No use of SA, 5, 7 and 9 mM of SA. All the treatments, including the control, received fungicide and waxing applications in postharvest; those materials, following that order, were administrated in dipping applications for ten seconds, just after the dipping on SA. The fungicide product used was Prochloraz in doses of 2 cc l^{-1}, while the waxing material employed was Sta-Fresh 2952 in doses of 74 g l^{-1}. Furthermore, the SA concentrations were dissolved in a water container of 25 L; Besides, in this case SA was also mixed with 1% (v/v) of ethanol and 0.01% (v/v) of tween 20 (emulsifier). The dipping in SA was done for five minutes. This dipping time and the concentrations implemented were selected based on Lu *et al.* (2011) and Cano-Reinoso *et al.* (2022 b). They proved that postharvest dipping applications around five minutes should have a minimum concentration of 2 mM to cause a positive impact on pineapple fruit. Finally, the summary of the treatments implemented pre and postharvest in this research are presented in Table 2.

Table 1 - Physical and mineral composition of the soil in the first part of the experiment

Properties	Content
Texture	
Clay (%)	9.00
Loam (%)	36.56
Sand (%)	50.42
Chemical properties	
pH (H_20)	7.62
C (%)	4.60
N (mg kg^{-1})	853.00
P (mg kg^{-1})	5.29
K (mg kg^{-1})	9.68
Ca (mg kg^{-1})	76.39
Mg (mg kg^{-1})	109.00
Na (mg kg^{-1})	12.84

The N, P, K, Ca, Mg and Na represent the available mineral content in the soil.

Table 2 - Summary of the treatments implemented in pre and postharvest stage of the experiment

Treatment	Description
A	Control: No use of SA
B	5 mM SA pre/postharvest
C	7 mM SA pre/postharvest
D	9 mM SA pre/postharvest

* Preharvest salicylic acid (SA) was sprayed at 8, 6, 4 and 2 weeks before harvest until fruits were wet to runoff. Postharvest SA was administrated with dipping of 5 minutes, and complemented with fungicide and waxing applications. Pre and postharvest SA solutions were mixed with: 1% (v/v) ethanol, and 0.01% (v/v) tween 20 (emulsifier).

Fruit quality evaluation

Total soluble solids (TSS) and total acidity (TA) in the fruit

The total TSS and TA content was calculated in every fruit selected of every treatment from each replication, employing the method described in Shamsudin *et al.* (2020). TSS was measured using a hand-held refractometer (MASTER-53 α; Atago; Japan), while TA was determined by titration to pH 8.1 with 0.1 M NaOH employing phenolphthalein indicator and expressed as a percentage of citric acid.

Fruit respiration rate

The respiration rate was calculated in a fruit selected from eight until 40 days of cold storage in each replication of every treatment implemented. A similar method previously reported in Bhande *et al.* (2008) and Cano-Reinoso *et al.* (2022 b) was implemented. For this case, changes in the CO_2 concentration were measured in a sealed glass container having 31 cm height x 24 cm wide with 9 l of capacity. The device used for this procedure was a portable AZ7788A carbon dioxide detector (CO_2 range: 0-5000 mg kg^{-1}, 10-95 % RH, 0-50°C; AZ Instrument Corp; Taiwan). Moreover, before beginning the procedure, the fruit weight was calculated using a weighing scale, as described in Shamsudin *et al.* (2007). Once finished this process, the CO_2 detector and the fruit were arranged inside the container, avoiding any air introduction or scape. The changes in the CO_2 concentration were measured during one hour in every fruit, and after that, the respiration rate was determined using the following expression:

$$RF = \left[\frac{(FCO_2)h-(FCO_2)h+1}{\Delta t}\right]\frac{V}{FW} \quad (1)$$

Where, respiration of the fruit (*RF*) is the respiration rate in ml CO_2 $kg^{-1}\cdot h^{-1}$, FCO_2 is the CO_2 gas concentration in ml l^{-1}, *h* is the storage time in hours, Δt the time difference between two CO_2 gas measurements, *V* is the free volume of the container in l and *FW* is the weight of the fruit in kg. The free volume of the container was obtained as the total volume of the container minus the volume occupied by its content at the moment of the measuring, using a water displacement method as described in Bhande *et al.* (2008). Figure 1 shows a picture of the arrangements carried out to implement the respiration rate method of this experiment already described.

Ascorbic acid (AsA), translucency and internal browning incidence determination

These variables were measured in each of the fruits per replication of every treatment. The AsA content was calculated using dye, 2,6-dichlorophenol-indophenol titration method described in Ding and Syazwani (2016) and Ojukwu and Nwobi (2017). First, diluted pineapple juice was pipetted into a conical flask and mixed with glacial acetic acid, titrating the solution until faint permanent pink colour. Next, the titrated value was recorded; then, the titration was repeated, boiling and cooling with distilled water for a blank and a standard ascorbic acid solution. The titrations obtained were repeated twice, and the average value was calculated and expressed as mg kg^{-1} fruit fresh weight.

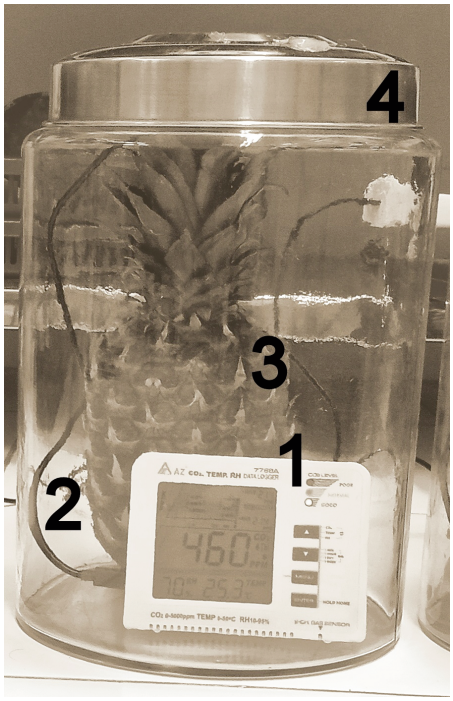

Fig. 1 - Picture showing the respiration rate method employed. 1) CO_2 detector used, 2) Cable of electricity to activate the detector introduced by the top of the container, 3) Fruit and 4) Sealed container.

On the other hand, the translucency internal browning incidence was obtained accounting number of fruits affected from the total examined, since harvest until 40 days of cold storage. The results are expressed in percentage.

Statistical analysis

The statistical analyses were performed using SPSS Version 22.0 software (SPSS Inc., Chicago, IL, USA). All data were analysed by an analysis of variance of one-way (ANOVA). Mean significant differences at $p<0.05$ were determined by Duncan's multiple range tests.

3. Results

Fruit physicochemical quality

The TSS, TA and TSS/TA ratio did not show significant differences after 40 days of cold storage in the implemented treatments. The TSS and TA content was on average 15 and 0.70%, respectively; mean-

while, for the TSS/TA the mean values ranged around 21 (Table 3). For these variables, high SA concentrations were not clearly associated with more elevated mean results. On the other hand, the AsA results did not demonstrate significant differences in this experiment after 40 days of cold storage. Nevertheless, it is important to notice that the treatment using 9 mM of SA in pre and postharvest obtained the highest mean value (526.75 mg kg^{-1}), which was linked to the more superior content of TA (0.80%) (Table 3). Moreover, in figure 2 it is possible to observe the trend of AsA through the postharvest time of the experiment. AsA continually elevated and reduced its level, with the treatment employing 9 mM of SA in pre and postharvest having the most inferior peak change, especially between 32 and 40 days of cold storage.

Furthermore, in the case of the respiration rate, this variable exposed significant differences after 40 days of cold storage. The control treatment (no use of SA) provided the highest value, while the treatment utilizing 9 mM of SA in pre and postharvest had the most reduced one (8.24 and 6.32 ml CO_2 $kg^{-1} \cdot h^{-1}$, respectively). Figure 3 shows the respiration rate trend during postharvest. In this figure, it is evidenced that the treatments employing a higher SA concentration, like the treatments with 7 and 9 mM of SA in pre and postharvest, had a steadier trend and lower change during cold storage. On the other hand, the control treatment and the one using 5 mM of SA in pre and postharvest suffered a remarkable increase, primordially in 16 days, suggesting a representative metabolic change at that moment in the fruit. On top of that, high concentrations of SA were associated with a lower respiration rate and more elevated AsA content at harvest.

Internal browning and flesh translucency in the fruit

The internal browning and flesh translucency provided significant differences in the results obtained.

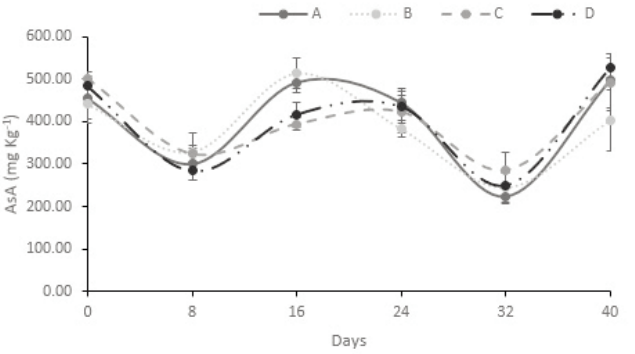

Fig. 2 - Effect of the treatments implemented on the ascorbic acid (AsA) content during 40 days of cold storage. A) control [No use of salicylic acid (SA)], B) 5 mM SA pre/postharvest, C) 7 mM SA pre/postharvest, and D) 9 mM SA pre/postharvest. Values are the mean four replicates, and vertical bars represent ± SE.

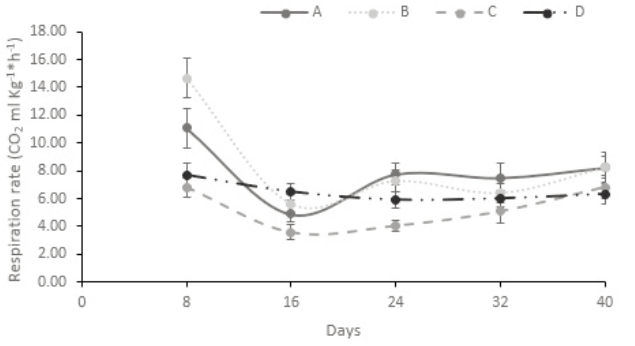

Fig. 3 - Effect of the treatments implemented on the fruit respiration rate during 40 days of cold storage. A) control [No use of salicylic acid (SA)], B) 5 mM SA pre/postharvest, C) 7 mM SA pre/postharvest, and D) 9 mM SA pre/postharvest. Values are the mean four replicates, and vertical bars represent ± SE.

Regarding the internal browning, this had its most elevated severity and incidence in the treatments using 5 and 7 mM of SA in pre and postharvest (3.75 and 6.25%, respectively), while the control treatment

Table 3 - Impact of the treatments implemented on the fruit quality characteristics after 40 days of storage

Treatment	TSS (%)	TA (%)	TSS/TA	AsA (mg Kg^{-1})	Respiration rate (ml CO_2 Kg-1 h^{-1})	Browning (%)		Translucency (%)	
						Severity	Incidence	Severity	Incidence
A	14.80 ± 0.22 a	0.70 ± 0.07 a	21.73 ± 1.85 a	496.25 ± 62.86 a	8.24 ± 0.40 a	0.00 b	0.00 b	1.85 ab	16.67 a
B	15.40 ± 0.50 a	0.67 ± 0.07 a	24.10 ± 3.65 a	402.50 ± 71.81 a	8.23 ± 0.92 a	3.75 a	6.25 a	0.64 b	8.33 b
C	15.80 ± 0.29 a	0.75 ± 0.05 a	21.43 ± 1.53 a	492.50 ± 65.97 a	6.83 ± 0.65 ab	3.75 a	6.25 a	0.35 b	4.17 b
D	15.15 ± 0.31 a	0.80 ± 0.06 a	19.13 ± 1.23 a	526.75 ± 23.47 a	6.32 ± 0.53 b	0.00 b	0.00 b	0.00 b	0.00 c

* Mean values ± SE in each column followed by the same lower-case letters are not statistically different by Duncan's multiple range test, and Kruskal-Wallis test (for the internal browning and translucency data) ($p < 0.05$).

and the one employing 9 mM of SA in pre and postharvest did not expose representative symptoms after 40 days of cold storage (0% for the severity and incidence in both cases) (Table 3). Moreover, this lowest browning severity and incidence outcome was related to the most inferior respiration rate and the highest AsA content at harvest, primordially in the treatment utilizing 9 mM of SA in pre and postharvest. Concerning the flesh translucency, the outcomes of this experiment also revealed that this previous treatment is the one causing the lowest severity and incidence (0% in both cases), while the control treatment was the least efficient in this aspect (1.85 and 16.67%, respectively) (Table 3). Similar to the internal browning results, the most elevated AsA content and lowest respiration rate at harvest in the treatment employing 9 mM of SA in pre and postharvest were linked to the most inferior translucency incidence and severity.

4. Discussion and Conclusions

Typically, MD2 pineapple displays 12% or higher values for TSS during cold storage (Paull and Chen, 2018; Cano-Reinoso *et al.*, 2021). The values obtained in this research were higher than 14%, especially for those using SA applications. TSS represents the sucrose content in pineapple fruit primordially; also, a change in its content commonly is associated with modifications in the cell wall invertase (CWI) activity (Saradhuldhat and Paull, 2007; Paull and Chen, 2018). The results of this experiment infer that the pre and postharvest treatments employed did not cause a representative impact on the sucrose content and the sugar metabolizing enzymes, essentially a negative impact, to cause a change in the TSS level at the end of the cold storage.

In the case of the TA, due to its low acid properties, MD2 has evidenced TA values among 0.4-0.7% at harvest, which is much lower than acid hybrids like smooth cayenne (Ding and Syazwani, 2016; Paull and Chen, 2018). Nevertheless, a small increase in this range has been reported during cold storage of pineapple due to the rise of the citric acid content (Mandal *et al.*, 2015; Ding and Syazwani, 2016). Citric acid is considered a source for several metabolizing process in fruits, like antioxidant scavenging and respiration; also, it is the main acid influencing the TA measuring in pineapple (Paull and Chen, 2018; Yang *et al.*, 2019). Therefore, in concomitance with the fruit decay, this acid could has speed up its accumulation as a reflect of this physiological deterioration, influenced by the need of a scavenger agent to cope with this condition. This fact can explain why despite the lack of significant differences, in mostly all the treatments, TA had higher values than the minimal recommended for MD2, after 40 days of cold storage. On top of that, as also the control treatment (no use of SA) had an ideal TA outcome, it could be suggested that the waxing formula used was ideal to decrease the fruit metabolism in order to avoid a high consumption of citric acid as a substratum of several physiological process, generating the respective TA content.

Furthermore, a small increase in the TSS and TA content has been observed by SA applications in pineapple (Lu *et al.*, 2011; Mandal *et al.*, 2015). Moreover, some studies have demonstrated that pre and postharvest implementations of SA tend to suppress the TSS and TA degrading, especially during postharvest, like in mango and grape (Champa *et al.*, 2014; Hong *et al.*, 2014). This information could explain why the TSS and TA values were in the optimal content for consumption or slightly superior in almost all the treatments employing SA. Besides, MD2 has more elevated TSS/TA ratios than other hybrids, especially because of its lower acid content, ranging between 20-30 in mostly all cases. (Chen *et al.*, 2009; Ding and Syazwani, 2016). Overall, values between this range were observed in the results of this experiment (Table 3).

On the other hand, regarding AsA, this is considered a powerful plant antioxidant and one of the most representative in pineapple fruit (Kongsuwan *et al.*, 2009; Noichinda *et al.*, 2017). This information infers that the treatment utilizing 9 mM of SA in pre and postharvest can provide a more elevated antioxidant production, as it impacts the AsA level positively and the previous described TA content associated with citric acid. MD2 delivers AsA values as minimal as 300 mg kg^{-1} at harvest, much higher than pineapple acid hybrids (Lu *et al.*, 2014; Paull and Chen, 2018). This value tends to increase during postharvest to maintain optimal physiological conditions, essentially when a stress factor affects the fruit (Lu *et al.*, 2011; Mandal *et al.*, 2015). The variability of the AsA exposed in figure 2 could be associated with the activity of the ascorbic peroxidase (APX) and monodehydroascorbate reductase (MDHAR) enzymes. For example, APX is an optimal scavenger of H_2O_2, which is generated with the progress of the fruit

senescent. However, this enzyme can cause the oxidation of AsA into monodehydroascorbate (MDHA). Therefore, MDHA have to be recycled into AsA by the glutamic acid pathway employing MDHAR as cofactor, in order to maintain an ideal level of AsA to cope with impact of the fruit decay. As a result, these two enzymes can be used as an indicator of the AsA content and metabolization (Gallie, 2013; Akram *et al.*, 2017). Based on that, the treatment working with 9 mM of SA in pre and postharvest could be considered the one causing more influence on these enzymes, catalyzing their activities during cold storage. Similar patterns in the AsA production during postharvest has been reported in pineapple by Lu *et al.* (2010) and Cano-Reinoso *et al.*, (2022 b), where higher SA concentrations were associated with a more superior AsA content.

In the case of the respiration rate, values between 5 and 20 ml CO_2 $kg^{-1} \cdot h^{-1}$ have been reported for pineapple in previous studies (Lu *et al.*, 2011; Hu *et al.*, 2012; Cano-Reinoso *et al.*, 2022 b). The outcomes obtained in this experiment provided results among that recommended range. On the other hand, SA has been associated with an increase in antioxidant scavenger enzymes like catalyze (CAT) and superoxide dismutase (SOD), which, together with the APX, reduced the impact of the fruit decay symptoms during postharvest, eliminating singlet of oxygen-derived from reactive oxygen species (ROS), essentially the already mentioned H_2O_2 (Goñi *et al.*, 2017; Noichinda *et al.*, 2017). On top of that, Lu *et al.* (2011) and Cano-Reinoso *et al.* (2022 b) demonstrated that SA solutions with concentrations higher than 5 mM, essentially with postharvest implementations, can generated a low respiration rate, with a similar trend pattern of this experiment. Besides, they determined that this low respiration rate was associated with a more superior activity of the CAT, SOD and APX, especially between 16 and 24 days of cold storage. That period of time was regarded as the moment when more ROS were produced in the fruit; therefore, the higher activity of these enzymes to cope with the damage caused. According to that, in this experiment the treatments using 7 and 9 mM of SA in pre and postharvest could be suggested as the ones causing a more elevated scavenger activity of CAT, SOD, APX, generating less ROS, decreasing the respiration rate, and promoting an ideal fruit physiological condition.

Regarding the internal browning, this physiological condition in fruits is associated with a high activity of phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) enzymes during postharvest, concomitant with an elevated level of ROS and low antioxidant content (Youryon *et al.*, 2018; Paull and Chen, 2019). This finding suggests that SA solutions with concentrations of 5 and 7 mM applied pre and postharvest can catalyze PAL and PPO activity, increasing the ROS content, creating higher browning symptoms in the flesh. In the case of the treatment employing 9 mM of SA in pre and postharvest, its lowest internal browning severity and incidence was linked to the lowest respiration rate and the most elevated antioxidant content. This fact infers that those high concentrations of SA (9 mM), on the contrary, can inhibit the PAL and PPO activity, reduce the ROS level, generating positive effect on the fruit metabolism.

On the other hand, these results contradict the outcomes of Lu *et al.* (2011) and Mandal *et al.* (2015), where concentrations of 5 mM or higher applied pre and postharvest caused the lowest internal browning incidence and severity, as in this experiment the control treatment, produced positive results in this matter. Furthermore, despite having a high respiration rate, the control treatment delivered an ideal AsA and TA content. This situation exposes that the fruits of this treatment were harvested with optimal physiological characteristics, necessary to delay any internal browning symptoms until the last day of cold storage. Besides, some authors have suggested that MD2 pineapple, due to its higher antioxidant content, could extend its postharvest shelflife up to 45-50 days, depending on the fruit quality condition collected from the field (Paull and Chen, 2018, 2019).

Conversely, translucency is a physiological disorder of pineapple fruit characterized by water soaking symptoms, being MD2 a susceptible hybrid (Chen and Paull, 2017; Paull and Chen, 2018). Furthermore, the low calcium content in the fruit and possible low assimilation of Ca^{2+} into the cell wall matrix have been linked to this physiological disorder's exhibition (Paull and Chen, 2015, 2018). These facts suggest that SA applications employed in pre and postharvest, essentially at high concentrations like in the treatment working with 9 mM of SA in pre and postharvest, could enhance the calcium ion assimilation and concentration in flesh tissues, reducing the exhibition of translucency. These results agree with

the findings of Mandal *et al.* (2015), where implementing SA with concentrations of 5 mM after 15 days of cold storage got to reduce translucency incidence, compared to treatments without SA. On top of that, similar with the internal browning, the lowest translucency severity and incidence was associated with the most inferior respiration rate and highest AsA content. As a result, after analyzing all the variables measured in this experiment, it is possible to suggest that this treatment could be considered as the more appropriate to obtain an ideal pineapple quality after the 40 days of cold storage.

Finally, the current available information about the SA effect on pineapple exposes that low preharvest concentrations, between 2 and 5 mM, mixed with more elevated ones in postharvest, provide the best results regarding the fruit quality (Lu *et al.*, 2010, 2011; Cano-Reinoso *et al.*, 2022 b). Nevertheless, this experiment got to demonstrated that elevated concentrations of SA employed preharvest could also benefit the fruit. For example, a more superior calcium uptake, and AsA level and recycling can be encourage with those high SA concentrations (primordially between 7 and 9 mM). Because of that, future experiments in pineapple should be focus on these concentrations, prioritizing the impact on the fruit mineral status and antioxidant content. Also, deeper researches concerning the determination of the ideal pre harvest frequency of SA application should be done, as former studies still lack on this matter. These preliminary results demonstrated that four preharvest applications can be more beneficial than two, complementing former experiments.

In conclusion, SA treatments applied pre and postharvest affected the MD2 pineapple quality. The treatment employing SA solutions with a concentration of 9 mM pre and postharvest provided the best results, essentially because it had the lowest and regular postharvest trend and outcome of respiration rate, and the highest value of AsA and TA content. Also, this treatment obtained the most reduced severity and incidence of internal browning and flesh translucency after 40 days of cold storage.

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Effect of far-red light applied at the end of the day in red and green leaf lettuce cultivars grown under two types of white LED

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Key words: cultivar difference, end of day, far-red LED, stem elongation.

Abstract: The growth and yield of 13 red and green leaf lettuce (*Lactuca sativa* L.) cultivars were evaluated under two types of white LED irradiation. There was a difference in growth under the two types white LEDs, specifically in the fresh total weight, fresh leaf weight and dry weight in all cultivars. In addition, the main stem elongation was confirmed for red and green lettuce cultivars under all treatments, but some cultivars promoted the growth of the main stem and the others were inhibited by treatment with far-red light applied at the end of the day (EOD-FR). Furthermore, the EOD-FR treatment affected the characteristic reactions due to white LED light quality in each of the cultivars. These results showed that it is necessary to investigate the selection of white LEDs with and without EOD-FR treatment for each lettuce cultivar.

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1. Introduction

Lettuce (*Lactuca sativa* L.), which is often cultivated not only in greenhouses but also in indoor plant factories, has become a model plant for studying the response of plants grown under LEDs (Lin *et al*., 2013; Yan *et al*., 2019). Among them, there are many reports of growth differences by irradiating red and blue LEDs with a monochromatic color, mixing red and blue LEDs, and irradiating them in combination, such as changing the irradiation time. In particular, depending on the quality of the LED light used for irradiating the plants, differences in morphological growth such as fresh and dry weight and leaf area, and in the amount of substances such as anthocyanins and vitamins in plants have been reported (Bleiss and Smith, 1985; Jieun *et al*., 2012; Jishi *et al*., 2016; Bian *et al*., 2018; Ishii *et al*., 2018). It is also known that light quality and quantity not only promotes plant photosynthesis, but also regulates plant growth (Chen *et al*., 2016). However, it is reported that blue lights, which affect photomorphogenesis and red, which affects photosynthesis, are

highly efficient for plant growth (Goto, 2005).

Moreover, far-red (FR) light, along with red light, is known to have a significant effect on plant growth and has been shown to affect seed germination, plant growth, and flowering (Hisamatsu *et al.*, 2002). It has been reported that FR LED treatment after sunset (end of day-far red: EOD-FR) promotes shoot elongation in poinsettia (*Euphorbia pulcherrima*) and chrysanthemum (*Chrysanthemum sp.*) (Lund *et al*., 2007; Islam *et al*., 2014), and also extends the hypocotyl axis in the rootstock of pumpkin (*Cucurbita maxima*) seedlings (Yang *et al*., 2012). In komatsuna (*Brassica rapa* var. *perviridis*) and pak choi (*Brassica rapa* var. *chinensis*), EOD-FR treatment has been found to increase fresh weight, dry matter weight, and leaf area (Akutsu *et al*., 2017). It has been reported that the fresh and dry weights and leaf length of baby leaf lettuce grown under white light irradiation with FR light increased by more than 10% compared to those grown without the FR light irradiation (Li and Kubota, 2009). In Japan, most indoor plant factories growing lettuce use red or red-blue mixed LEDs. However, white LEDs are used extensively in our daily life and are easily available. If white LEDs can be used for lettuce production in an indoor plant factory, it may reduce the unit price spent on LEDs and consequently increase cost effectiveness. In addition, white LEDs have begun to be used for growing leafy vegetables such as leaf lettuce in plant factories, but it might be difficult to optimize the light wavelength and intensity for plant cultivation as they are originally used for indoor lighting in households. Compared to using white LED irradiation alone for plant production, it is possible to optimize the light intensity for each wavelength by combining monochromatic LED lighting (Watanabe *et al*., 2016), but we confirmed a difference in the growth using white LED irradiation and whether changes were made by FR LED irradiation. In this study, we investigated the effects of EOD-FR treatment on lettuce using 13 leaf lettuce cultivars under irradiation with two types of white LEDs. Plants were grown hydroponically in growth chambers to maintain environmental conditions for air temperature, humidity and concentration of CO_2 other than light conditions.

2. Materials and Methods

The 13 cultivars of leaf lettuce used in this study are listed in Table 1. The seeds of each cultivar were sown on urethane cubes (M Hydroponic Research Co. Ltd., Aichi, Japan) with distilled water and then germinated for one week in a growth chamber (TGE-5-2L; Espec Corp., Osaka, Japan) at 25°C, 70% relative humidity, and 600 ppm CO_2 for 16 h under continuous illumination at 100 µmol/m^2/s cool white fluorescent lamps (FHF32EX-D-HX-S; NEC Corp., Tokyo, Japan). Subsequently, the germinated seeds were transferred into a commercial A treatment nutrient solution suitable for lettuce cultivation (OAT Agrio Co., Ltd., Tokyo, Japan) and grown for an additional week. After that, eight seedlings were transferred to containers (293 mm × 211 mm × 106 mm) with 6 L of commercial A treatment nutrient solution (OAT Agrio Co., Ltd., Japan). The growth of the seedlings was observed for three weeks under the following four

Table 1 - Lettuce cultivars used in this experiment

Kinds of lettuce	Cultivar name	Name of seedling company
Red leaf lettuce	Leaf lettuce red	Sakata Seed Corp., Japan
	Red wave	Sakata Seed Corp., Japan
	Bancyu sun bright	Nakahara Seed Co. Ltd.,Japan
	Sun bright	Nakahara Seed Co. Ltd.,Japan
	Fancy red	Nakahara Seed Co. Ltd.,Japan
	Red fire	Takii Seed Co. Ltd., Japan
	Bancyu red fire	Takii Seed Co. Ltd., Japan
	Sun marino	Takii Seed Co. Ltd., Japan
	Calbee red	Nakahara Seed Co. Ltd.,Japan
Green leaf lettuce	Summer green	Sakata Seed Corp., Japan
	Fancy green	Nakahara Seed Co. Ltd.,Japan
	Green wave	Takii Seed Co. Ltd., Japan
	Yakiniku lettuce	Sakata Seed Corp., Japan

irradiation treatments: white LED (White A; 16 h white photoperiod, Fluorescent lamp-type LED for growing plants, Espec Corp., Osaka, Japan), FR LED irradiation for 3 h after irradiation with white A (White A + FR; 16 h white + 3 h FR photoperiod, FR LED: Valore Corp., Kyoto, Japan), another white LED (White B; 16 h white photoperiod, Fluorescent lamp-type LED for growing plants, Espec Corp., Osaka, Japan), and FR LED irradiation for 3 h after irradiation with white B (White B + FR; 16 h white + 3 h FR photoperiod). The intensity of irradiation in white LED treatments was 100 µmol/m^2/s and FR LED treatment was 13.2 µmol/m^2/s. The wavelengths for all LEDs are shown in figure 1. During cultivation, to their roots were given sufficient air using an air pump (Kotobuki Kougei Co., Ltd., Japan) to avoid root rot. Once a week, all solutions were replaced with fresh ones, and the electric conductivity (EC) value adjusted to 1.2 dS/m^1. Twenty-one days after the start of cultivation, all plants were harvested and their fresh weight, root weight, maximum leaf length, number of leaves, and SPAD value (SPAD-502; Konica Minolta Holdings Inc., Tokyo, Japan) were measured. After drying for more than three days at 70°C, the dry leaf weight and dry root weight were also measured. The plants on day 14 were alternately harvested, and all cultivation experiments were repeated twice. All data were evaluated by one-way ANOVA (analysis of variance) using the Statcel add-in (OMS Publishing Inc., Saitama, Japan) in Excel (Microsoft Corp., Redmond, WA), followed by Tukey's multiple post-hoc comparison test.

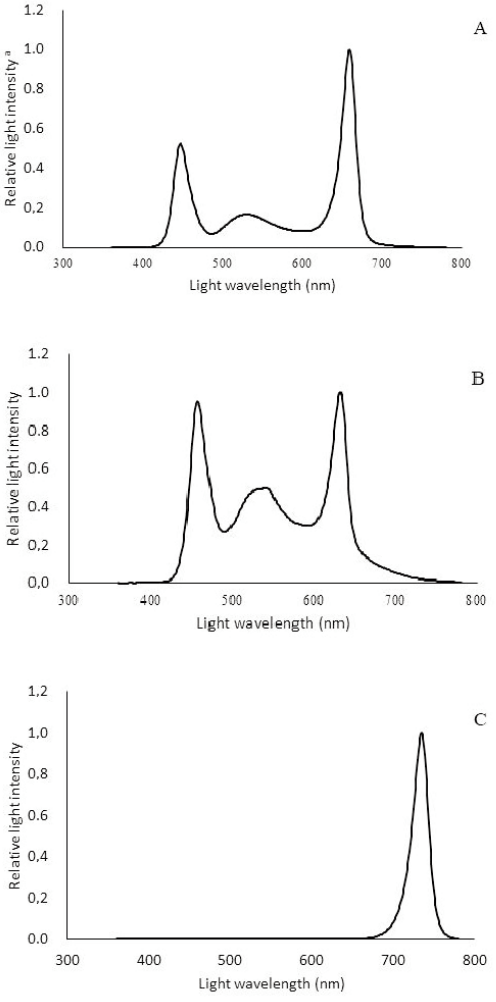

Fig. 1 - Wavelength distribution characteristics of various LED light sources used in this experiment. A= White A LED, B= White B LED, C= FR LED. [(a)] It was indicated relative value with the maximum peak taken as 1 against the measured light intensity.

3. Results

Among the red leaf lettuce cultivars, the effect of white LED irradiation was observed on the fresh total weight, fresh leaf weight, root weight, and dry weight of 'Leaf lettuce red' (Table 2). Fresh and dry weights of 'Red wave', 'Fancy red', and 'Calbee red' cultivated under White A and White A + FR irradiation were significantly greater than those cultivated under White B and White B + FR. The fresh total weight, fresh leaf weight, stem weight, and maximum leaf length in 'Bancyu sun bright' and 'Sun bright' grown under White A + FR and White B + FR tended to be more, and the fresh leaf weight and dry total weight in 'Sun bright' and the fresh total weight, fresh leaf weight, and stem weight in 'Sun bright' were significantly greater than those cultivated under White A and White B. The fresh total weight, fresh leaf weight, and dry total weight of 'Red fire' and 'Sun marino' cultivated under White A + FR tended to be more, and the fresh leaf weight in 'Red fire' and the fresh total weight in 'Sun marino' were significantly greater than those cultivated under the other LEDs. All investigated items except the number of leaves and SPAD in 'Bancyu red fire' grown under White A and White B showed a higher value, especially for the dry total weight, which was significantly greater than that grown under White A + FR and White B + FR.

Among the green leaf lettuce cultivars, in 'Summer green', the effect of white LED irradiation was observed on the fresh total weight, fresh leaf weight, and dry total weight (Table 3). In 'Fancy green', the fresh total weight, fresh leaf weight, and

Table 2 - The growth difference in red lettuce cultivars by two kinds of white LED and FR light treatment

Cultivars		Kinds of LED	Fresh total weight (g)	Fresh leaf weight (g)	Stem weight (g)	Root weight (g)	Maximum leaf length (cm)	Main stem length (cm)	Number of leaf	SPAD	Dry weight (g)
Leaf lettuce red	1	White A	5.7±1.0 ab	3.7±0.3 b	1.5±0.3 b	0.4±0.0 b	13.3±0.6	18.6±0.4b	8.1±0.5	22.6±2.6a	0.31±0.05 b
	2	White A+FR	4.3±0.6 b	3.0±0.4 b	1.3±0.2 b	0.3±0.1 b	13.0±0.4	19.4±0.3b	7.4±0.6	16.8±1.5b	0.23±0.03 b
	3	White B	8.7±0.7 a	6.3±0.5 a	2.4±0.2 a	1.0±0.2 a	13.2±0.6	21.2±0.3ab	8.8±0.4	16.9±0.8b	0.50±0.07 a
	4	White B+FR	4.3±0.2 b	2.8±0.8 b	2.0±0.5 ab	0.4±0.1 b	13.6±0.8	21.7±0.3a	7.4±0.5	18.2±1.3ab	0.38±0.07 a
Red wave	5	White A	9.1±1.5 a	8.5±1.4 a	0.6±0.2	1.7±0.3 a	16.7±0.6	3.5±0.7b	6.7±0.3	17.2±2.0	0.50±0.08 a
	6	White A+FR	9.0±1.5 a	8.1±1.3 a	0.8±0.2	1.2±0.2 a	17.3±0.7	4.7±0.9b	6.0±0.3	15.1±1.7	0.50±0.08 a
	7	White B	7.0±1.3 ab	6.1±1.1 ab	0.9±0.3	0.9±0.1 ab	17.4±0.4	6.8±0.5a	6.5±0.5	17.8±3.1	0.36±0.06 ab
	8	White B+FR	3.5±0.7 b	3.1±0.7 b	0.5±0.1	0.4±0.1 b	15.7±0.6	7.4±0.2a	6.1±0.4	12.8±1.2	0.20±0.04 b
Bancyu sun	9	White A	6.8±1.1 b	6.2±1.0 b	0.6±0.1 b	0.7±0.2 b	16.6±1.2b	8.3±0.3ab	9.1±0.4	17.5±1.7	0.35±0.06 b
	10	White A+FR	13.3±2.0 a	12.3±0.9 a	1.0±0.2 a	1.8±0.2 a	20.1±0.4a	6.4±0.3b	9.9±0.5	16.0±1.2	0.51±0.10 a
	11	White B	6.5±1.2 b	5.8±1.2 b	0.8±0.1 ab	0.9±0.2 b	19.0±0.5ab	9.5±0.6a	9.5±0.6	14.4±1.7	0.30±0.04 b
	12	White B+FR	11.3±0.9 a	10.5±0.8 a	0.9±0.1 a	1.6±0.1 a	20.8±0.7a	7.3±0.7b	10.4±0.5	16.9±1.5	0.54±0.05 a
Sun bright	13	White A	10.1±0.7 b	8.9±0.6 b	0.9±0.0 b	1.2±0.2 b	15.6±0.5c	7.4±0.4b	9.6±0.3	15.2±1.3b	0.47±0.03 b
	14	White A+FR	23.5±1.3 a	18.4±1.1 a	2.0±0.1 a	2.0±0.3 a	20.0±0.6b	9.5±0.7ab	9.6±0.3	17.0±2.4ab	0.62±0.03 a
	15	White B	11.3±0.9 b	11.6±0.3 b	1.0±0.1 b	1.1±0.1 b	17.7±0.5 bc	8.7±0.8 b	9.9±0.4	15.9±2.0 b	0.47±0.07 b
	16	White B+FR	22.7±3.7 a	15.7±0.9 a	2.6±0.2 a	1.1±0.2 b	26.1±0.9 a	11.5±0.6 a	9.1±0.4	24.0±1.4 a	0.59±0.05 a
Fancy red	17	White A	9.1±0.6 a	9.3±0.4 ac	0.6±0.2	1.7±0.3 a	16.7±0.6	3.5±0.7 b	8.2±0.3	17.2±1.0 a	0.49±0.10 ab
	18	White A+FR	8.9±0.6 a	9.5±0.3 a	0.8±0.2	1.2±0.2 a	17.3±0.7	4.7±0.9 b	8.3±0.5	15.1±0.7 ab	0.52±0.07 a
	19	White B	3.5±0.7 b	3.1±0.7 b	0.5±0.1	0.4±0.1 b	15.7±0.6	7.4±0.2 a	8.0±0.4	12.8±1.2 b	0.20±0.03 c
	20	White B+FR	6.6±1.1 c	5.3±0.8 c	0.6±0.1	0.9±0.2 a	17.4±0.4	4.9±0.6 b	8.2±0.3	17.8±1.1 a	0.36±0.06 b
Red fire	21	White A	10.9±0.9 c	9.4±1.0 b	1.5±0.1 b	1.4±0.1	19.2±0.3 b	6.9±0.6 c	6.5±0.3 b	14.4±1.7	0.71±0.08 b
	22	White A+FR	20.7±1.3 a	15.1±1.7 a	5.6±0.6 a	2.0±0.6	22.5±0.5 a	19.9±0.8 b	8.3±0.4 a	13.2±0.9	1.17±0.15 a
	23	White B	17.9±0.8 bc	13.0±1.2 ab	4.9±0.6 a	2.0±0.4	22.1±0.6 a	22.3±1.3 ab	7.4±0.3 ab	14.9±1.1	0.91±0.11 ab
	24	White B+FR	16.7±0.6 b	11.6±1.1 b	5.1±0.6 a	1.3±0.2	21.4± 0.6 a	24.7±1.6 a	8.5±0.3 a	14.3±0.7	0.93±0.10 ab
Bancyu red fire	25	White A	9.5±0.6 a	8.6±0.4 a	1.0±0.3 a	1.5±0.3	19.1±0.8 ab	7.0±0.7 a	8.4±0.5	16.5±2.3	0.48±0.08
	26	White A+FR	5.6±0.7 b	5.4±0.7 b	0.3±0.1 b	0.9±0.1	16.3±0.8 b	3.4±0.6 c	8.1±0.3	19.8±2.4	0.34±0.05
	27	White B	8.5±0.6 a	8.0±0.5 a	0.6±0.1 ab	1.2±0.2	20.7±0.8 a	5.3±0.4 bc	8.5±0.7	15.5±2.0	0.47±0.03
	28	White B+FR	6.0±0.9 ab	6.0±0.9 ab	0.2±0.0 b	1.0±0.1	15.3±0.8 b	3.1±0.4 c	8.6±0.4	18.8±2.6	0.38±0.06
Sun marino	29	White A	3.7±0.3 b	3.6±0.3 ab	0.1±0.0 b	1.1±0.2	10.0±0.3 b	2.5±0.6	8.1±0.3	14.1±1.4 b	0.20±0.02 b
	30	White A+FR	6.3±0.8 a	5.5±0.9 b	0.3±0.1 a	0.8±0.2	11.8±0.7 ab	2.8±0.5	8.3±0.5	17.0±1.0 a	0.34±0.03 a
	31	White B	4.2±0.6 b	3.6±0.7 ab	0.2±0.0 a	0.8±0.2	12.1±0.3 a	2.5±0.3	7.8±0.3	17.9±1.1 a	0.33±0.05 a
	32	White B+FR	2.7±0.2 b	2.7±0.3 a	0.4±0.1 a	0.7±0.1	10.8±0.7 ab	3.5±1.3	7.5±0.3	12.5±1.4 b	0.24±0.06 ab
Calbee red	33	White A	13.2±1.5 a	8.5±0.9 a	1.0±0.4 b	1.3±0.2 ab	16.5±0.4 b	5.9±1.2 c	7.7±0.2	19.8±0.9 a	0.73±0.10 a
	34	White A+FR	13.1±0.9 a	8.7±0.5 a	2.9±0.2 a	1.6±0.2 a	20.3±0.6 a	16.2±0.6 a	7.8±0.3	18.5±0.5 ab	0.64±0.04 a
	35	White B	4.6±0.7 b	4.0±0.6 b	1.7±0.3 b	0.9±0.1 ab	17.6±0.4 b	13.8±0.5 b	6.7±0.5	19.4±1.2 ab	0.26±0.03 b
	36	White B+FR	7.5±0.6 b	4.6±0.5 b	1.7±0.3 b	0.6±0.1 b	18.0±1.4 ab	17.7±0.7 a	6.6±0.3	16.0±1.14 b	0.38±0.06 b

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

maximum leaf length tended to be more when grown under White A + FR and under White B. The main stem was significantly greater than that grown under White A, but the SPAD was lower than that grown under the other LEDs. In 'Green wave', there was no difference in fresh total weight grown under White A, White B, and White B + FR, and the fresh leaf weight and dry total weight tended to be more when grown under White A. In 'Yakiniku lettuce', there was no difference in fresh total weight, stem weight, maximum leaf length, and dry total weight when grown under White A + FR, White B, and White B + FR. The root weight, stem weight, number of leaves, and dry total weight of plants grown under White A tended to

Table 3 - The growth difference in green lettuce cultivars by two kinds of white LED and FR light treatment

Cultivars		Kinds of LED	Fresh total weight (g)	Fresh leaf weight (g)	Stem weight (g)	Root weight g)	Maximum leaf length (cm)	Main stem length (cm)	Number of leaf	SPAD	Dry weight (g)
Summer green	37	White A	14.4±0.8 a	12.2±0.6 a	2.1±0.4 ab	1.7±0.2	18.1±0.6	9.5±0.8 b	14.8±1.3	32.2±3.2	0.42±0.09 ab
	38	White A+FR	12.7±1.1 b	8.6±1.0 b	2.3±0.3 ab	1.6±0.2	18.3±0.6	10.6±0.5 b	15.3±0.9	39.2±3.0	0.31±0.05 b
	39	White B	12.4±0.6 b	10.4±0.4 b	2.0±0.2 b	1.6±0.4	17.7±0.8	11.6±0.5 b	16.1±1.1	36.3±1.9	0.33±0.08 ab
	40	White B+FR	13.8±1.1 ab	12.1±0.5 a	3.1±0.4 a	1.6±0.5	19.3±0.3	16.3±1.1 a	14.1±1.0	28.4±4.5	0.63±0.10 a
Fancy green	41	White A	14.1±0.4 b	11.8±0.7 b	1.6±0.1 ab	1.0±0.1 b	19.3±0.5 b	10.4±0.4 a	10.0±0.4	16.4±1.5 b	0.49±0.04 b
	42	White A+FR	17.5±1.3 a	15.2±0.4 a	1.3±0.2 bc	1.5±0.3 ab	23.5±0.7 a	6.4±0.4 b	9.9±0.6	24.9±1.3 a	0.57±0.06 a
	43	White B	19.5±0.7 a	16.6±0.7 a	1.9±0.1 ab	1.8±0.2 ab	24.7±0.7 a	7.3±0.5 b	9.1±0.7	27.8±2.7 a	0.63±0.07 a
	44	White B+FR	10.4±0.7 c	9.2±0.7 b	0.7±0.1 c	2.1±0.5 a	19.7±0.6 b	5.7±0.6 b	9.8±0.3	30.3±1.4 a	0.49±0.05 b
Green wave	45	White A	6.2±0.4 a	3.9±0.9	2.3 ±0.5	0.6±0.1 a	21.7±0.8 b	13.5±0.5	7.0±0.5	24.3±2.8	0.57±0.11
	46	White A+FR	4.4±0.5 b	2.7±0.3	1.7 ±0.2	0.4±0.1 b	21.5±1.3 b	13.4±0.5	6.7±0.6	22.9±0.9	0.47±0.06
	47	White B	5.1 ±1.1 ab	2.8±0.7	2.3±0.5	0.6 ±0.1 a	25.9±1.4 a	13.8±0.6	7.0±0.3	22.3±1.1	0.50±0.09
	48	White B+FR	5.1 ±0.6 ab	3.0±0.4	2.1± 0.3	0.4±0.1 b	26.8±1.6 a	14.3±0.5	6.6±0.3	22.3±1.1	0.53±0.03
Yakiniku lettuce	49	White A	2.5±0.4 b	1.8±0.3 b	0.3±0.1 b	0.4±0.1 b	10.3±0.5 b	7.4±0.6 b	6.3±0.5	18.6±1.3 a	0.16±0.02 b
	50	White A+FR	4.1±0.3 a	3.4±0.3 a	0.7±0.1 a	0.7±0.1 a	12.0±0.4 ab	9.1±0.5 ab	6.5±0.4	20.8±1.9 a	0.39±0.04 a
	51	White B	3.4±0.6 ab	2.0±0.3 b	0.7±0.1 a	0.3±0.1 b	13.7±0.8 a	11.1±0.9 a	6.6±0.6	14.8±1.5 b	0.31±0.01 a
	52	White B+FR	4.0±0.2 a	3.3±0.2 a	0.7±0.1 a	0.5±0.1 ab	12.9±0.3 a	10.3±0.5 a	6.8±0.6	15.7±1.2 ab	0.32±0.04 a

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

decrease, and a significant difference was observed in the stem weight and dry total weight. Although the total light intensity was higher in White A + FR and White B + FR than in White A and White B, White B + FR showed no significant decrease in fresh leaf weight and dry weight only in 'Fancy green' grown under white B, suggesting that FR LED had little effect on the total light intensity in this experiment. However, it would be necessary to investigate the effects of similar total light intensity with and without FR irradiation in the future.

Principal component analysis was conducted to divide the cultivars into red lettuce cultivars and green lettuce cultivars to make it easier to understand the tendency. The average values of various traits for each LED treatments for each cultivar were used, and the parameters are shown in Tables 4 and 5. The contribution rates of the first principal components of the red lettuce cultivars and green lettuce cultivars were 47.34% and 38.23%, respectively. The first principal component in red lettuce cultivars showed a positive factor loading for all traits except the main stem length, and a negative factor loading for only the main stem length. The first principal component in green lettuce cultivars showed a positive factor loading for fresh total weight, fresh leaf weight, stem weight, maximum leaf length, and main stem length, and a negative factor loading for root weight, number of leaves, SPAD, and dry total weight. Furthermore, the second main component in red lettuce cultivars showed a positive factor loading for stem weight and main stem length, and a negative factor loading for the others. The second main component for green lettuce cultivars showed a positive factor loading for fresh total weight, and fresh

Table 4 - Eigen value, contribution and factor loading of 1st, 2nd and 3rd principal components in red leaf lettuce cultivars

Characteristics	Component No.		
	1	2	3
Fresh total weight (g)	0.460	-0.011	0.049
Fresh leaf weight (g)	0.458	-0.027	-0.028
Stem weight (g)	0.327	0..541	0.179
Root weight (g)	0.411	-0.154	-0.234
Maximum leaf length	0.333	0.281	0.425
Lengh of main stem (cm)	-0.038	0.648	-0.124
No. of leaves	0.266	-0.347	-0.332
SPAD	0.071	-0.385	0.738
Dry weight (g)	0.337	-0.051	-0.244
Eigen value	3.287	1.423	0.868
Contribution	47.34%	20.49%	12.51%
Cumulative contribution	47.34%	67.84%	80.34%

Table 5 - Eigen value, contribution and factor loading of 1st, 2nd and 3rd principal components in green leaf lettuce cultivars

Characteristics	Component No.		
	1	2	3
Fresh total weight (g)	0.354	0.418	0.059
Fresh leaf weight (g)	0.285	0.442	-0.015
Stem weight (g)	0.473	0.188	0.143
Root weight (g)	-0.101	0.462	0.330
Maximum leaf length	0.449	0.004	-0.227
Lengh of main stem (cm)	0.427	-0.256	0.196
No. of leaves	-0.030	-0.189	0.875
SPAD	-0.357	0.380	-0.041
Dry weight (g)	-0.216	0.368	0.095
Eigen value	2.752	2.297	0.890
Contribution	38.23%	31.93%	12.37%
Cumulative contribution	38.23%	70.13%	82.5%

leaf weight, stem weight, root weight, maximum leaf length, SPAD, and dry total weight, and a negative factor loading for the others. The cumulative contribution rates of the first and second principal components were 67.84% and 70.13% in the red and green lettuce cultivars, respectively. Principal component analysis was performed because the values were considered to be effective.

For red lettuce cultivars, the scatter diagram of the types of LEDs and each cultivar for the first (Z1) and second main components (Z2) showed that the fresh total weight, fresh leaf weight, stem weight, root weight, and dry total weight in 'Red wave', 'Bancyu sun bright', 'Sun bright', 'Fancy red', 'Red fire', 'Sun Marino' and 'Calbee red' grown under White A + FR was higher (Fig. 2). In addition, it showed an opposite tend for the fresh total weight, fresh leaf weight, and dry weight in 'Leaf lettuce red' and 'Bancyu red fire' grown under white B. Also, for 'Bancyu sun bright', 'Sun bright', 'Fancy red', and 'Calbee red' grown under White B, the fresh total weight, fresh leaf weight, and dry weight tended to be less. While the maximum leaf length for these cultivars grown under White B tended to be less, the main stem length tended to be more. On the other hand, for green lettuce cultivars, 'Summer green' grown under White A + FR and White B, 'Yakiniku lettuce' grown under White A, and 'Green wave' grown under White A + FR showed a tendency for the fresh weight, fresh leaf weight, stem weight, and dry weight and the maximum leaf length to be lower as shown in the scatter diagram for the first and second main components (Fig. 3).

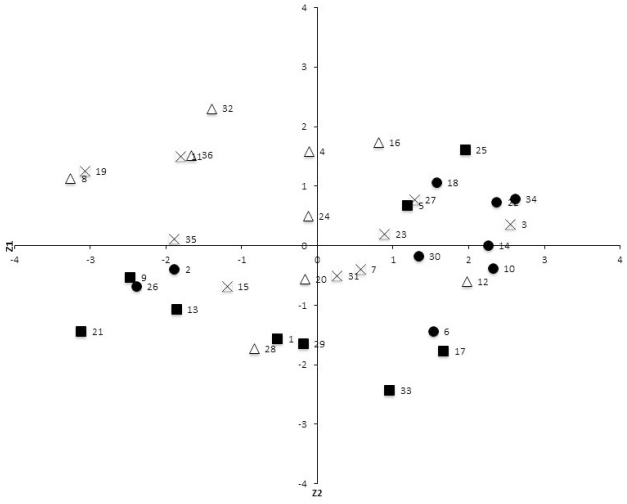

Fig. 2 - The scatter diagram in Z1-Z2 plane nine characteristics in red lettuce cultivars arranged by the principal component analysis. ■: White A irradiation, ●: White A + FR irradiation, ×: White B irradiation, △: White B + FR irradiation *Numbers were shown in Table 2.

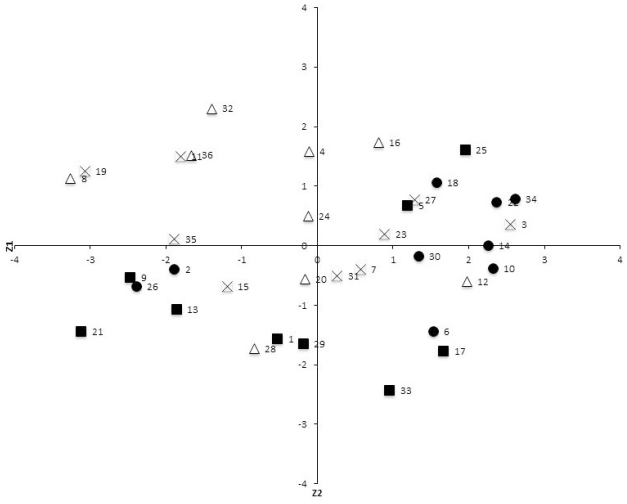

Fig. 3 - The scatter diagram in Z1-Z2 plane nine characteristics in red lettuce cultivars arranged by the principal component analysis. ■: White A irradiation, ●: White A + FR irradiation, ×: White B irradiation, △: White B + FR irradiation. * Numbers were shown in Table 3.

4. Discussion and Conclusions

The fresh total weight, fresh leaf weights, and dry total weight cultivated under White A and B with or without FR LED in lettuce cultivars tested in this study increased or decreased. It was considered that the

type of LED irradiated in the photoperiod was deeply related to the effect of the FR irradiated in the dark period, as this phenomenon was similar to the elongation of the main stem (Tables 2 and 3). For the cultivars 'Leaf lettuce red', 'Bancyu red fire', 'Calbee red', and 'Green wave', it was considered better to use only white LEDs without FR treatment because white LED irradiation with FR LED did not increase leaf weight and the running cost was more. However, white LEDs that are generally sold have different wavelengths depending on the manufacturer, and hence this inference may not apply to all white LEDs.

In general, it has been reported that FR light treatment promotes the elongation of the main stem or hypocotyl in cucumber (*Cucumis sativus* L.), lily *(Lilium longiforum* Thumb.), and chrysanthemum (*Chrysanthemum moriforlium* Ramat.) (Blom *et al*., 1995; Xiong *et al*., 2002; Hisamatsu *et al*., 2008). However, in Brassicaceae, Akutsu *et al*. (2016) reported that komatsuna and red mustard (*B. juncea*) were unable to extend the main stem, while pak choi and 'shimana' (a cultivation of *B. juncea*) easily extended the main stem regardless of the cultivation period and the light intensity of FR LEDs; thus, the effect differed depending on the plant species and cultivars being treated with the FR LED. Furthermore, the effect of FR treatment on leaf area and main stem in komatsuna and pak choi differed depending on the variety (Akutsu *et al*., 2017). The elongation of the main stem causes quality deterioration in terms of appearance and taste in leafy vegetables such as lettuce. The main stem was elongated regardless of the FR treatment for all lettuce cultivars tested in this experiment. However, it was found that the relationship between the main stem elongation and FR treatment can be divided into several patterns regardless of whether they were green or red lettuce cultivars. Under White A irradiation, FR treatment did not promote the main stem elongation in 'Leaf lettuce red', 'Red wave', 'Fancy red', 'Sun marino', 'Summer green', 'Green wave', and 'Yakiniku lettuce', while it promoted the main stem elongation in 'Sun bright', 'Red fire', and 'Calbee red'. In addition, FR treatment suppressed the main stem elongation in 'Bancyu sun bright', 'Bancyu red fire', and 'Fancy green'. On the other hand, under White B irradiation, FR treatment did not have an effect on the main stem elongation in 'Leaf lettuce red', 'Red wave', 'Bancyu sun bright', 'Red fire', 'Sun marino', 'Fancy green', 'Green wave', and 'Yakiniku lettuce', while it promoted the main stem elongation in 'Sun bright', 'Calbee red' and 'Summer green'. In addition, FR treatment suppressed the main stem elongation in 'Fancy Green' and 'Bancyu red fire'. Thus, it was considered that the relationship between FR irradiation and main stem elongation depends on the variety and cultivar. In addition, some cultivars such as 'Fancy red', 'Red fire', 'Summer green' and 'Fancy green', which showed an increase or a decrease in the main stem elongation after FR treatment, depending on if they were cultivated under White A or White B irradiation. There are also reports that FR treatment did not have any effect on main stem elongation in lettuce (Mickens *et al*., 2018; Lin *et al.*, 2020). Therefore, it was found that the effect of FR treatment on the main stem elongation differs depending on the wavelength of the LED irradiated in the photoperiod and on the cultivars. On the other hand, some cultivars such as 'Sun bright', 'Sun marino', and 'Fancy green', which an increase in SPAD after FR treatment depending on if they were cultivated under White A or White B irradiation, there was no effect of FR irradiation on SPAD in the other cultivars investigated in this experiment (Table 2 and 3). Thus, it was considered that no relationship between FR irradiation and SPAD depends on the variety and cultivar.

Furthermore, based on the results of the main component analysis for fresh and dry weights, the fresh total weight and the fresh leaf weight increased as the Z1 axis (the first principal component) became positive in all lettuce cultivars tested in this experiment, and the dry weight increased as the Z2 axis (the second principal component) became positive in red lettuce cultivars and as the Z2 axis became negative in green lettuce cultivars. Under White A LED irradiation, fresh total weight and fresh leaf weight were increased in all red lettuce cultivars except 'Leaf lettuce red' and 'Bancyu red fire' on treatment with FR LED, while the fresh total weight and fresh leaf weight were increased in only 'Fancy green' in green lettuce cultivars. This suggests that the effect of FR treatment cultivated under White A irradiation may be higher in red lettuce cultivars than in green lettuce cultivars. On the other hand, under White B irradiation, fresh total weight and fresh leaf weight were increased in only 'Bancyu sun bright' and 'Sun bright' of red lettuce cultivars and in 'Summer green' and 'Yakiniku lettuce' of green lettuce cultivars on the treatment FR LED. This suggests that the effect of FR treatment when cultivated under White A may be higher than when cultivated under White B in red let-

tuce cultivars. Therefore, it is necessary to select lettuce cultivars that match the type of white LED used as a source of light in the photoperiod, and depending on the lettuce cultivar used, select whether to treat with FR LED.

The main stem, which is unsuitable for sale was remarkably elongated under the two kinds of white LEDs either with or without FR treatment for the 13 leaf lettuce cultivars tested in this experiment. In general, white LEDs have different intensity, spectrum, and shade by adjusting the monochromatic emission of red, yellow, green, and blue (Chang *et al*., 2012), as shown in white A and B, respectively, used in this experiment. Also, In lettuce, the effect of FR light is clearly intensity-dependent, and the intensity required for maximum response depends on the trait (Zou *et al*., 2021). Furthermore, the effect of FR light has been found to depend on the type of photosynthetic photon flux density (PPFD, 400-700 nm) radiation and light intensity (Meng and Runkle, 2019). This suggests that it might be possible to obtain the effect of FR light by changing the type and intensity of the white LEDs used in this experiment, or by changing the intensity of FR light, even in lettuce cultivars that were investigated in this study and did not show the FR light effect. Therefore, it would be necessary to investigate the characteristics of the White LEDs used and the plants before growing plants. Furthermore, as used in a previous report (Ishii *et al*., 2018), it would be necessary to investigate the effect of FR light on lettuce cultivars that did not elongate the main stem when they were cultivated under monoclonal red or blue, or mixed red-blue LEDs.

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