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'*Momordica charantia*' introducing a new rootstock for grafted cucumber under low-temperature stress

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Key words: *Cucurbita maxima,* female flower, Karela, photosynthesis, rootstock, yield.

Abbreviation: Rma= grafted onto *Cucurbita maxima;* Rmo= grafted onto *Momordica charantia*; Rn= non-grafted; Rs= self-grafted; Tc= control temperature; Ts= stress temperature.

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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: Cucumber is a sensitive vegetable to low temperatures. Grafting vegetables on different rootstocks can decrease the harmful effects of environmental stresses, including low-temperature stress. An experiment was performed to evaluate grafting cucumbers on different rootstocks at low temperatures. Cucumber growth and yield and photosynthesis traits were examined. Treatments were the optimum temperature (25±2°C), and cold temperature (15±3°C, Ts), and rootstocks, were *Momordica charantia* (Rmo), *Cucurbita maxima* (Rma), non-grafted (Rn) and self- grafted (Rs) with 4 replications. Shoot fresh and dry weight, chlorophyll, RWC, transpiration, decreased with temperature stress. The number of female flowers, electrolyte leakage, photosynthesis, stomatal conductance increased with Ts. First fruit emergence per plant, N, P, K, Mg concentration decreased with Ts stress. Transpiration, female flower, RWC, and stomata conductance, N, P, K, Ca, and phenol increased in Rma and Rmo. Mg was at the highest concentration in Rma and Na in Rn. All in all, using Rmo as well as Rma is recommended for rootstock as it causes more reproductive growth.

1. Introduction

Recently, the greenhouse culture of plants has widely expanded (Yan *et al.,* 2013). Cucumbers (*Cucumis sativus*) are commonly grown in the greenhouse. The optimal temperature for aerial growth and roots are about 28-23/18-15°C (day/night) and 20°C, respectively (Ikeda and Kawashiro, 2005). Thus, cucumbers are cold-sensitive vegetables. Low-temperature stress can occur in plastic culture or non-equipped greenhouses, which are very common in Iran. It may also affect field culture cucumbers and spring culture cucumbers. Low temperatures have differ-

ent deleterious effects on cucumbers, including affecting the nutrient uptake of plants (Pregitzer and King, 2005), and inducing greater H_2O_2, MDA, and soluble sugar content in cucumbers, causing damage to plants and inhibiting plant growth (Qiu-yan *et al.,* 2013).

There are several ways to control low-temperature damage to plants, including controlling root temperatures by warming nutrient solution or soil. Still, these ways require excess energy (Willits and Peet, 1998). Finally, excess chemical nutrient applications for promoting growth can prevent damage to plants, which unfortunately involves environmental risks. Therefore, vegetable grafting can be a proper way to increase plant resistance to environmental stresses in addition to expanding its yield, quality, and growth. Furthermore, if any energy-efficient way is introduced, which can raise the growth rate of plants in greenhouse conditions at lower temperatures, vast amounts of energy and money will be saved. Vegetable grafting has been used to increase plant tolerance under environmental stresses, including high temperatures (Rivero *et al.,* 2003), low temperatures (Rivero *et al.,* 2003), salinity stresses (Estan *et al.,* 2005), drought stresses (Bhatt *et al.,* 2002) and heavy metal stresses (Edelstein *et al.,* 2005).

Fig leaf gourd and bur cucumbers (*Sicyos angulatus*) are used for increasing resistance against low-temperature stresses for cucumber rootstocks (Venema *et al.,* 2008). These rootstocks increase plant resistance using different ways. First, low-temperature stresses decrease Co_2 assimilation in cucumbers and this reduction is improved by grafting (Zhou *et al.,* 2009). Secondly, a previous study done by Zhou e*t al.* (2007) showed that fig leaf gourd rootstocks enhanced vegetative growth and yield of cucumbers under low-temperature stresses.

There is a hypothesis stating that grafted plant responses to temperature stresses are related to scion species (Venema *et al*., 2005). Many researchers have shown beneficial effects of vegetable grafting on the growth, fruit yield, and quality of plants. Some of the studies have used different rootstocks for cucumbers under different stresses showing better growth rate for grafted plants under stress conditions compared to non-grafted ones like: heavy metal absorption (Rouphael *et al*., 2008; Kumar *et al*., 2015), low soil temperatures (Tachibana, 1982), salinity (Huang *et al*., 2010), improve NaCl and $CaCl_2$ tolerance in Cucumber (Colla *et al.,* 2013), Al toxicity in cucumber (Rouphael *et al.,* 2016).

Different rootstocks were used in normal and stress conditions to improve yield and growth traits like different kinds of squash (Yang *et al*., 2006), (Massai *et al*., 2004), (Rouphael *et al*., 2008), Fig leaf guard (Tachibana, 1982), pumpkins and bottle gourd and P360 (*Cucurbita maxima* Duch.×*Cucurbita moschata* Duch.) (Colla *et al*., 2010 and 2013). To the best of our knowledge, few studies have investigated the use of *Momordica charantia* as a rootstock for cucurbits, or more explicitly grafting, to examine the possibility of cucumber cultivation at low temperatures in the whole growth period. Hence, the goal of the present study was the use of *Momordica charantia* and *Cucurbita maxima* as rootstocks and *Cucumis sativus* var. DavosII*,* which is a common variety of cucumbers in Iran as a scion under low-temperature stress.

2. Materials and Methods

Experimental design and plant preparation

This experiment was conducted in a plastic greenhouse in the Department of Horticulture Science at the Isfahan University of Technology, Isfahan, Iran. An investigation was arranged as a combined analysis involving data from two locations, simultaneously collected, based on CRD with 4 replications. Treatments were optimum (25±2°C, Tc) and low temperatures (15±3°C, Ts). Cucumbers *(Cucumis sativus* var. DavosII*)* were grafted to *Momordica charantia* (Rmo), *Cucurbita maxima* (Rma). Non-grafted (Rn) and self-grafted (Rs) consider as a control. Scion seeds had been cultivated 10 days before rootstock seeds in cocopeat: perlite 1:1. Scion plants and rootstocks were cut beneath and above the first true leaves, respectively. Hole-insertion grafting was used and grafted plants were transferred to a recovery greenhouse with high relative humidity. Plants were kept for 2 weeks in recovery conditions and gradually adapted to normal greenhouse conditions. Grafted plants were transferred to 5kg pots, including soil. Irrigation was used when the plant needed it. Chemical fertilizer NPK (20, 20, 20) 2 mg/pot was applied every 10 days. Plants were conducted to wire above the greenhouse, and there was no use of any pesticide.

Plant growth and fruit properties

The male and female flower, node numbers, and shoot numbers were counted during the experiment;

root and internode length was determined with a ruler. The time and node of male and female flower emergence were recorded. Fruit diameter, fruit firmness, and TSS were measured with a caliper (Mitutoyo Corp, Japan), Pentameter (model OSK-I-10576), and portable Refractometer (PAL^{-1} Brix, Japan), respectively (Raeisi *et al.,* 2014).

Shoots were excised from the roots using a steel blade and then fresh weights of roots and shoots were measured. All the samples were oven-dried at 70˚C for 48 hours and the dry weights were estimated. During the experiment and finally 124 days after transplanting, fruits were harvested and washed using tap water and were weighed by an analytical balance.

SPAD value and photosynthesis trait assay

Chlorophyll content was measured using a chlorophyll meter (SPAD-502 plus, Minolta, Japan). Fv/Fm was measured by chlorophyll fluorescence (OS- 30, USA) after 3 weeks. Photosynthetic properties were determined from the youngest fully-expanded leaf for 3 replications per treatment by Portable photosynthesis systems for gas exchange and chlorophyll fluorescence measurements (LI-COR-6400, USA) from 10:00 to 11:00 AM on a clear day (without clouds). The measurements were conducted with photosynthetically active radiation (PAR) intensity of 1000 µmol m^{-2} s^{-1} and CO_2 concentration of 350 µmol·mol. Mesophyll conductance (µmol $m^{-2}s^{-1}$) was calculated by dividing the photosynthetic rate by the sub-stomatal CO_2 level (Ahmadi and Siosemardeh, 2005).

Antioxidant activity

Antioxidant activity was measured and expressed as gallic acid (equivalents l of gallic acid/g) with UV-VIS spectrophotometer (Shimadzu UV160A-Japan). Three mg of sample were dissolved in 5 mL methanol stock, and 1.4 ml of this solution was blended with 0.6 mL of DPPH solution. After 30 min, the absorbance of the solution was recorded at 515 nm by the spectrophotometer (UV 160A- Shimadzu Corp., Kyoto, Japan) against methanol as a blank. The 0.2 mM of DPPH solution in methanol was used as a stock of DPPH for the determination of the free radical scavenging activity of the samples (Koleva *et al.*, 2002).

Phenolic content

Total phenolic content was determined using the Folin-Ciocalteu method. The absorbance was measured at 725 nm with a spectrophotometer (UV 160A- Shimadzu Corp., Kyoto, Japan). The results were expressed in gallic acid equivalents (mg/100 g fresh weight) using gallic acid (0-0.1 mg/mL) standard curve (Singleton *et al*., 1965).

Proline

Proline accumulation was determined using the method proposed by Bates *et al*. (1973). After the extraction of toluene, the clear phase was recovered and spectrophotometrically estimated at 520 nm using toluene as a blank. Purified proline was used for standardization (0-50 mg/mL) and was expressed as mol proline g^{-1} fresh weight.

Electrolyte leakage

Electrolyte leakage (EL) was measured using an electrical conductivity meter employing the method described by Lutts *et al.* (1995)

Relative water content

Relative water content (RWC%) was determined using ten 7 mm-diameter leaf discs. The leaf discs of each treatment were weighed (FW). They were then hydrated until saturation was reached (constant weight) for 48 h at 5°C in darkness (TW). The leaf discs were dried in an oven at 105°C for 24 h (DW). Relative water content was calculated according to the following equation (Filella *et al*., 1998):

$$RWC\% = (FW - DW)/(TW - DW) \times 100$$

The determination of total nitrogen in the leaf samples was based on the Kjeldahl method (Estan *et al.,* 2005). The concentrations of K, Ca, Mg, and P were measured (Shield Torch System, Agilent 7500a). Meanwhile, phosphorus was estimated by the vanadomolybdo phosphoric acid colorimetric method at 460 nm (Estan *et al.,* 2005). P was colorimetrically determined using a spectrophotometer (UV 160A- Shimadzu Corp., Kyoto, Japan).

Statistical analysis

All data were analyzed using two-way ANOVA (Statistix 8 software) (Tallahassee FL, USA) and the means were compared for the significance by the least significant difference (LSD) test at $P<0.05$.

3. Results and Discussion

Analysis of variance of temperature and rootstock on some characteristics of cucumber

Temperature effect shoot fresh and dry weight, chlorophyll index, and the number of the female flower, photosynthetic rate, transpiration, stomatal

conductance, RWC, EL, phenol content, and all nutrient concentration (Table 1 a, b). Rootstock changes all cucumber characteristics except for average fruit weight, fruit diameter, TSS, firmness, mesophyll conductance, proline, and antioxidant (Table 1 a, b). The interactive effect of temperature and rootstock showed that all measured parameters change significantly (Table 1 a, b).

The main effect of temperature on cucumber

Shoot fresh and dry weight, chlorophyll, RWC, transpiration, decreased with temperature stress. The number of the female flower, EL, photosynthesis, and stomatal conductance increased with Ts (Table 2 a). First fruit emergence per plant, N, P, K, Mg concentration decreased with Ts stress (Table 2 b). Shoot fresh and dry weight and fresh root weight was high-

Table 1 a - Analysis of variance of the effect of temperature and rootstock on some characteristics of cucumber

Source	df	Shoot fresh weight	Shoot dry weight	Root fresh weight	ChI	Number of female flowers	Number of male flowers	Fruit number	Fruit yield	First fruit per plant	Average fruit weight	Fruit diameter	TSS	Firmness
Temperature	1	3203.42 *	230. 9 **	244.56 NS	43.12 **	78.12 *	6.12 ns	230. 9 NS	1441.5 NS	152.91 *	1971.09 NS	0.002 NS	0.30 NS	7.65 NS
Rootstock	3	2467.99 *	23.45 *	150.01 *	35.93 **	602.45 **	13.08 **	23.45 *	19335 *	92.57 *	2094.59 NS	0.10 NS	0.18 NS	2.07 NS
T×R	3	1327.25 *	26.54 **	111.77 **	4.65 **	81.12 **	4.20 *	26.54 **	11769.1 **	4.81 **	2133.88 *	0.04 **	0.01 **	1.23 **
Error	18	708.05	5.27	67.38	3.23	76.32	1.35	5.27	5110.2	17.23	1292.36	0.04	0.12	4.26
CV		10.30	25.62	13.04	15.50	13.97	14.38	25.62	18.03	26.05	18.65	6.77	13.40	25.25

NS= no significant, * significant at 5% and ** significant at 1%.

Table 1 b - Analysis of variance of the effect of temperature and rootstock on some characteristics of cucumber

Source	df	Photo-synthetic rate	Tran-spiration	Stomata conduc-tance	Mesophyll conduc-tance	RWC	EL	Proline	Anti-oxidant activity	Phenol content	N Conc.	P Conc.	K Conc.	Ca Conc.	Mg Conc.	Na Conc.
Temperature	1	47.52 *	10.52 **	0.002 **	240063 NS	0.006 **	257.36 **	3.27 NS	0.06 NS	55824.1 **	0.72 **	0.036 **	0.06 **	0.15 **	0.16 **	0.039 **
Rootstock	3	25.08 *	2 **	0.001 *	26980 NS	0.001 **	60.38 **	1.31 NS	0.006 NS	5561.8 **	1.39 **	0.03 **	2.06 **	0.65 **	0.07 **	0.01 **
T×R	3	14.25 **	1.12 **	0.002 **	4898 **	0.0008 **	72.71 **	2.82 **	0.02 **	4553.6 **	0.54 **	0.01 **	0.62 **	0.54 **	0.06 **	0.008 **
Error	18	6.17	0.97	0.001	14354	0.00003	9.38	1.05	0.03	52.2	6.53	4.71	5.08	3.85	1.05	2.78
CV		14.41	14.82	17.80	38.05	0.74	7.56	11.03	5.81	5.01	12.1	26.7	21.5	7.87	6.23	16.9

NS= no significant, * significant at 5% and ** significant at 1%.

Table 2 a -The effect of temperature on some characteristics of cucumber

Temperature (° C)	Shoot fresh weight (g)	Shoot dry weight (g)	ChI (SPAD value)	Number of the female flower	El (%)	RWC (%)	Transpiration (mmol H_2O m^{-2} s^{-1})	Photosynthetic rate (mmol H_2Om^{-2} s^{-1})	Stomata conductance (mmol H_2Om^{-2} s^{-1})
Tc	76.52 a	11.71 a	12.7 a	2.18 b	37.1 b	0.79 a	2.83 a	4.16 b	61.31 b
Ts	55.53 b	6.21 b	10.4 b	3.06 a	43.91 a	0.75 b	1.57 b	7.02 a	177.34 a

Tc= optimum temperature, Ts= low temperature.
Within a column means followed by the same letter are not significantly different at P<5% according to LSD test.

Table 2 b - The effect of temperature on some characteristics of cucumber

Temperature (° C)	First fruit emergence per plant (day)	Phenol (mgg^{-1} FW)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Na (%)
Tc	19.57 a	17.69 b	3.51 a	0.43 a	3.11 a	1.54 b	0.74 a	0.14 b
Ts	12.30 b	271.06 a	3.37 b	0.39 b	2.54 b	1.66 a	0.68 b	0.21 a

Tc= optimum temperature; Ts= low temperature.
Within a column means followed by the same letter are not significantly different at P<5% according to LSD test.

est in Rma, the number of male flower increased in Rn and Rs and female flower increased in Rmo and Rma.

The main effect of rootstock on cucumber

The highest El and photosynthesis were observed in Rmo. The RWC and stomata conductance increased in Rma and Rmo, however, transpiration increased in Rs, Rmo, and Rma (Table 3 a). N, P, K, Ca, and phenol increased in Rma and Rmo. Mg was at the highest concentration in Rma and Na in Rn (Table 3 b). Rma has the highest fruit number, fruit yield, although, first fruit emerges in Rmo (Table 4).

The interaction effect of temperature and cucumber grafting on some growth characteristics of cucumber

K concentration was the highest in Rmo in both temperatures and P concentration increased in Rma×Ts. The highest N, K and Ca, concentrations were seen in Rma×Ts. P concentration increased in Rma and Rmo at Ts. Mg increased in Rs×Tc and Na in Rs×Ts (Table 5).

Table 3 a -The effect of rootstock on some characteristics of cucumber

Rootstock	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Number of male flowers	Number of female flower	El (%)	RWC (%)	Photosynthetic rate (µmol CO_2 m^{-2} s^{-1})	Transpiration (mmol H_2O m^{-2} s^{-1})	Stomata conductance (mmol H_2O m^{-2} s^{-1})
Rn	61.28 b	8.23 b	16.78 b	23.50 a	2.37 b	38.98 bc	0.76 b	4.94 b	1.65 b	78.79 b
Rs	55.48 b	9.29 ab	17.96 ab	23 a	1.75 b	41.67 ab	0.76 b	3.83 b	2.69 a	71.94 b
Rma	92.34 a	11.17 a	18.21 a	5.5 b	4.5 a	37.10 c	0.78 a	4.84 b	1.86 ab	89.96 ab
Rmo	55b	7.14 b	17.95 b	12.75 b	3.87 ab	44.38 a	0.78 a	8.75 a	2.61 ab	126.62 a

Rn= nongrafted (*Cucumis sativus* var. DavosII);
Rs= self grafted;
Rma= *Cucurbita maxima*;
Rmo= *Momordica charantia*.
Within a column means followed by the same letter are not significantly different at P<5% according to LSD test.

Table 3 b - The effect of rootstock on some characteristics of cucumber

Rootstock	Phenol (mgg^{-1} FW)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Na (%)
Rn	124.54 bc	3.19 c	0.45 b	2.52 d	1.05 d	0.70 b	0.19 a
Rs	112.19 c	3.08 d	0.38 c	2.55 c	1.32 c	0.69 c	0.20 a
Rma	133.90 b	3.85 a	0.47 a	3.65 a	2.11 a	0.51 d	0.14 c
Rmo	206.87 a	3.64 b	0.33 d	2.57 b	1.89 b	0.93 a	0.18 b

Rn= nongrafted (*Cucumis sativus* var. DavosII);
Rs= self grafted;
Rma= *Cucurbita maxima*;
Rmo= *Momordica charantia*.
Within a column means followed by the same letter are not significantly different at P<5% according to LSD test.

Table 4 - The effect of rootstock on some characteristics of cucumber

Rootstock	Fruit number	Fruit yield (g)	First fruit emergence per plant (day)
Rn	0.8 b	63.88 b	21.51 a
Rma	2.00 a	159.50 a	15.29 ab
Rmo	1.37 ab	91.88 ab	11.00 b

Rn= nongrafted (*Cucumis sativus* var. DavosII);
Rs= self grafted did not have any fruit;
Rma= *Cucurbita maxima*;
Rmo= *Momordica charantia*.
Within a column means followed by the same letter are not significantly different at P<5% according to LSD test.

Shoot fresh weights decreased in Rn and Rs at low temperatures (Ts), but shoot dry weights did not significantly change in Rma and Rmo at both temperatures (Fig. 1). Shoot dry weights followed the same trend as the fresh shoot weight was the highest in Rn, Rs, Rma at Tc (Fig. 1). Root fresh weights decreased in Rn, Rs, and Rma at Ts compared with Tc, but did not significantly change in Rmo at both temperatures, although the fresh root weight was the lowest in Rmo at Tc. The highest root fresh weight was seen in Rma×Tc (Fig. 2 a).

SPAD value was the highest in Rs, Rma, and Rmo at Tc (Fig. 2 b). The interaction effect of the tempera-

Table 5 - The interaction effect of temperature and cucumber grafting on some elements concentration (%) of leaves

Rootstock	N		P		K		Ca		Mg		Na	
	Tc	Ts	Tc	Ts	Tc	Ts	Tc	Ts	Tc	Ts	Tc	Ts
Rn	3.36 d	3.03 e	0.37 c	0.35 c	2.50 f	2.75 e	1.28 c	0.83 g	0.81 b	0.58 c	0.18 c	0.21 b
Rs	3.08 e	3.08 e	0.33 c	0.34 c	3.00 d	2.15 g	1.65 b	1.00 e	0.88 a	0.51 e	0.12 f	0.28 a
Rma	3.78 c	4.2 a	0.34 c	0.52 a	3.45 b	3.69 a	1.24 d	1.75 a	0.51 e	0.57 d	0.12 f	0.14 e
Rmo	3.98 b	3.78 c	0.42 b	0.48 a	3.25 c	3.10 cd	0.83 g	0.87 f	0.56 d	0.50 e	0.09 g	0.16 d

Rn= nongrafted (*Cucumis sativus* var. DavosII);
Rs= self grafted;
Rma= *Cucurbita maxima*;
Rmo= *Momordica charantia*.
Within a column means followed by the same letter are not significantly different at P<5% according to LSD test.

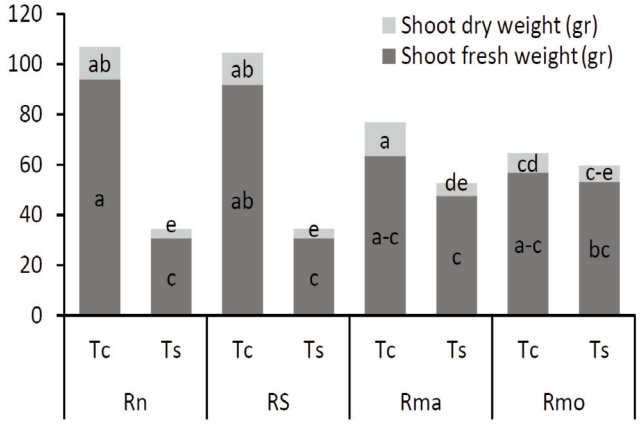

Fig. 1 - The interaction effect of temperature and cucumber grafting on shoot fresh and dry weight. Tc= optimum temperature; Ts= low temperature; Rn = nongrafted (*Cucumis sativus* var. DavosII); Rs= self grafted; Rma= *Cucurbita maxima*; Rmo= *Momordica charantia*.

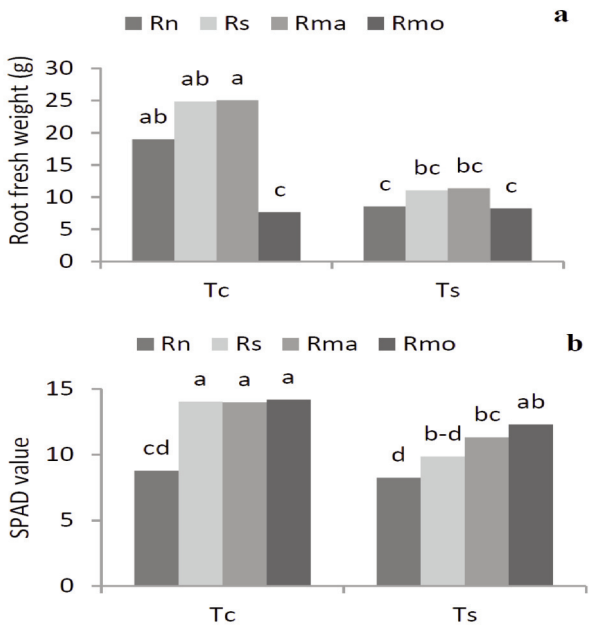

Fig. 2 - The interaction effect of temperature and cucumber grafting on root fresh weight (a) and SPAD value (b) Tc= optimum temperature; Ts= low temperature; Rn = nongrafted (*Cucumis sativus* var. Davos II); Rs= self grafted; Rma= *Cucurbita maxima*; Rmo= *Momordica charantia*.

ture on grafted cucumber did not significantly affect the time of the emergence of the first female flowers (data did not show). The highest and the lowest number of the male flower were seen in Rs×Tc and Rma, respectively. The female flower was affected by temperature variations in different rootstocks. The female flower increased in the Rn and Rma at Ts and did not change in Rs and Rmo at Tc and Ts (Fig. 3).

Fruit number was the highest in Rma×Tc, although the same results were seen in Rmo×Tc and Rmo and Rma at Ts (Fig. 4 a). Fruit yield was the highest in Rma×Tc; the same result was statically seen in Rma ×Ts (Fig. 4 b).

First, fruit emergence time significantly decreased in grafted plants at this temperature (Fig. 5). The highest fruit weight was in Rma×Tc; fruit diameter did not significantly change between treatments (Fig. 6). TSS increased in Rn and Rmo at Ts (Fig. 7 a). Firmness decreased in Rmo×Ts (Fig. 7 b) results, Goreta *et al.* (2008) found that shoot weight reduction in watermelon grafted onto *Cucurbita maxima* Duch.× *Cucurbita moschata* Duch was less) than nongrafted plants under salt stresses. The grafted cucumber plants showed less change in shoot and root weights under Cu stress conditions and this might be due to the lower accumulation of Cu in leaves through their squash rootstocks (Rouphael *et al.,* 2008).

The lowest male flower was seen in Rma at both temperatures (Fig. 3). Conversely, the highest number of female flowers was observed in Rma at Ts and Tc compared with other rootstocks at each temperature. On the other hand, at the optimum temperature (Tc), each rootstock had a lower female flower

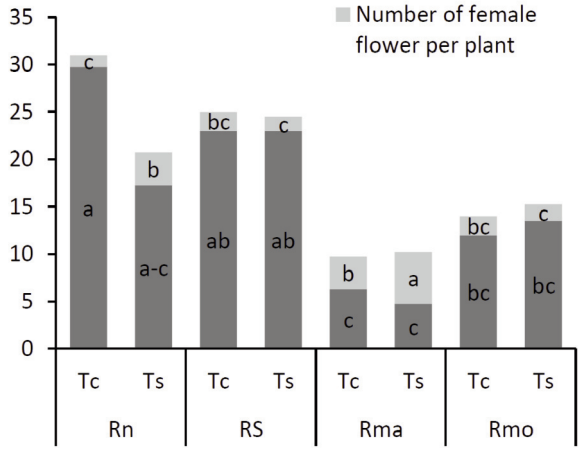

Fig. 3 - The interaction effect of temperature and cucumber grafting male and female flower number. Tc= optimum temperature; Ts= low temperature; Rn = nongrafted (*Cucumis sativus* var. DavosII); Rs= self grafted; Rma= *Cucurbita maxima*; Rmo= *Momordica charantia*.

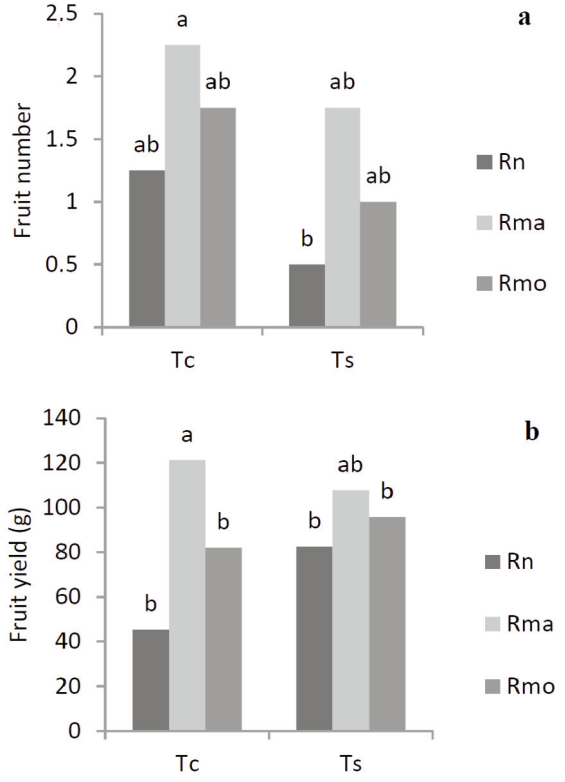

Fig. 4 - The interaction effect of temperature and cucumber grafting on fruit number (a) and fruit yield (b) *Momordica charantia* Rs did not have fruit. Tc= optimum temperature; Ts= low temperature; Rn = nongrafted (*Cucumis sativus* var. DavosII); Rs= self grafted; Rma= *Cucurbita maxima*; Rmo= *Momordica charantia.*

showed the highest male flower. Van der Ploeg and Heuvelink (2005) reported that low temperatures reduced the tomato fruit set through poor pollen quality and increased the period between anthesis and fruit maturity resulting in lower fruit yields. The result of Khah *et al.* (2006) showed that tomatoes cv. Big Red grafted onto cv. Heman and Primavera produced more fruit at low temperatures compared to non-grafted plants in greenhouse conditions. In our study, fruit number decreased at Ts and grafting had a beneficial effect on fruit number, but did not influence fruit yields. In agreement with our study, commercial tomato grafting was not able to improve the reduction of tomato yields under low light stresses by shading (Krumbein and Schwarz, 2013).

The economic increase of yield imparted by select vigorous commercial rootstocks (Kyriacou *et al*., 2017) like increasing tomato yields was observed by

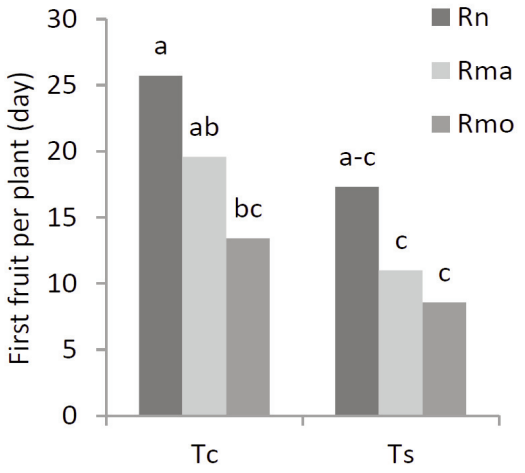

Fig. 5 - The interaction effect of temperature and cucumber grafting on the first fruit emergence of a plant. Tc= optimum temperature; Ts= low temperature; Rn = nongrafted (*Cucumis sativus* var. DavosII); Rs= self grafted; Rma= *Cucurbita maxima*; Rmo= *Momordica charantia*.

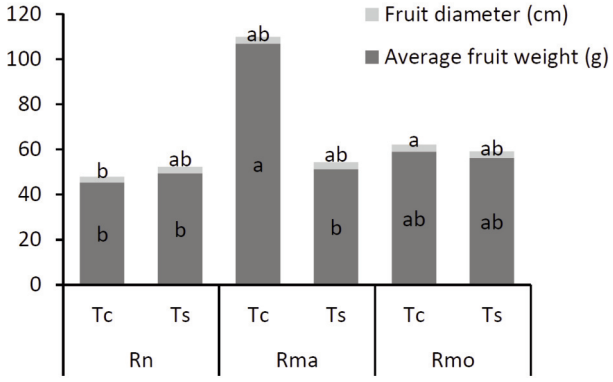

Fig. 6 - The interaction effect of temperature and cucumber grafting on average fruit weight and fruit diameter. Tc= optimum temperature; Ts= low temperature; Rn = nongrafted (*Cucumis sativus* var. DavosII); Rs= self grafted; Rma= *Cucurbita maxima*; Rmo= *Momordica charantia*.

grafting tomatoes onto Kagemusia and Helper's rootstocks by Lee *et al.* (2007). Santa-Cruz *et al.* (2001) grafted tomato cv. Moneymaker onto Pera and observed that growth and yield of tomato increased under salt stress conditions. Similar results were observed by Estan *et al.* (2005), who found that tomato yield increased through grafting under salt stresses and also reported that fruit yield in hetrografted plants increased more compared with self-grafted plants. Ruiz *et al.* (1997) believed that increase yield and growth of the grafted plants might be due to an increase in the nutrient and water uptake fed by vigorous rootstock. Huang *et al.* (2010) reported that cucumbers grafted had a higher fruit yield than non-grafted plants under salt stresses. Colla *et al.* (2010) found that increasing CO_2 assimilation by cucumber grafting increased fruit yields.

The interaction effect of temperature and cucumber grafting on some physiological characteristics and nutrient concentration of cucumber

The photosynthesis rate decreased in Rn and Rs at Ts compared with Tc. However, it did not significantly change in Rma and Rmo (Fig. 8 a). Transpiration decreased at Ts in all rootstocks compared with Tc (Fig. 8 b). Stomata conductance gradually increased in Rs, Rma, and Rmo at Ts compared with Tc and reached the highest in Rmo×Ts (Fig. 8 c). Mesophyll conductance did not significantly change in each rootstock at Tc and Ts, except for ungrafted cucumbers in which it increased at Ts compared with Tc (Fig. 8 d).

El and RWC were generally the lowest and the highest at TS, respectively. the highest EL was seen in Rmo×Ts. The maximum RWC, at Ts, was observed in Rn, Rma, and Rmo (Fig. 9 a, b). Antioxidant increased in Rn and Rs at Ts and did not change in other treat-

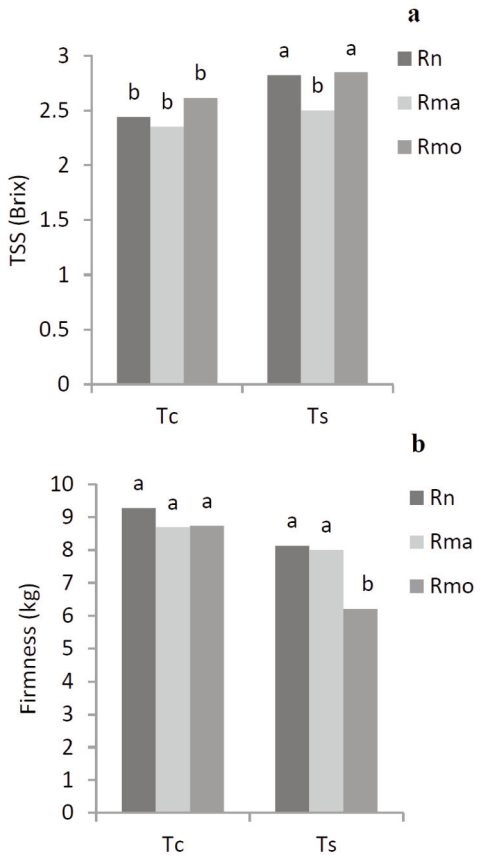

Fig. 7 - The interaction effect of temperature and cucumber grafting on TSS (a) and firmness (b). Tc= optimum temperature, Ts= low temperature; Rn = nongrafted (*Cucumis sativus* var. DavosII); Rs= self grafted; Rma= *Cucurbita maxima*; Rmo= *Momordica charantia.*

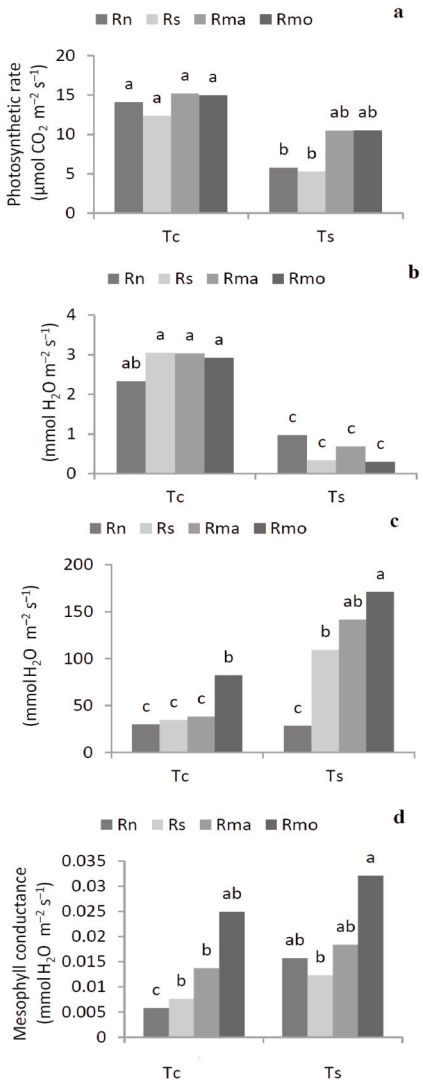

Fig. 8 - The interaction effect of temperature and cucumber grafting on photosynthesis (a), transpiration (b), stomata conductance (c), mesophyll conductance (d) Tc= optimum temperature; Ts= low temperature; Rn = nongrafted (*Cucumis sativus* var. DavosII); Rs= self grafted; Rma= *Cucurbita maxima*; Rmo= *Momordica charantia*.

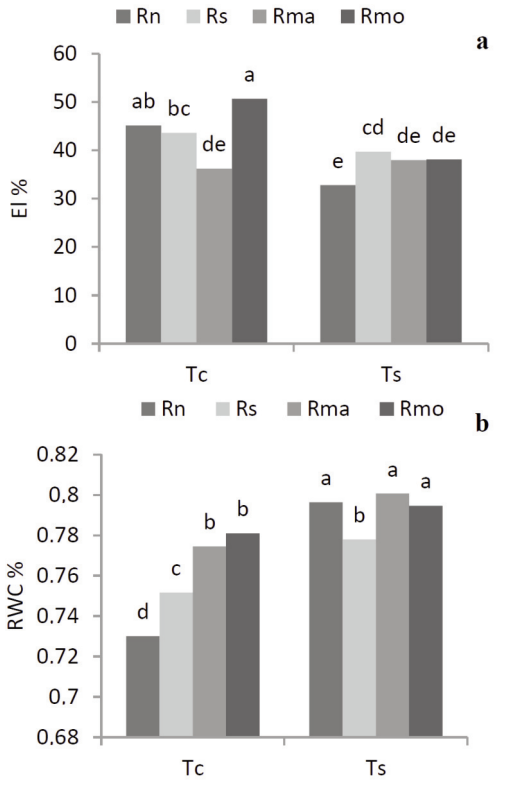

Fig. 9 - The interaction effect of temperature and cucumber grafting on EI (a) and RWC (b) Tc= optimum temperature, Ts= low temperature, Rn = nongrafted (*Cucumis sativus* var. DavosII); Rs= (self grafted); Rma= *Cucurbita maxima*, Rmo= *Momordica charantia*.

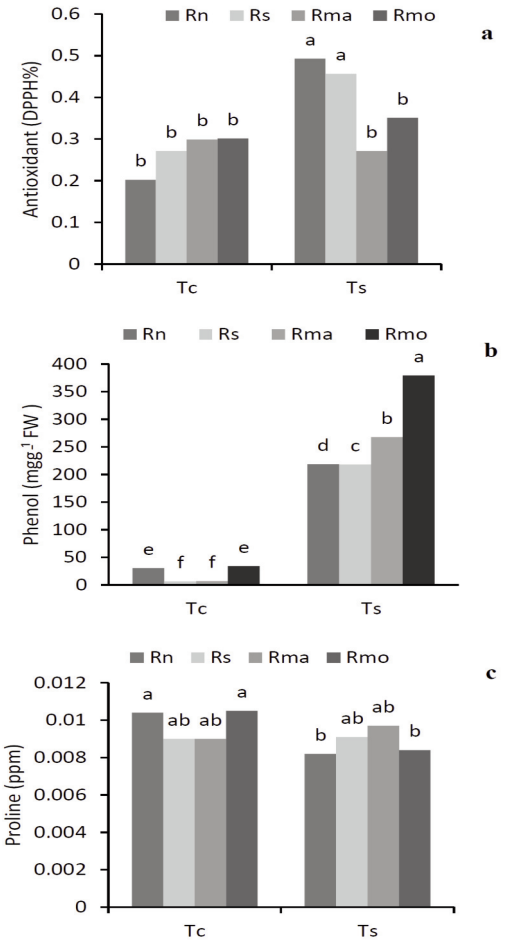

Fig. 10 - The interaction effect of temperature and cucumber grafting on antioxidant (a) and phenol (b) and proline (c) Tc= optimum temperature; Ts= low temperature; Rn = nongrafted (*Cucumis sativus* var. DavosII); Rs= self grafted; Rma= *Cucurbita maxima*; Rmo= *Momordica charantia*.

ments (Fig. 10 a). Phenol content increased in all plants at Ts and reached the highest point in Rmo×Ts (Fig. 10 b). Proline did not significantly change among treatments (Fig. 10 c).

Increasing antioxidant content with using rootstock was reported by Rouphael *et al*. (2018); this may help grafted plant to have a better function under different stress.

The photosynthesis rate and SPAD value showed that the SPAD value did not change in Rmo. Thus, photosynthesis did not change. However, the same result was not seen in Rma. Therefore, it seems that another mechanism interfered with the stability of photosynthesis in Rma in this condition rather than changes of chlorophyll content (Fig. 6 a and 7a).

Some researchers demonstrated that the photosynthesis rate was influenced by leaf area, stomatal conductance, and chlorophyll content. In our study, low-temperature stress caused a reduction in SPAD value and resulted in photosynthesis reduction. On the other hand, the photosynthesis in grafted cucumbers having high SPAD value and stomata conductance improved under low-temperature stresses. Davis *et al.* (2008) reported that grafting increased photosynthesis under salt stress conditions too.

Grafted cucumbers showed higher photosynthesis rates, stomatal conductance, and intercellular CO_2 under salt stresses compared with non-grafted plants (Yang *et al.,* 2006). Massai *et al.* (2004) and Moya *et al.* (2002) reported that grafting improved photosynthesis under salt stress conditions, too. Rootstock-induced changes in stomatal development, as reduced transpiration relates to lower stomatal density in grafted plants (Kumar *et al*., 2017).

Zhou *et al.* (2009) reported that increasing the photosynthesis rate by grafting plants onto a different rootstock might be due to a decrease in the ROS concentration in the leaves. Beside, grafting increased water and nutrient uptake of the plant and could enhance its photosynthesis (Martinez-Ballesta

et al., 2010). Salehi *et al.* (2010) reported that melon grafting had high CO_2 assimilation due to increasing stomata conductance and Ci and resulted in an increase in photosynthesis.

The photosynthesis rate and the fresh shoot weight of Rmo and Rma did not significantly change at both temperatures. Thus, low temperatures could not affect the photosynthesis rate in Rmo and Rma. On the other hand, decreasing the fresh weight was seen at Ts in Rn due to a decrease of photosynthesis rate in these rootstocks (Fig. 7).

Overall, low stomata and mesophyll conductance of un-grafted cucumbers which have adverse effects on water relation, mineral nutrient uptake and transport (Kumar *et al*., 2017), carbohydrate and hormone relationship, photosynthesis and respiration rates result in a decline in yields (Kozlowiski, 1984; Barrick and Noble, 1993; Bacanamwo and Purcell, 1999). These results were in line with our results at low-temperature stresses. Still, when cucumbers were grafted onto Karella they were kept under proper conditions for more efficient photosynthesis, and consequently, a better yield was produced.

The result of the study showed that salinity stresses reduced the K concentration in melons and cucumbers and grafted plants had higher nutrient concentrations than non-grafted plants. Many rootstocks are capable of increasing the uptake and translocation of nutrients (Kumar *et al*., 2017). In agreement with our result, Colla *et al.* (2013) reported that cucumber grafting had no influence on K and P concentrations in the leaves, root, and fruit under salt stresses. Our result showed that the highest K concentration was in Rmo at both temperatures and P concentration increased by grafting at Tc.

4. Conclusions

It seems that the photosynthetic traits of grafted cucumbers were not affected mainly by Rmo and Rma at stress conditions. On the other hand, hormonal changes or nutrient uptake of these rootstocks seemingly caused lesser root and additional vegetative growth but stimulated more fruit and yields. The effect of these rootstocks on productivity growth resulted in more male flowers than female ones, which was predictable according to the productive growth model of Cucurbitaceae in which male flowers and then female flowers emerged in the bush. Accordingly, if this experiment lasted more, it might result in more female flowers, suggesting that further investigations might be needed. All in all, using Rmo as well as Rma is recommended after testing the fruit quality and the economic yield.

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Kale seed priming with red seaweed biostimulant

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Key words: *Brassica oleraceae* var. *acephala*, germination, rhodophyta, *Solieria* sp., thermal stress.

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Abstract: Seed priming is a treatment that can contribute to improve seed physiological potential and increase its tolerance to abiotic stresses. Thus, this work evaluated the effect of kale seed priming with red seaweed biostimulant on physiological seed potential, seed health and tolerance to high temperature at germination. The experimental design was completely randomised, with a 2 x 4 factorial scheme. Treatments consisted of doses of 0, 0.25, 0.50 and 1.0 mL L^{-1} of red algae *Solieria* sp. biostimulant, and temperatures of 20 and 30°C. The biostimulant used was subjected to chromatographic analysis to detect bioactive compounds. Seed imbibition curves were used to determine priming duration procedure. Treatments effects were evaluated by seed health, germination, root and shoot length, and dry mass, under ideal (20°C) and stress (30°C) temperatures. The results were submitted to analysis of variance, Tukey's test (temperatures) and regression (doses). The 22-h imbibition period is adequate for kale seed priming with *Solieria* sp. biostimulant. Kale seed priming with *Solieria* sp. does not interfere with seed health. The temperature of 30°C reduces kale seed germination index, as seedling root growth. The use of *Solieria* sp. biostimulant does not promote kale seed physiological potential.

1. Introduction

Kale (*Brassica oleracea* var. *acephala*) is a vegetable crop belonging to the Brassicaceae plant family, originating from the European continent, with excellent adaptation to mild temperatures. However, there is a demand for this vegetable throughout the year. Considering the tropical climate conditions on several countries, studies of the tolerance to high temperatures in kale production are necessary.

The use of seeds for kale seedlings production has been increasing in recent years, especially considering the increasing release of hybrid cultivars, which do not emit lateral shoots, preventing asexual propagation (Trani *et al*., 2015).

Seedlings production is one of the most important stages for horticul-

tural production systems, which requires high-quality seeds. A key component of the performance of crop seeds is the complex trait of seed vigour. Crop yield and resource-use efficiency depend on successful plant establishment in the field, and it is the vigour of seeds that defines their ability to germinate and establish seedlings rapidly, uniformly and robustly across diverse environmental conditions (Finch-Savage and Bassel, 2016).

One procedure that can be used to improve seed quality is physiological conditioning, also known as seed priming. Seed priming is a pre-sowing treatment that partially hydrates seeds without allowing radicle emergence. Consequently, primed seeds demonstrate rapid germination and improved germination rate and uniformity. Moreover, seed priming is often implicated in improving the stress-tolerance of germinating seeds (Chen and Arora, 2013). Some examples of success using this technique are verified in the literature, as exemplified in the use of seed priming in spinach seed treatment that resulted in high germination rates, seedling emergence, growth, maturity and yield; in this research the maximum seed vigour was obtained when seeds were treated with 6% concentration of *Sargassum wightii* (Brown Seaweed) (Takoliya *et al*., 2018).

Studying physiological and biochemical mechanisms involved in heat stress-tolerance in rice seeds, Hussain *et al*. (2016) found that the best performance and greater rice seedlings tolerance obtained from primed seeds, by different methods (hydropriming, osmopriming, redox priming, chemical priming, and hormonal priming) is associated with increased starch metabolism, high respiratory rate, lower peroxidation and increased capacity of the antioxidant defence system. In addition, other experimental results have shown that the higher germination efficiency and vigour (seedling growth) occur due to the mobilisation of reserves and the activation of genes responsible for the synthesis of vital enzymes during the priming procedure (Lal *et al*., 2018).

Seed priming can be performed with algae extracts or formulations (Sharma *et al*., 2014). Some researches demonstrated beneficial effects of seed priming with several seaweed species, for several horticultural crops, for example, tomato seeds primed with *Anabaena minutissima*, *Ecklonia maxima* and *Jania adhaerens* (Righini *et al*., 2021) and with *Ulva lactuca* and *Padina gymnospora* (Santacruz-Ruvalcaba *et al*., 2019); pepper seeds with *Kappaphycus alvarezii* (K-sap) and *Gracilaria edulis* (Dutta *et al*., 2019).

Seaweeds are green, brown and red marine macroalgae. Extracts of brown seaweeds are widely used in horticulture crops, mainly for their plant growth-promoting effects and ameliorating effect on crop tolerance to abiotic stresses, such as salinity, extreme temperatures, nutrient deficiency and drought (Battacharyya *et al*., 2015). Red seaweeds (Rhodophyta) are sources of carrageenans, which are sulphated linear polysaccharides that represent major cellular constituents of this algae; Carrageenans improve plant growth by regulating various metabolic processes, such as cell division (Shukla *et al*., 2016).

Studying the effects of extracts and isolated molecules of two species of *Gracilaria* (Gracilariales, Rhodophyta) on early growth of lettuce, Torres *et al*. (2018) found a promoting effect of the aqueous extracts in lettuce root length. Nonetheless, there is a lack of studies testing red seaweeds for seed treatment, especially in seed priming protocols.

Therefore, the objective of this work was to evaluate the effect of kale seed priming with red algae biostimulant in seed performance under adequate conditions and thermal stress.

2. Materials and Methods

The experiment was carried out in a Seed and Phytopathology Laboratory, in two stages. In the initial stage, the imbibition curves were performed to determine the ideal time for priming. Kale seeds of the Brazilian cultivar Butter were used, and a *Solieria* sp. biostimulant, which has 7.5% Mn and 13% S; a density of 1.3 g cm^{-3}, and is rich in carrageenans. The experimental design used was completely randomised, in a 2 x 4 factorial scheme (temperatures x doses), with four replications.

To determine the bioactive compounds present in the seaweed extract, a chromatographic analysis was performed, according to the methodology described below.

Methodology for the analysis of phenolic compounds by liquid chromatography high efficiency (HPLC)

The analyze were performed on HPLC equipment brand HP model 1100, Lichrospher RP18 column (5 µm) equipped with 210 nm UV detector and quaternary pump system. The reverse phase analysis consisted of: solvent A - Milli-Q water with 1% phosphoric acid and solvent B - Acetonitrile. The mobile phase

pumping system was gradient, with 90% of solvent A from 0 to 5 min, 60% of A from 5 to 40 min and 90% of A from 45 to 50 min. The standard flow was maintained at 0.5 mL/min according to Morelli (2010). The samples were filtered through Nylon membranes of 0.45 µm pore diameter. The phenolic compounds were identified according to their order of elution and by comparing their retention time with those of their pure standards. Quantification was performed by the external standardization method, by correlating the area (mAU *s) of the compound peak to the standard curve performed with each standard evaluated (gallic acid, epigallocatechin, catechin, epicatechin, epigallocatechin gallate, rutin, ferulic acid, naringin, hesperidin, myricetin, resveratrol, quercetin, apigenin and canferol). The result is expressed in µg/mL of extract.

Seed imbibition curves

Seed imbibition curves were constructed to define the priming period, performed using a method adapted from Ferreira *et al*. (2013). Briefly, four replicates of ±0.1 g of seeds per treatment were soaked in solutions of 0, 0.25, 0.50 and 1.00 mL L^{-1} *Solieria* sp. biostimulant in a plastic box (11 x 11 x 3.5 cm). Each box contained 50 mL of solution in the bottom, and a metallic screen on the top, with the seeds placed between four sheets of germitest paper previously wet (at five times its weight). The boxes were placed in a germination chamber (BOD) at 20°C until protrusion of the primary root.

To determine the amount of solution absorbed, seeds were removed from the germination chamber and the gerbox, dried with paper towels and weighed on a digital analytical balance, with an accuracy of 0.001 g. After weighing, seeds were placed again in the gerbox and brought to the germination chamber. The evaluations were made within 60 min, and when protrusion of the primary root occurred, the process was interrupted, registering the corresponding period. Afterwards, the results obtained in the imbibition curves were analysed, and the appropriate period for priming was determined, which must be previous to the protrusion of the primary root (Bewley *et al*., 2013).

Seed priming

In the second research stage, *Solieria* sp. biostimulant doses for seed priming were tested. The experimental design was completely randomised in a 2 x 4 factorial scheme (temperatures x doses) with five replications. Seed priming occurs at 20°C for 22 h (defined in the previous stage), given the evaluated doses of 0, 0.25, 0.50 and 1.00 mL L^{-1}, according to the method described in the step of the seed imbibition curves. After priming, seeds were evaluated for health, germination (percentage and velocity index), root and shoot seedling length and dry seedling mass.

Seed health

Seed health was evaluated by the blotter test, with eight replicates of 25 seeds placed in plastic boxes (11 x 11 x 3.5 cm) containing three sheets of filter paper moistened with distilled water in a ratio of 2.5 times the dry paper weight. The seeds were incubated at 25°C for 7 days under a 12-h photoperiod. After incubation, the seeds were examined individually under a stereomicroscope and optical microscope, counting the percentage of incidence, and the pathogens were identified based on their morphological characteristics (MAPA, 2009 b).

Germination test

The germination test was carried out at the ideal temperature for the species (20°C) and the stress temperature (30°C), separately. Four replicates of 50 seeds, already conditioned, were distributed on paper for germination ("germitest"), previously moistened with distilled water and kept in a germination chamber. The percentage of germination was evaluated on the fifth day (first count) and 10 days after sowing (DAS) (final count), according to the criteria established in the Rules for Seed Analysis (MAPA, 2009 a).

Germination velocity index

The germination velocity index was determined in conjunction with the germination test, with daily counts, counting seeds with protrusion of 2 mm of primary root according to the protocol proposed by Matthews and Powell (2011).

Seedling length

Seedling length was determined at the end of the germination test (10 DAS), with 20 normal seedlings per experimental unit, from which the shoot length and root length were determined with a ruler graduated in centimetres (Nakagawa, 1999).

Seedlings dry mass

The same seedlings used to evaluate the length were separated in shoots and roots and dried in a forced-air circulation oven at 65°C for 72 h. Once dry, the samples were removed from the greenhouse and

placed in a desiccator, and then weighed on a 0.001g precision scale (Nakagawa, 1999).

Statistical analysis

The results were submitted to analysis of variance. Tukey's test ($p \leq 0.05$) was used for the temperature factor, and polynomial regression was realised for the dose factor. All analyses were performed using Sisvar software (Ferreira, 2011).

3. Results

Chromatographic analysis of the algae extract revealed the presence of gallic acid, at a concentration of 63.9 µg/L, as can be seen in Table 1.

Kale seed imbibition curves with red seaweed (*Solieria* sp.) biostimulant showed an increase in the wet mass accumulation up to 14 h, and the highest absorption peaks were obtained between 12 and 14 h. This period defines germination phase I (Fig. 1 a). Between 14 to 28 h of imbibition, the absorption was constant, without a significant increase in the control treatment, and this was denominated as phase II. After 28 h, phase III germination started. Using the doses of 0.25, 0.5 and 1.0 mL L^{-1}, the germination phase I occurred until 17, 13 and 17 h, respectively, and in all cases, the root protrusion occurred with 28 h of soaking (Fig. 1 b, c and d).

Table 1 - Identification (possible compound) and quantification of phenolic compounds

Compound	Quantification (µg/L)	Retention time (min)
Gallic acid	63. 79	5.877

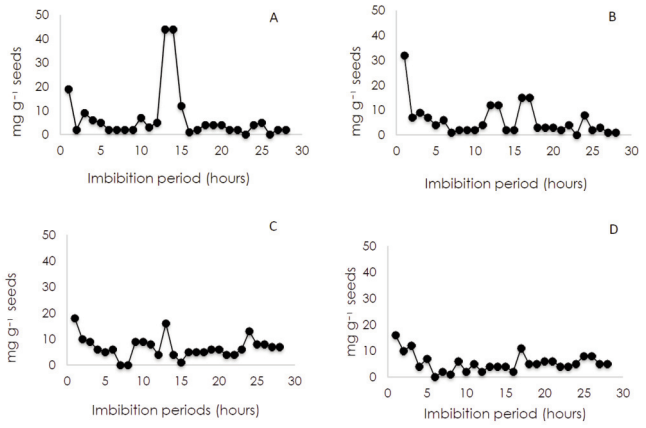

Fig. 1 - Inibibition curves of kale seeds primed with 0 (A), 0,25 (B), 0,50 (C) and 1 (D) mL L^{-1} of *Solieria* sp. biostimulant.

For seed health, no difference was observed between treatments (Table 2). There was a low incidence of fungi, such as *Penicillium* spp., *Rhizopus* spp. and *Cladosporium* spp., and absence of *Fusarium* spp. However, although no statistical difference was observed, 0.37% of *Trichoderma* spp. was detected in the treatment with 0.25 mL of red algae.

Table 2 - Fungi incidence on kale seeds primed with doses red seaweed (*Solieria* sp.) biostimulant

Fungi	Incidence (%)			
	Doses (mL L^{-1})			
	0	0.25	0.5	1
Alternaria spp.	4.1 NS	5.2	3.7	2.4
Penicillium spp.	0.37	0.12	3.37	0.37
Rhizopus spp.	0.12	0	0	0
Cladosporium	0.12	0.12	0	0
Trichoderma spp.	0	0.37	0	0.12

NS = not significant by the Tukey test ($p<0.05$).

Regarding the germination seed performance, differences between temperatures, without dose effects, were observed for the percentage and velocity index (Table 3).

There was a significant difference in seedling root length between the temperatures used, with a pro-

Table 3 - Means of germination count (G), radicle protrusion velocity index (RPVI), root length (RL), dry root (DRM) and shoot (DSM) seedling mass from kale seeds primed with red seaweed (*Solieria* sp.) biostimulant, submitted to different germination temperatures

Temperature	Doses (mL L^{-1})			
	0	0.25	0.5	1
G (%)				
20	82.5 a*	85.5 a	88.0 a	86.0 a
30	82.5 a	80.8 a	79.0 b	70.8 b
RPVI				
20	82.8 a	85.9 a	87.5 a	86.0 a
30	52.6 b	48.0 b	47.0 b	48.9 b
RL (cm)				
20	4.8 a	5.2 a	5.1 a	6.0 a
30	2.7 b	2.5 b	2.5 b	2.4 b
DRM (mg seedling^{-1})				
20	4.8 a	2.9 a	5.0 a	4.8 a
30	2.3 b	4.0 a	3.8 a	3.1 a
DSM (mg seedling^{-1})				
20	3.2 b	3.2 a	2.9 a	3.8 a
30	4.6 a	3.5 a	3.5 a	4.8 a

* Means followed by the same letter in the column, for each variable, do not differ from each other by Tukey test ($p<0.05$).

nounced reduction in root size when the seeds were exposed to 30°C. However, the doses of biostimulant evaluated in this study did not differ and were not able to attenuate the effects of thermal stress (Table 3). In relation to shoot seedling growth, the opposite response was observed, with higher mean lengths at 30°C compared with 20°C (Fig. 2). In examining the effect of the biostimulant doses, at 30°C, there was growth increment until the dose of 0.25 mL L^{-1}, after which, the growth declined (Fig. 2b).

In regards to the accumulation of dry mass in roots and shoots of seedlings, no effects of biostimulant doses were observed (Table 3). For the temperature, a reduction in root length and increase in shoot length was verified in the control, which reinforces the hypothesis of the compensatory effect, related to plasticity.

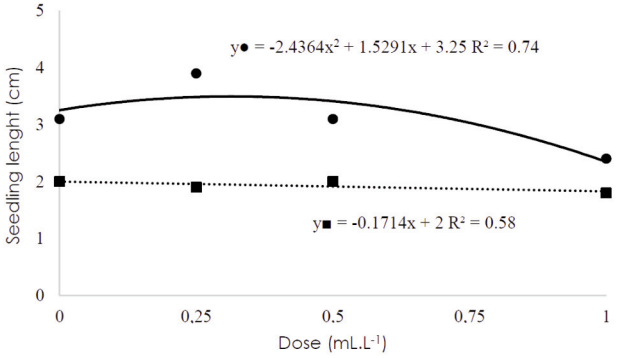

Fig. 2 - Averages of kale seedling shoot length, from primed seeds with red seaweed (*Solieria* sp.) biostimulant, submitted to 20°C (■) and 30°C (●) during germination.

4. Discussion and Conclusions

Regarding results of chromatographic analysis, which revealed the presence of gallic acid, it is important to consider that gallic acid is a secondary metabolite present in most plants and algaes (Cotas *et al*., 2020). Considered one of the major phenolic acids, gallic acid (or gallate) is a benzoic acid of great importance for the formation of a so-called galatotanin-hydrolyzable tannins group formed by a unit of sugar and a variable number of phenol acid molecule. This metabolite is known to exhibit a range of bioactivities including antioxidant and antimicrobial (Fernandes and Salgado, 2016).

Considering results of seed health, is important to mention that according to Machado *et al*. (2012), some *Trichoderma* strains are used to control phytopathogens and promote plant growth due to their versatility of action, such as parasitism, antibiosis and competition, as well as acting as inductors of plant resistance against diseases. Melo *et al*. (2017) observed progressive increases in phytoalexin concentration in soybean and sorghum seeds treated with increasing algae doses, demonstrating a high correlation between dose used and the amount of phytoalexin produced. Phytoalexins are compounds that have been studied a few years ago, which present mainly antimicrobial activity (Arruda *et al*., 2016).

The highest phytopathogenic fungi observed was *Alternaria* spp. (Table 2), however no differences between treatments were found. This fungus is a pathogen that can be transmitted by seeds and interferes with the germination and development of seedlings (Torres-Córtes *et al*., 2019).

Regarding the germination seed performance, differences between temperatures, without dose effects, were observed for the percentage and velocity index (Table 3). In general, germination capacity was reduced at 30°C, indicating an abiotic stress effect in this process. Temperature exerts a marked effect on germination because it influences the performance of enzymes involved in the mobilisation of reserves, as well as enzymes associated with hormonal regulation.

In *Arabidopsis*, a model species in studies of physiology and genetics, and of the same botanical family of kale, it was verified that when seeds are exposed to high temperatures during germination, there is a stimulus to biosynthesis of abscisic acid (ABA) and, consequently, a repression of gibberellin synthesis (Toh et *al*., 2008). The balance between ABA and gibberellins is responsible for the occurrence of germination or maintenance of dormancy (Kucera *et al*., 2005). Furthermore, it should be noted that gibberellins stimulate the synthesis and production of hydrolases, especially alpha-amylase, resulting in seed germination (Miransari and Smith, 2014).

However, the inhibitory effects of ABA on seed germination include the impedance of radicle expansion and endosperm weakening, as well as increased expression of transcription factors, which may adversely affect the seed germination process (Graeber *et al*., 2010).

The absence of the effect of red algae on germination might indicate that this response is associated with the composition of the biostimulant and to the moment of application. One of the main compounds

found in red algae is carrageenans, sulphated linear polysaccharides. Recent research has uncovered the biological activity of carrageenans and their oligomeric forms as plant growth promoters and elicitors of defence responses (Shukla *et al*., 2016).

However, carrageenans have shown a relatively greater effect when applied to plants at adult developmental stages. For example, according to Gonzales *et al*. (2013), oligo-carrageenans obtained by depolymerisation of red algae carrageenans increase the growth of tobacco plants by increasing photosynthesis and nitrogen assimilation, as well as stimulate the growth of 3-year-old *Eucalyptus globulus* plants.

Regarding results of seedling root length (Table 3) is worth mention that temperature stress has a detrimental effect on plant metabolism by interrupting cell homeostasis, and the direct result of cellular changes is increased accumulation of toxic compounds in cells, including reactive oxygen species (Essemine *et al*., 2010). According to Tsukagoshi (2016), reactive oxygen species regulate the activity of the root meristems and root development. Therefore, this mechanism is probably involved in the reduced growth of the kale seedling root accompanying the elevation of temperature, as verified in this work.

However, in relation to shoot seedling growth, the opposite response was observed (Fig. 2). This response might be explained by a mechanism of plasticity, in an attempt to balance the growth of the seedling, due to the reduction in root size. Hernandez-Herrera *et al*. (2014) found that there was a stimulatory effect of algae extracts on the length of plumule in tomato seedlings.

In regards to the accumulation of dry mass in roots and shoots of seedlings, no effects of biostimulant doses were observed (Table 3). As mentioned by Mašková and Herben (2018), the ratio of biomass partition between roots and shoots is essential for the ability of plants to compensate for the limited resources in the environment and thereby to survive and succeed in competition. Allocation plasticity is an important process for seedlings, and this is one of the most vulnerable phases of the breeding cycle, for most species. In this context, a rapid allocation response may have a direct impact on its survival (Lloret *et al*., 1999).

Regarding the biostimulant doses evaluated, it is possible that there were no effects on the accumulation of seedling dry mass because this process is more related to the issue of mobilisation and partition of the reserves, with little influence exerted by the compounds present in algae. However, considering that to date, there are no scientific studies to prove the direct effect of algae biostimulants in the process of mobilising seed reserves and partitioning of biomass in seedlings, additional studies in this area are necessary.

In conclusion, the 22-h imbibition period is adequate for kale seed priming with *Solieria* sp. biostimulant. Kale seed priming with *Solieria* sp. biostimulant does not interfere with seed sanity. The temperature of 30°C reduces velocity germination index and kale seedling growth, obtained from seeds primed with *Solieria* sp. biostimulant. The use of *Solieria* sp. biostimulant does not promote improvements in kale seed physiological potential under the conditions in which this research was carried out.

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Assessment of pesticide residues level in watermelon fruits [*Citrullus lanatus* (Thunberg) Matsumura and Nakai] in Lower, Central and Upper Badibou Districts in North Bank Region, of the Gambia

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Key words: maximum residue limit, pesticide, pesticide residue, watermelon.

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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: Field and laboratory studies were conducted late 2019 in three districts of the Gambia's North Bank Region; namely, Central Badibou, Lower Badibou and Upper Badibou to ascertain the pesticide residue level in watermelon fruit, determine the insect pest control methods, types of pesticide, frequency of application, and pre-harvest interval observed. Multistage sampling technique was used in selecting the research respondents. Eighty-five (85) farmers were identified; forty-five (45) were randomly selected as research respondents. Data was collected using structured questionnaires. SPSS Software was used to analyse the questionnaires and Gas Chromatograph to determine the pesticide residue level. Data obtained were analysed and compared with the European Union Maximum Residue Limit (MRL). The results of the analysis revealed that the farmers used chemical control method in watermelon production, and applied at frequency of once in every two weeks. Furthermore, the results indicated that the chemical applied at flowering stage and the pre-harvest interval (17-21 days) ranked the highest. The pesticides residues found in the watermelon samples were Dimethoate, Profenophos, Dicofol, Cypermethrin, Lambda-cyhalothrin, Permethrin and Deltamethrin and most were above the MRL. The presence of pesticides residues in the watermelon samples calls for strict regulation on the use of pesticides on watermelon. Further study is recommended in other fruits and vegetables grown in the country especially in the studied region.

1. Introduction

The developing countries are seriously affected by the sky-rocketing of unemployment rates. Agriculture is the backbone of all the developing countries' economies. The production of watermelon by Gambian Farmers has featured prominently irrespective of the uncountable num-

ber of challenges faced by farmers amongst other hosts of issues that militate against this lucrative enterprise (Department of Planning Agriculture, 2016). The government's attention should, therefore, be drawn towards addressing challenges faced in watermelon production particularly pesticides and its use.

The sector contributes positively to the Gross Domestic Product (GDP) and employs about 70% of the labour force (Jobarteh and Selmani, 2020). According to the ANR policy (2019-2026), the consumption trend of fruits and vegetables of various kinds in the country is fairly high and likely to continue. Fruits are a concentrated source of natural components, and these natural components are plant-derived materials performing a key role in maintaining human health, especially in disease prevention, growth, and development. Today, plants, and plant-based compounds are the basis of modern pharmaceuticals used for the treatment of various dreadful diseases (Reetu and Tomar, 2017). Fruits such as mango, organs, guava, pear, and watermelon are life-enhancing medicines packed with vitamins, minerals, and antioxidants to human as well as cash crops for export by the growers in The Gambia. Watermelon does not only have the potential of enhancing the health of consumers but also increases the income of farmers (Adeoye *et al.*, 2011).

The production of watermelon is mainly carried out in the rainy season from September to December. It is produced in all the regions of the country; however, North Bank Region and Central River Region are the major growing areas of watermelon in The Gambia. In 2016, a total of one hundred and seventy-four (174) tonnes of watermelon fruits were produced (Department of Planning Agriculture, 2016). The crop is generally susceptible to many insects and diseases but no systematic study has been carried out to determine the damage level. Research has revealed that factors such as the prevalence of insects and diseases, climatic factors such as rainfall, temperature, and soil types hinder the production of watermelon (Adojutelegan *et al.*, 2016). Amongst the problems faced by watermelon farmers, insect pests ranked the highest (Chamo *et al.,* 2016). Farmers used different strategies such as attractants (cocoa butter), food baits, sanitation, botanicals, and chemicals to control fruit flies. Synthetic pesticides are known to be one of the most effective agents for controlling insects and diseases. Amongst various categories of pesticides, the insecticide is considered as the most toxic (Kodandaram *et al.,* 2013). It is becoming a dominant agent for controlling insects and diseases of crops.

Farmers use pesticides such as Carbofuran, chlorpyrifos, diazinon, dimethoate and metalaxyl in the production of crops (Wanwimolruk *et al.,* 2015). Watermelon is very susceptible to insects and diseases such as fusarium wilt, anthracnose, downy mildew virus diseases, gummy stem blight, powdery mildew, bacterial fruit blotch, damping-off, root-knot nematodes, adult striped, spotted cucumber beetle and squash bugs as a result, farmers use pesticides in controlling them (Shrefler *et al*., 2015). The system of continuous application of pesticides may lead to a high level of pesticide residue in the crops (Nguyen *et al.,* 2018). This could have a counterproductive effect on the quality of products to be consumed and placing a farmer's life at risk.

Cases of indiscriminate use of pesticides and non-adherence to good agricultural practices are very common. For example, some farmers apply chemicals on their fields in the afternoon and pick the fruits early in the next morning for sale in the local markets. These observations suggest that the fruits sold in the markets may have serious pesticide contamination. Most of the farmers in The Gambia, do not consider the so-called Maximum Residue Limit. Thought the Maximum Residue Limit regulation exists in the country, but it is not adhered to.

The production and consumption of watermelon are rapidly increasing in The Gambia. During the peak season of watermelon, the price is affordable to all categories of the population due to the high supply in the market. Most of the children and even some adults when eating watermelon eat it to the core. Sometimes, the remains are given to animals such as goats and sheep. To my knowledge no formal research has been done in the country to determine pesticide residue in watermelon. As a result, it is necessary to assess the pesticide residue in watermelon fruits as this crop in question have numerous health benefits such as it preventing cancer and diabetes due to its high content of lycopene, an antioxidant, lower blood pressure due to its richness in an amino acid. Therefore, this study aimed to determine the methods used in controlling insect pest of watermelon and to assess the concentration of pesticide residue in the watermelon fruits at harvest in the study area.

2. Materials and Methods

The study location

The study was conducted in North Bank Region of the Gambia, located in the Northern part of the River Gambia from October to November 2019. The area is located between latitude 16.01° West and longitude 13.52° North. The Region is one of the five administrative Regions of The Gambia. Its capital is Kerewan and subsequently reorganized as the Kerewan Government Area Council, with none change within the area covered. The area is divided into seven districts, namely, Central Baddibu, Lower Baddibu, Upper Baddibu, Jokadu, Lower Niumi, Upper Niumi and Sajal. The area is peculiar for watermelon production and a large percentage of the population practices farming at a large scale (Figs. 1, 2, and 3).

Research design

The study used qualitative and quantitative methods to collect data. The research methods used were as follows:

Step one: the researcher conducted interviews with the watermelon farmers in the study area (North Bank Region).

Step two: Watermelon samples were collected for pesticide residue analysis.

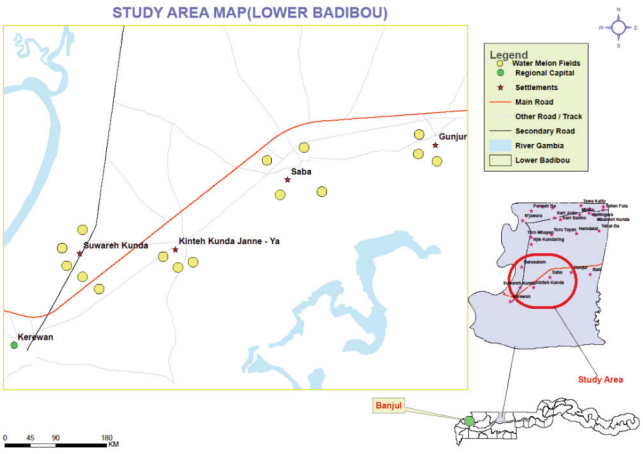

Fig. 1 - Map showing the study areas in Lower Badibou District.

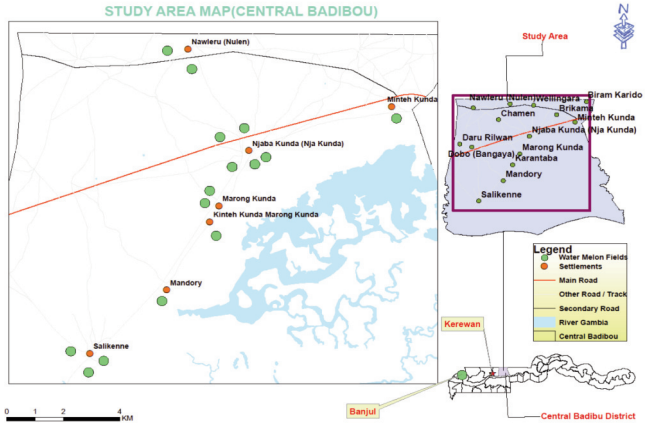

Fig. 2 - Map showing the study areas in Central Badibou District.

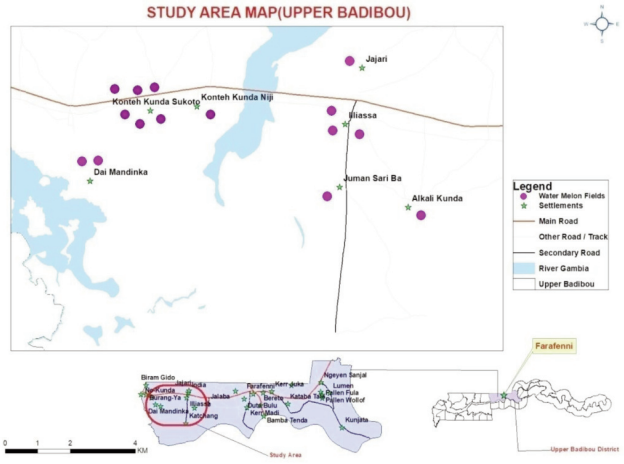

Fig. 3 - Map showing the study areas in Upper Badibou District.

Sampling procedures and sample sizes

Multistage sampling techniques were used in selecting the research respondents (contact farmers). Before the study, the Regional Directorate was contacted for information regarding the production trend of watermelon in the Region. Based on the findings, three districts (Central Badibou, Lower Badibou and Upper Badibou) with the highest number of watermelon growers were selected using purposive sampling. The second stage was the purposive sampling of 37 major watermelon growing villages in the sample districts. Populations of 85 farmers were identified out of which forty-five (45) farmers were randomly selected as research respondents 15 from each district.

Samples of 10 watermelon fruit due for harvesting were randomly collected from (10) different watermelon fields for pesticide residue analysis. The pesticide residues found in the fruits were compared with the European Union Pesticide Maximum Residue Limit standard (Table 1).

Data collection

Data were collected using structured questionnaires designed in English and administered orally in the local languages (Wolof, Mandinka, Fula, and Serer etc.). Before the actual data collection, the questionnaires were validated pre-tested with few

Table 1 - European Union Pesticide Database on maximum residue limit standard

Sample pesticide	Maximum residue limit (MRL) (mg/Kg)
Dimethoate	0.01
Dicofol	0.02
Lambda-Cyhalothrin	0.06
Profenophos	0.01
Deltamethrin	0.02
Permethrin	0.05
Cypermethrin	0.20

Source: European Union Pesticide Database on MRL Standard Latest Update, 2016.

watermelon farmers and adjusted. The demographic characteristics of the farmers (age, education, farming experience, labour source, farm size) and pest control method used by farmers were observed. One watermelon fruit was collected from a total of 10 farmers. During the collection, foil paper, tissue, markers were used. The tissue paper was used to clean the watermelon fruits and wrapped with foil paper to avoid contamination. Samples were labelled using the board marker. The watermelon samples were sent to Ceres Locustox in Dakar for pesticide residue analysis. The field coordinates were taken with GPSMap 60CS GARMIN.

Sample preparation and storage

Individual watermelon fruit samples were cut and chopped with a knife to about 3 cm cube in size. The process was carefully carried out to ensure that the test samples were homogeneous. The samples were blended with a Robot coupe blender 6 to ensure homogeneity. Each time the blender was used for a sample, the content in the machine was cleaned together with all the other equipment used in the process with detergent before another sample was blended to avoid contamination. Precautionary measures were employed to avoid any losses of juice or flesh.

Test portion

Individual test portion sufficient for one analysis was taken as sub-sample from the main test sample. These were immediately prepared for analysis. 10 g of the solution was measured from the homogeneous sample and put into 50 g centrifuge tube. The solution was extracted with the help of the acetonitrile.

Extraction

To determine the concentrations of pesticide residues, the extraction procedure was performed as described by Institut Luxembourgeois de la Normalisation, de l'Accreditation, de la Securite (ILNAS, 2018). The samples were prepared as follows: Ten millilitre (10 ml) of acetonitrile and 100 µl of PCB28 were separately measured and put into the test tube containing the test sample, and agitated by hand vigorously for a minute. Four grams (4 g) of Magnesium sulfate was added for the removal of residual water together with, 1 g of Sodium chlorine, 0.5 g of Na2Hcitrates-squihydrate and 1 g of Na3citrates- dehydrates. The solution was agitated vigorously with the flash shaker for one minute and centrifuged for phase separation for five minutes at a speed of 3000 m/s.

Purification

One millilitre of the solution was pipette and separately placed into 2 ml test tubes containing 150 mg $MgSO_4$ and 25 mg Primary Secondary Amine Sorbent (PSA). The solutions were centrifuged for five minutes at the speed of 3000 revolution/min. One (1 ml) was then transferred to another test tube and 5% solution of formic acid added to improve the storage stability of certain base sensitive pesticides. In addition, a control was prepared alongside, which has the entire additive except the watermelon sample.

Data analysis

Data were analyzed using Statistical Package for Social Science (SPSS) windows software version 20. The pesticides residue analysis was done using the Gas Chromatograph as described by Institut Luxembourgeois de la normalisation, de l'accreditation, de la securite (ILNAS, 2018).

3. Results and Discussion

Demographic characteristics of the watermelon farmers in the study area

The demographic characteristics of the research respondents were presented in Table 2. The result derived shows that all respondents were 100% male. Gender is very fundamental when it comes to the production of a certain type of crops in The Gambia. Traditionally men are attributed to watermelon production due to its tediousness. This finding is similar with Adeoye *et al.* (2011) who indicated the dominance of male fork in watermelon production in

Nigeria. The watermelon growers were predominantly youths and middle-aged individuals as 62% of the respondents were not more than 50 years old (Table 2). Age determines strength, therefore; it plays an important role in any meaningful production. Watermelon production is very tedious and rigorous as a result the middle-age individuals are the most people venturing into it. During the research, it was found out that some old age had to reduce their farm size due to the tediousness of the work. This is in agreement with (Adeoye *et al.,* 2011) who stated that age is crucial to carrying out activities such as mounting knapsack sprayer at the back, which most of the farmers used in the study area.

Majority of the research respondents did not have formal education, amongst them Arabic education ranked the highest 60%, followed by secondary education, (24.4%), primary education (6.7%), tertiary education (4.4%) and illiterate (4.4%). This shows that the level of conventional education is very low among the watermelon farmers in the study area (Table 2). Most of the pesticides are labelled either in English or in French; as a result, the respondents could not read the pesticide label. This might be responsible for the high concentration of pesticide residue in the watermelon fruits from the study area. Mubushar *et al.* (2019) reported the inappropriate use of pesticide due to high level of illiteracy.

Watermelon production was dominated by less experienced farmers, 1-5 years experience (55.6%), 6-10 years (24.4%), 11-15 years (11.1%) and 16 years above (8.9%). This could be attributed to the fact that the majority of the watermelon farmers in the study area are in the middle age. Therefore, the inexperience of the watermelon farmers might have caused poor handling and frequent application of pesticides (Eyhorn *et al.,* 2015), which could have led to the high concentration of pesticide residue in watermelon fruits.

Table 2 - Demographic characteristic of the watermelon farmers

Personal characteristics	Categories	Percentage (%)
Sex	Male	100
	Female	0
Age	20-30 years	13.3
	31-40 years	31.1
	41-50 years	31.2
	51-60 above	24.4
Educational qualification	Primary	6.8
	Secondary	24.4
	Illiterate	4.4
	Tertiary	4.4
	Arabic	60
Experience	1-5 years	55.6
	6-10 years	24.4
	11-15years	11.1
	16 above	8.9

NS = not significant by the Tukey test ($p<0.05$).

Control methods of insect pest practiced by watermelon farmers in the study area

The various methods of pest management practised by respondents are shown in figure 4. Chemical control method had the highest in the study area (88.9%) followed by Botanical (6.7%), and Integrated Pest Management (4.4%). This was attributed to the believed that chemical is readily available and required less labour as compared to other control methods (Fig. 4). The results manifested that chemical control method was predominantly practised. According to the farmers, the pesticides are easy to use and readily available. Some farmers practised other control methods but they claimed to be difficult and time-consuming. For example, to have enough of neem solution to cover a hectare of land is not easy, which compelled majority of the farmers to practised chemical control method. This in line with Padmajani *et al.* (2014) who reported the easy use agro-chemical by farmers despite knowing other control methods.

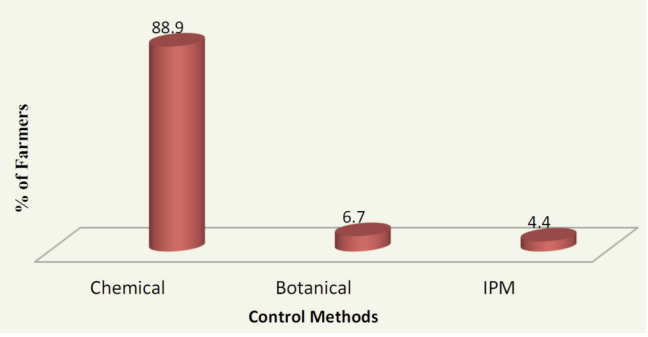

Fig. 4 - Control methods of insect pest practiced by watermelon farmers.

The frequency of application of the pesticides by watermelon farmers in the study area

Figure 5 revealed the frequency of pesticide application during watermelon production in the study area. The result revealed that majority of the respondents applied pesticide once every two weeks

(44.4%) followed by those who applied once every week (31.1%). The respondents who applied pesticide once every three weeks were 17.8% and the lowest percentage (6.7%) of respondents were found to applied pesticide once in a month.

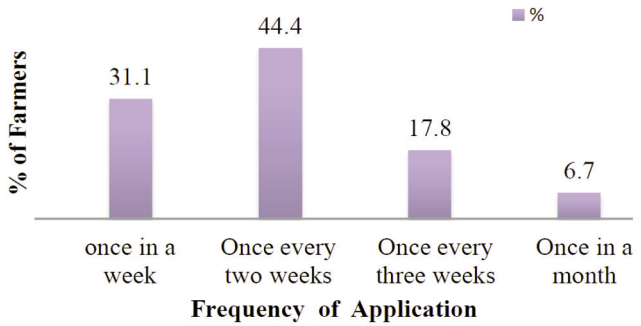

Fig. 5 - Percentage frequency of pesticide application in the study area.

The pre-harvest interval observed by the watermelon farmers in the study area

Figure 6 showed the pre-harvest interval observed in the watermelon growing areas. Most of the respondents (46.7%) observed pre-harvest interval of 16-20 days, followed by 17.8% of the respondents who observed pre-harvest interval above 21 days. The lowest percentages (11.1%) of respondents were found to observe pre-harvest interval of 6-10 days.

Pesticide residue concentration levels detected in the watermelon samples

The potential contamination of watermelon fruits with the different pesticides used in the study area was investigated. The results detected seven pesticides that happen to fall in the same class of pesticides, insecticide in the watermelon fruits that were subjected to analysis. The insecticides detected were; Dimethoate, Profenophos, Dicofol, Cypermethrin, Lambda-cyhalothrin, Permethrin, and Deltamethrin.

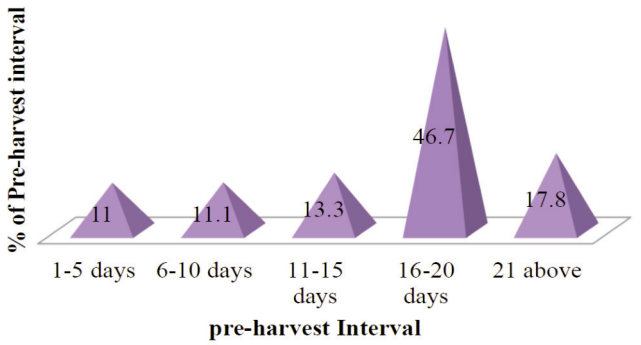

Fig. 6 - Pre- harvest intervals observed in the study area.

The Dimethoate and Profenophos are in the pesticide group of organophosphate, whereas Dicofol belongs to the organochlorine. Cypermethrin, Lambda-cyhalothrin, Permethrin, and Deltamethrin belong to the pyrethroids.

The various levels of dimethoate residues detected in the watermelon samples are shown in figure 7. The test result indicated that there was high pesticide residue concentration of dimethoate in all the watermelon samples compared to the European Union Maximum Residue Limit (0.01 mg/kg) except in sample 6 which recorded (0.01 mg/kg). In samples 1 and 2 the residue level recorded were 0.8 mg/kg, whilst samples 4 and 10 recorded 1 mg/kg. The highest concentration of Dimethoate was found in samples 3, 5, 7, 8, and 9 with 2 mg/kg. The present of dimethoate residue in watermelon was reported by Omoyajowo *et al.* (2017) in his research on the Assessment of Pesticide Residue Levels in common fruits consumed in Lagos State, Nigeria. If the MRL exceeds its limit it can cause various health problems to consumers. Ngoula *et al.* (2014) stated that high dose of dimethoate disrupts spermatogenesis and reduce fertility in test animal.

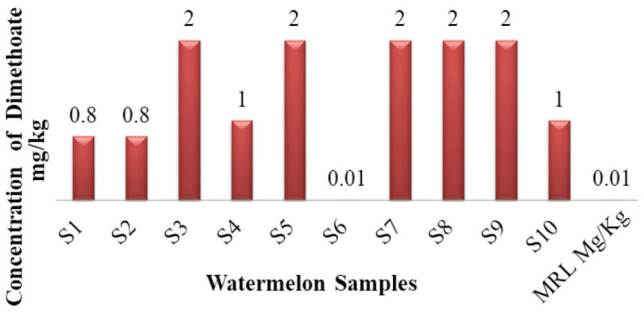

Fig. 7 - Residue level of dimethoate in watermelon samples.

Figure 8 presents the dicofol residues detected in the various watermelon sample analysed for pesticide residue. The findings revealed that watermelon samples were all found to have some amount of insecticide residues of dicofol and were all above the European Union Maximum Residue Limit which is 0.02 mg/kg. Sample 2 and 10 registered (0.3 mg/kg), sample 8 registered (0.4 mg/kg), sample 1 registered (0.5 mg/kg), sample 9 registered (0.7 mg/kg), samples 3, 4, 6, and 7 registered (0.8 mg/kg) and sample 5 registered the highest pesticide residue of (1 mg/kg).

Figure 9 presents the residue of lambda-cyhalothrin detected in the various watermelon sam-

ples. Lambda-cyhalothrin residue was found in all the watermelon samples that were subjected to analysis. Sample 1 recorded (0.006 mg/kg) while sample 2, 3, 4, 5, 6, 7, 8, 9 and 10 recorded 0. 009 mg/kg and they were all found to be below the European Union Maximum Residue Limit which happened to be 0.06 mg/kg.

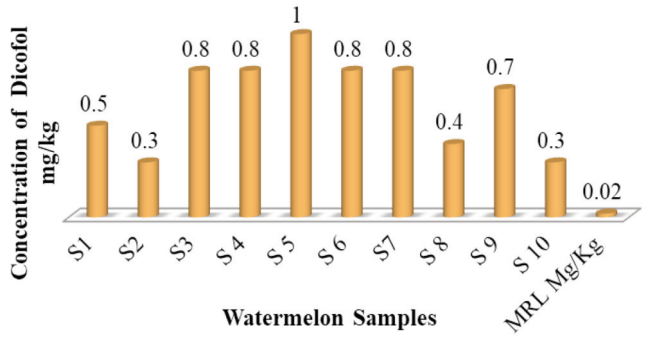

Fig. 8 - Pesticide residue level of dicofol in watermelon samples.

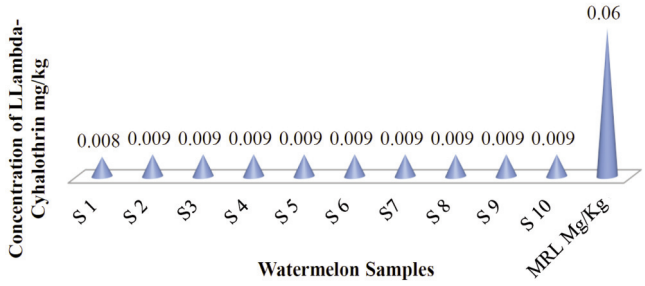

Fig. 9 - Pesticide residue level of lambda-cyhalothrin in watermelon samples.

The Profenophos residue level detected in the watermelon samples is shown in (Fig. 10). Profenophos was found in all the watermelon subjected to analysis. Samples 3, 4, 6, 7, 8, 9, and 10 had pesticides residue concentration level of 0.06 mg/kg, while sample 1, 2, and 5 had 0.05 mg/kg, when compared to the Maximum Residue Limit standard of 0.01 mg/kg of the European Union, they were all found to be above the residue limit.

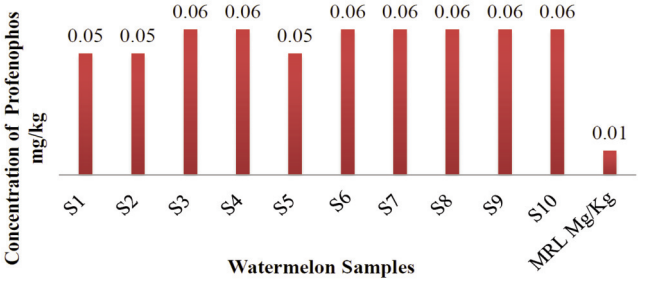

Fig. 10 - Pesticide residue levels of profenophos in watermelon samples.

Figure 11 indicates the deltamethrin residue level detected in the watermelon samples. Deltamethrin residues were present in all the watermelon samples; Sample 1 recorded (0.1 mg/kg), samples 2, 6, 8, and 10 recorded (0.2 mg/kg), samples 3, 4, 5, and 7 recorded (0.3 mg/kg). Sample 9 recorded the highest concentration level of (1 mg/kg). Compared with the European Union MRLof 0.02 mg/kg; all the samples were above the MRL.

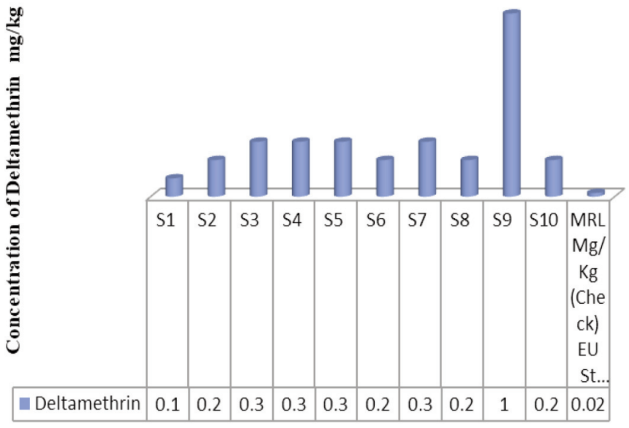

Fig. 11 - Pesticide residual level of deltamethrine in watermelon samples.

The Permethrin residue detected in the various watermelon samples are presented in figure 12. The Permethrin was found in all the watermelon samples and there concentrations were above the European Union Maximum Residue Limit of 0.05 mg/kg. The highest concentration of Permethrin was observed in sample 5 with (1.5 mg/kg), followed by sample 8 (1.4 mg/kg), while samples 4, 9 and 10 recorded 1 mg/kg (Fig. 12).

Figure 13 presents the residue of Cypermethrin detected in the various watermelon samples. Cypermethrin was found in all the watermelon sam-

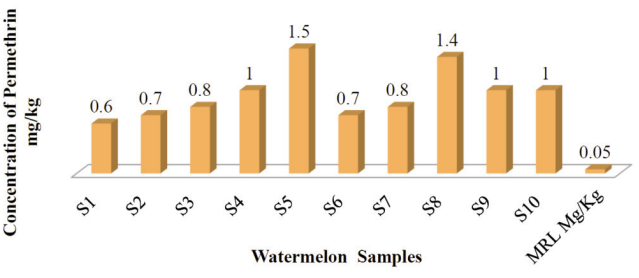

Fig. 12 - Pesticide residue level of permethrine in watermelon samples.

ples and were all below the European Union MRL of 0.2 mg/kg. The concentration of cypermethrin varied from 0.008 mg/kg to 0.08 mg/kg.

Effects of frequency of pesticide application and pre-harvest interval on pesticide residue level in the watermelon fruits.

In figure 14 the effect of the frequency of application of dimethoate on pesticide residue detected in watermelon fruits are shown. The watermelon fruit samples that received one application of dimethoate in every week had the highest concentration (1.5 mg/kg). The application of pesticides once every month had the lowest residue with 0.8 mg/kg. Frequency of application has great impact on the pesticide residue found in the watermelon fruits. This shows that before the dose of one application is neutralised another dose is applied again. This might be one of the main reasons why some of the watermelon fruits were highly contaminated with pesticides.

In figure 15 the effect of observing pre-harvest interval on pesticide residue in watermelon fruits are shown. Pre-harvest interval, which is the period between the time of application and period of harvest for the consumption of plant product, is recommended for each and every pesticide used on crops. The highest residue of dimethoate was found in fruits with pre-harvest interval 1 to 5 days (1.8 mg/kg) while the lowest residue of dimethoate was observed in the watermelon samples with pre-harvest interval of 21 days above (0.7 mg/kg). Most of the chemical products used by farmers on watermelon have pre-harvest interval of three to four weeks. Islam and Haque (2018) reported the importance of observing proper pre-harvest interval as it reduces the pesticide residue. Therefore, pre-harvest intervals have a great influence on the residue concentration of fruits and vegetables.

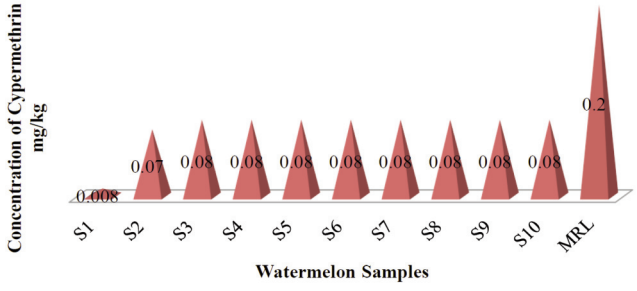

Fig. 13 - Pesticide residual level of cypermethrin in watermelon samples.

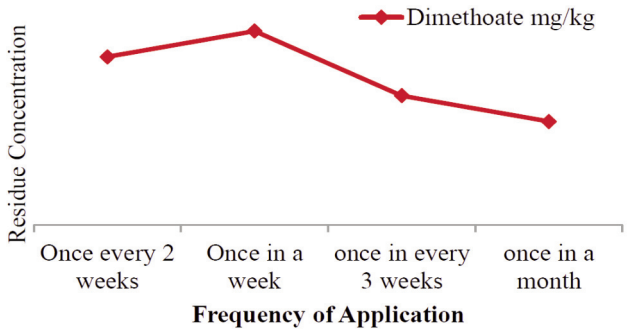

Fig. 14 - Effect of the frequent application of pesticide on pesticide residue in watermelon fruit.

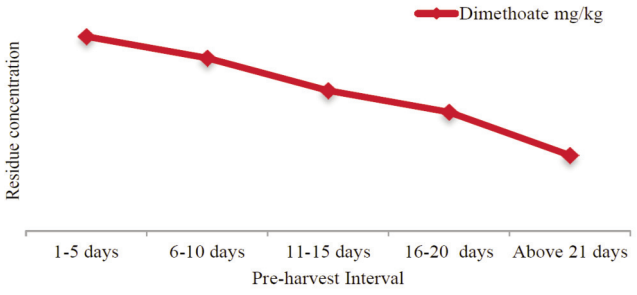

Fig. 15 - Effect of pre-harvest interval on the pesticide residue

4. Conclusions

Few pesticides residues were detected in all the watermelon samples analysed for pesticides residue the study and it was an indication that most of the fruits were contaminated with pesticides such as Dimethoate, Profenophos, Dicofol, Cypermethrine, Lambda-cyhalothrin, Permethrin and Deltamethrin. The residue level of most these insecticides were found to be above the recommended MRL. The frequency of pesticide application and the pre-harvest interval were found to have influence on the pesticide residue concentration in the watermelon fruits from the study area. The study further revealed that most of the respondents were illiterate and has less experience in watermelon production. Base on the findings from the study the following recommendation are made:

- farmers observe a pre-harvest interval of above 21 days to avoid high residue concentration in the watermelon fruits.
- farmers to use Cypermethrin and Lambda-cyhalothrin in the production of watermelon because they are easily degradable by the heat.

further research on other fruits and vegetables grown in the country should be conducted by encouraging the student to take up such research topics.
farmers should be educated and encouraged on other alternative pest control methods by setting up demonstration plot beside farmer field to demonstrate effectiveness of other methods.
the extension workers and farmers must be trained on the judicious use of pesticide through farmer field schools initiatives.
the promotion of the use and production of non-persistent pesticides and put more tax on persistent pesticides to reduce its importation
Responsible Sectors/Agency to put strict measures in the illegal entry of pesticide by closely monitoring the entry points and pesticide vendors.
pesticides residue regulation policy should be formulated and make sure it is strictly implemented by the responsible agencies.
the laboratory of the National Research Institute and the University of The Gambia be equipped with all relevant equipment to facilitate research in the country.

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Effect of biostimulants and media compositions on growth and yield of *Capsicum annuum* L. under drought stress conditions

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Key words: chili pepper, drought stress, growth stimulant, organic farming, sustainable agriculture, *Trichoderma*.

Abstract: The study was conducted at the Teaching and Research Farm of Agricultural Faculty, University of Jambi, Indonesia, from April through to September 2019. The aim of this study was to investigate the effect of biostimulants and media compositions on the growth and yield of chili pepper during restricted soil water content. The study was arranged in a split plot design with 3 replicates (groups). Different types of biostimulants (Citorin®, Hantu®, and a control) were designated as main plot, whereas media compositions (2:2:1, 2:1:1, 1:2:1 and 1:1:2) made of soil+trichocompost+rice husk charcoal were employed as sub plot. At the time of transplanting, soil water content was set to approximately 75% of field capacity to create stress conditions. The results showed that the proper choice of biostimulant and medium composition could increase nutrient status, total sugar and chlorophyll contents, and reduce proline level in plants grown under restricted water availability. Citorin® application on chili plants grown on organic media (soil+trichocompost+rice husk charcoal) with ratio of 2:1:1 could be recommended to support plant growth and production under drought stress conditions.

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1. Introduction

Chili pepper (*Capsicum annuum* L.) is one of important vegetable crops in Indonesia which is cultivated almost throughout the country. The demand of this commodity has significantly increased, but the production is not yet able to meet the requirement. Current average yield of chili pepper is about 8.47 ton ha^{-1}, and this was far lower than its potential yield that may reach 15-20 ton ha^{-1} (Statistics Indonesia, 2019). Therefore, efforts should be done to promote chili pepper production through the improvement of cultivation techniques and expansion of planting area.

The improvement of chili production through expansion of planting area both in dry and rainy season is restricted by uncertain condition of growing environment, resulting in poor plant growth and development.

Chili pepper growing during dry season is constrained by limited soil water availability, causing environmental stress to the plants. Study by Ichwan *et al.* (2017) revealed that chili pepper grown under 50% field capacity showed slow growth, reduced yield, high proline content, and low sugar content.

Efforts to improve the ability of chili plants to survive drought stress and to be able to grow and result in maximum yield can be done by applying effective biostimulants to plants grown under a mixture of soil, trichocompost and rice husk charcoal. The effect of biostimulant in enhancing plant growth and production had been reported by many authors (López-Bucio *et al.*, 2015; Rady and Ur Rehman, 2016; Niyokuri *et al.*, 2018; Drobek *et al.*, 2019). Also, the use of trichocompost or *Trichoderma*-enriched compost in supporting plant growth and development under limited soil water content had been studied with positive results (Bae *et al.*, 2009; Mastouri *et al.*, 2010; Khoshmanzar *et al.*, 2019).

The use of organic materials may increase plant tolerance to drought stress due to their ability to improve soil structure, increase aeration in root zone, reduce mass density, increase cation exchange capacity, and maintain primary nutrients such as N and P, in addition to increasing soil water holding capacity. Trichocompost (*Trichoderma* in compost) is one of organic materials that can be used to improve plant resistance to drought stress. In addition, the application of husk charcoal in growing media is another way to enhance soil organic matter and improve chemical, physical and biological properties of soil. Husk charcoal contains 0.32% N, 15% PO, 31% KO, 0.95% Ca, 180 ppm Fe, 80 ppm Mn, 14.1 ppm Zn and pH 6.8. Another characteristic of husk charcoal is light (specific gravity of 0.2 kg L^{-1}). Rice husk charcoal also increased field capacity and water content by increasing the porosity of the amended soil, and reduced the acidity of soil (Mishra *et al.*, 2017).

Rice husk charcoal treatments on pot grown lettuce (*Lactuca sativa*) and cabbage (*Brassica chinensis*) were found to increase the final biomass, root biomass, plant height and number of leaves in all the cropping cycles in comparison to charcoal free treatments (Carter *et al.*, 2013). Mannan and Shashi (2019) reported that the application of rice husk charcoal increased plant height, days to maturity, total dry weight, cob diameter, cob length, 100-grain weight, and yield of maize under drought conditions. Chlorophyll content was also found to increase, but proline content decreased due to rice husk charcoal application. Meanwhile, Imanda and Ketty (2018) claimed that rice husk charcoal along with cow manure was best composition for the growth of papaya plant. In chili pepper, however, study on the use of mixture of soil, trichocompost and rice husk charcoal, particularly under drought stress, is not yet well documented.

The application of biostimulant along with the improvement of growing media is expected to be synergistically improve the resistance of chili pepper against drought stress while sustaining best growth and yield. The purpose of this study was to obtain a proper combination of biostimulant and growing media compositions that enhance the growth and yield of chili pepper during drought stress.

2. Materials and Methods

Experimental design and plant handling

The study was conducted at the Teaching and Research Farm, Faculty of Agriculture, University of Jambi from April 2019 through to September 2019. A split plot design with three replications was employed in this study. The main plot consisted of different types of commercial mixed biostimulants (Citorin® and Hantu®) and a control, while the subplot consisted of different ratio of soil+trichocompost+rice husk charcoal (2:2:1, 2:1:1, 1:2:1, and 1:1:2). The biostimulant Citorin® contained gibberellic acid, P_2O_5, K_2O, MgO, Mn, antioxidant and vitamins (Amanah and Putra, 2018), and Hantu® contained gibberellic acid, indoleacetic acid, kinetin, zeatin, N, P, Na, Mg, Cu, Fe, Mn, Zn, Co, Cd, and Pb (Lidar and Mutryarny, 2017).

Seeds of chili pepper cv. Lado were sown on media consisted of soil+trichocompost+rice husk charcoal (2:1:1) on a seedbed. Seven days later seedlings were transferred to nursery, and left for 21 days before transplanting on individual pots with different media compositions according to the treatment. Soil water content in the media was set to 75% of field capacity to create drought condition, except those controls. This is in accordance with our previous investigation (Ichwan *et al.*, 2017).

Biostimulants were applied to the plants on weekly basis from week 2nd to week 12th after transplanting by foliar spraying. Fertilization and maintenance of plants were carried out in accordance with the standard of chili pepper cultivation (Zulkarnain, 2013).

Variables observed

Data on plant growth and yield were collected 10 weeks after transplanting (the time of fruit formation) and 14 weeks after transplanting (the time of first harvest). Variables observed were plant height, number of productive branches, total leaf area, dry weight (total and above-ground parts), fruit number, and total weight of fruits per plant.

Chemical analysis

Data on N, P, K^+, Ca^{2+} and Mg^{2+} content in plant tissues were also recorded as well as total sugar, chlorophyll content, and proline content within leaf tissues. Composite leaf samples were made by physically mixing individual leaves taken from 3 sample plants of 3 replicates into one homogenous sample. Compositing reduced the number of analyses to be performed and was designed to provide a representative sample of the treatment. Ten youngest mature leaves on main stem were collected at 10 weeks after transplanting (WAT). Dry and clean leaf samples were placed in a sample bag prior to laboratory analysis.

Total nitrogen content was determined by Kjeldahl method (Labconco Corporation, 1998), phosphorus concentration was determined by vanadium molybdate yellow colorimetric method (Karlberg and Pacey, 1989), whereas K^+, Ca^{2+} and Mg^{2+} were analysed using Atomic Absorption Spectrometry (AAS) method (The Perkin-Elmer Corporation, 1996). Total sugar was determined according to Irigoyen *et al.* (1992), chlorophyll content was determined according to Hall and Rao (1986), and proline content was analysed according to Bates *et al.* (1973).

Statistical analysis

Data were analysed statistically using Analysis of Variance (ANOVA) module (Petersen, 1985) to judge the significance of the effect of biostimulants and growing media compositions. If the result from ANOVA is significant (p<0.05), then the Fisher's Least Significant Different (FLSD) is applied to see the difference between two treatment means. Any difference larger than FLSD is considered a significant result.

3. Results

There was no significant effect of biostimulants on plant height at 10 weeks after transplanting (WAT). However, significant response was shown by plants grown on different media compositions. In the absence of biostimulant, the composition of 2:1:1, 1:2:1 and 1:1:2 were found to enhance plant height. When Citorin® was used as plant biostimulant, the medium composition of 1:1:2 was the best. However, with the use of biostimulant Hantu® none of medium composition showed significant effect on plant height (Table 1).

Table 1 - The effect of biostimulants on the height of chili pepper (cm) grown on different media compositions with limited soil water content (10 WAT)

Biostimulants	Plant height			
	Media compositions [z]			
	2:02:01	2:01:01	1:02:01	1:01:02
No Biostimulant	88.00 b	105.33 a	106.67 a	102.33 a
Citorin®	98.00 b	114.33 ab	99.00 b	118.00 a
Hantu®	102.00 a	108.00 a	95.33 a	104.67 a

[z] Media compositions= soil : trichocompost : rice husk charcoal. Numbers followed by the same lowercase in the rows are not significantly different according to Fisher's Least Significant Different test (α = 0.05).

The interaction between biostimulants and media compositions showed significant effect on the number of productive branches at 10 WAT (Table 2). The application of Citorin® on plant grown on medium composition of 1:1:2 resulted in the highest number of productive branches, followed by those grown on 2:2:1 medium composition, and they differed significantly from those grown on 1:2:1 medium composition. While in the use of Hantu®, medium composition of 2:2:1 was the best and the effect was significantly different from 1:2:1 and 1:1:2 compositions, but not 2:1:1 composition. In the absence of biostimulant, however, there was no significant difference in the number of productive branches on plants grown on different media compositions (Table 2).

Table 2 - The effect of biostimulants on the number of productive branches of chili pepper grown on different media compositions with limited soil water content (10 WAT)

Biostimulants	Number of productive branches			
	Media compositions [z]			
	2:02:01	2:01:01	1:02:01	1:01:02
No Biostimulant	88.00 aA	94.00 aA	90.66 aA	71.33 aB
Citorin®	106.66 aA	96.00 abA	78.66 bA	121.33
Hantu®	112.66 aA	100.00 abA	83.00 bA	79.00 bB

[z] Media compositions= soil : trichocompost : rice husk charcoal. Numbers followed by the same lowercase in the rows are not significantly different according to Fisher's Least Significant Different test (α = 0.05).

At 14 WAT, the number of productive branches was not affected by biostimulant application, but the composition of the media was found to affect this parameter significantly (Table 3). Hantu® was effective in promoting the number of productive branches on plants grown on medium composition of 2:1:1, 1:1:2 and 2:2:1, which were significantly different from those grown on 1:2:1 medium composition. With Citorin® application as well as in the absence of biostimulant, the effect of media compositions did not show any difference on the number of productive branches (Table 3).

Table 3 - The effect of biostimulants on the number of productive branches of chili pepper grown on different media compositions with limited soil water content (14 WAT)

Biostimulants	Number of productive branches			
	Media compositions [z]			
	2:02:01	2:01:01	1:02:01	1:01:02
No Biostimulant	161.00 a	176.67 a	129.00 a	170.67 a
Citorin ®	195.67 a	211.00 a	135.33 a	186.67 a

[z] Media compositions= soil : trichocompost : rice husk charcoal.
Numbers followed by the same lowercase in the rows are not significantly different according to Fisher's Least Significant Different test (α = 0.05).

The interaction between biostimulants and media compositions did not significantly affect total leaf area and total dry weight (Tables 4 and 5). The largest absolute total leaf area was found in the combination of Hantu® and 2:1:1 medium composition (2,644.66 cm^2) (Table 4). In addition, the greatest total dry weight was obtained in the combination of Citorin® and 1:1:2 medium composition (Table 5).

Significant effect of the interaction between biostimulants and media compositions was noted on dry weight of above-ground parts (Table 6), fruit number (Table 7) and fruit weight (Table 8). Data presented in Table 6 show that the dry weight of above-ground parts of plants treated with Citorin® on medium composition of 1:1:2 was significantly higher than those without biostimulant. In the absence of biostimulant, media composition of 2:1:1 and 1:2:1 were better than 2:2:1 or 1:1:2, while in the application of Citorin®, the composition of 1:1:2 and 2:1:1 were better than 2:2:1 or 1:2:1. However, when Hantu® was applied there was significant difference in the dry weight of above-ground parts of chili peppers grown on all media compositions (Table 6).

Table 4 - The effect of biostimulants on total leaf area of chili pepper (cm^2) grown on different media compositions with limited soil water content

Biostimulants	Total leaf area (cm^2)			
	Media compositions			
	2:02:01	2:01:01	1:02:01	1:01:02
No Biostimulant	1497.46	1930.62	1827.07	1784.26
Citorin®	2115.81	2285.40	1531.72	2361.31
Hantu®	1997.24	2644.66	1556.42	2413.54

[z] Media compositions= soil : trichocompost : rice husk charcoal.

Table 5 - The effect of biostimulants on total dry weight of chili pepper (g) grown under drought stress condition on different media compositions with limited soil water content

Biostimulants	Total dry weight			
	Media compositions [z]			
	2:02:01	2:01:01	1:02:01	1:01:02
No Biostimulant	10.11	13.68	13.34	10.41
Citorin®	11.38	14.31	11.51	16.44
Hantu®	13.58	15.38	12.48	13.04

[z] Media compositions= soil : trichocompost : rice husk charcoal.

Table 6 - The effect of biostimulants on dry weight of above-ground parts of chili pepper (g) grown on different media compositions with limited soil water content

Biostimulants	Dry weight			
	Media compositions [z]			
	2:02:01	2:01:01	1:02:01	1:01:02
No Biostimulant	4.73 bA	7.13 aA	6.93 aA	4.83 bB
Citorin®	5.03 bA	7.86 aA	5.93 bA	8.23 aA
Hantu®	6.60 aA	7.86 aA	6.46 aA	6.50 aAB

[z] Media compositions= soil : trichocompost : rice husk charcoal.
Numbers followed by the same lowercase in the rows and the same uppercase in the columns are not significantly different according to Fisher's Least Significant Different test (α = 0.05).

Data on fruit number presented in Table 7 show that plants grown on media with composition of 2:2:1 and treated with biostimulants produce more fruits than those without biostimulants. On 2:1:1 media composition, the application of Hantu® resulted in the greatest number of fruit, which was significantly different from either the application of Citorin® or no biostimulant treatment. On 1:1:2 media composition, however, the effect of Hantu® was significantly different to those plants grown in the absence of biostimulant only. On media composition of 1:2:1, the control treatment produced more fruits than those plants treated with Citorin® (Table 7).

Table 7 - The effect of biostimulants on the number of fruit of chili pepper grown on different media compositions with limited soil water content

Biostimulants	Fruit number			
	Media compositions [z]			
	2:02:01	2:01:01	1:02:01	1:01:02
No Biostimulant	25.00 bB	38.66 aB	41.66 aA	33.00 abB
Citorin®	42.00 aA	45.33 aB	24.66 bB	40.33 aAB
Hantu®	54.00 bA	73.66 aA	29.66 cAB	61.33 abA

[z] Media compositions= soil : trichocompost : rice husk charcoal. Numbers followed by the same lowercase in the rows and the same uppercase in the columns are not significantly different according to Fisher's Least Significant Different test (α = 0.05).

Table 8 - The effect of biostimulants on the weight of fruit of chili pepper (g) grown on different media compositions with limited soil water content

Biostimulants	Fruit weight			
	Media compositions [z]			
	2:02:01	2:01:01	1:02:01	1:01:02
No	54.89 cB	96.89 aA	81.08 abA	67.61 bcA
Citorin®	99.03 abA	107.75 aA	31.37 cB	76.77 bA
Hantu®	67.58 bB	106.95 aA	28.70 cB	71.47 bA

[z] Media compositions= soil : trichocompost : rice husk charcoal. Numbers followed by the same lowercase in the rows and the same uppercase in the columns are not significantly different according to Fisher's Least Significant Different test (α = 0.05).

Data presented in Table 8 show that biostimulant Citorin® significantly increased fruit weight when applied on plants grown on medium with 2:2:1 composition. On medium with 1:2:1 composition, the application of either Citorin® or Hantu® was found to result in less fruit weight significantly. In the absence of biostimulant, the composition of 2:1:1 significantly increased fruit weight than 2:2:1 and 1:1:2 media compositions. Also, in the application of Citorin®, the fruit weight of plants grown on medium with the composition of 2:1:1 was significantly heavier than of those grown on medium composition of 1:2:1. Further, in the application of Hantu® the fruit weight of plants grown on the media composition of 2:1:1 was significantly heavier than the weight fruits produced by plants grown on any other media compositions (Table 8).

The use of biostimulants and growing media containing soil+trichocompost+rice husk charcoal was able to maintain nutrient status within plant tissues to remain at optimal level during limited soil water availability, except for potassium which was at luxury consumption level and calcium which was below critical limit. Nutrient content of chili leaf tissues was measured when the plant was 14 weeks old after transplanting. The results of leaf tissue nutrient measurement is presented in Table 9.

Plants grown on different media compositions and treated with both Citorin® and Hantu® showed a higher total sugar and chlorophyll contents and lower proline level compared to those without biostimulant application (control). In spite of media compositions, plants treated with Citorin® show a higher total sugar and lower proline content in comparison to those

Table 9 - Leaf nutrient contents of chili pepper as affected by biostimulants and different media compositions during limited soil water availability

Biostimulants	Media [z] compositions	Leaf tissue nutrient content (%) [y]				
		N	P	K^+	Ca^{2+}	Mg^{2+}
No biostimulant	2:02:01	2.46	0.31	7.22	0.88	0.56
	2:01:01	1.94	0.37	6.22	1.18	0.73
	1:02:01	2.55	0.32	6.53	1.02	1.05
	1:01:02	3.01	0.26	6.13	1.03	1.02
Citorin®	2:02:01	3.21	0.33	4.37	1.01	0.86
	2:01:01	2.83	0.31	5.99	1.12	0.89
	1:02:01	3.12	1.34	8.24	0.85	0.44
	1:01:02	3.11	0.30	7.03	0.85	0.99
Hantu®	2:02:01	3.44	0.29	7.78	1.05	0.72
	2:01:01	3.65	0.31	5.83	1.08	0.61
	1:02:01	3.14	0.34	7.47	0.65	0.48
	1:01:02	3.02	0.30	7.67	0.86	0.93

[z] Media compositions= soil : trichocompost : rice husk charcoal.
[y] Leaf nutrient content was determined compositely by physically mixing individual leaves taken from each 3 sample plants of 3 repli-

treated with Hantu®. However, plants grown on medium composition of 1:1:2 and treated with Hantu® produced the highest chlorophyll content.

Total sugar, proline and chlorophyll content of leaves were measured when the plants were 14 weeks after planting, and the results are presented in Table 10.

4. Discussion and Conclusions

The application of biostimulants on chili pepper grown on different growing media compositions with limited soil water content was found to increased plant growth and yield. Our results proved that biostimulant application was important for chili pepper grown on media consisting of soil+trichocompost+rice husk charcoal but with limited water supply. Plants treated with biostimulants grew better than those without biostimulant on all growing media. Plants treated with either Citorin® or Hantu® and grown on soil+trichocompost+rice husk charcoal with ratio of 2:1:1 were taller and bigger than those grown on other media compositions without biostimulant application (Fig. 1 and Fig. 2). This increased in pepper growth and yield is presumably due to hormones, organic acids, and macro and micro nutrients contained within the biostimulants. Citorin® contains gibberellic acid (GA_3) along with nutrients such as P, K, Mg, Mn, antioxidants and vitamins (Amanah and Putra, 2018). Meanwhile, Hantu® contains the gibberellic acids (GA_3, GA_5, GA_7), IAA, kinetin and zeatin along with nutrients such as N, P, Na, Mg, Cu, Fe, Mn, Zn, Co, Cd, and Pb (Lidar and Mutryarny, 2017).

Hedden and Thomas (2012) claimed that GA physiologically acted as growth stimulant of plant organs through cell division and elongation. Further, Gupta and Chakrabarty (2013) suggested that gibberellic

Fig. 1 - The effect of biostimulants on the growth of chili pepper during limited water supply (A = no biostimulant; B = Citorin®; C = Hantu®).

Table 10 - Total sugar, proline and chlorophyll contents as affected by biostimulants and different media compositions during limited soil water availability

Biostimulants	Media [z] compositions	Total sugar [y] (mg g^{-1})	Proline [y] (mM g^{-1})	Chlorophyll [y] (cm^2 mL^{-1})		
				a	b	Total
No biostimulant	2:02:01	3.045	0.925	6.096	9.523	15.619
	2:01:01	3.136	0.592	6.688	10.175	16.863
	1:02:01	2.091	1.048	7.386	11.380	18.766
	1:01:02	3.091	0.304	7.527	10.695	18.222
Citorin®	2:02:01	3.136	0.439	7.731	11.723	19.455
	2:01:01	4.841	0.254	8.366	11.325	19.691
	1:02:01	2.614	0.921	5.252	7.109	12.361
	1:01:02	3.909	0.347	7.585	10.568	18.154
Hantu®	2:02:01	4.091	1.209	7.430	9.888	17.318
	2:01:01	3.727	0.803	6.681	9.217	15.899
	1:02:01	3.864	1.141	3.723	5.076	8.799
	1:01:02	2.455	0.566	8.695	12.011	20.706

[z] Media compositions= soil : trichocompost : rice husk charcoal.
[y] Total sugar, proline and chlorophyll contents was determined compositely by physically mixing individual leaves taken from each 3 sample plants of 3 replicates into one homogenous sample at 10 weeks after transplanting.

acids in plants played an important role in triggering the transition from meristem to shoot growth until mature organs. Various recent studies on the use of gibberellic acids indicated that these plant hormones could increase plant growth and development, improved yield, and increased tolerance to abiotic stresses such as drought, heat and salinity (Pal *et al.*, 2016; Sarwar *et al.*, 2017; Miceli *et al.*, 2019; Zhu *et al.*, 2019).

Fig. 2 - The effect of the ratio of soil+trichocompost+rice husk charcoal on the growth of chili pepper during limited water supply (A = 2:1:1; B = 2:2:1; C = 1:2:1; D = 1:1:2).

Growing media is one of important elements supporting plant growth and development. A good media should have good aeration, be able to hold water, and capable to store nutrients for plants. The mixture of soil+trichocompost+rice husk charcoal with the ratio of 2:1:1 or 1:1:2 produced better plant growth compared to others. The greatest plant height, total leaf area and dry weight of aboveground parts were achieved on plants grown on media with the ratio of 2:1:1 on all biostimulant applications, except Citorin® on 1:1:2 medium composition. Meanwhile, the highest number of productive branches (aged 10 and 14 WAT) was obtained on medium composition of 2:1:1 in all biostimulant applications. Based on these results it can be seen that media with more soil or more rice husk charcoal were preferable to produce better growth of chili pepper treated with biostimulants.

Trichocompost is *Trichoderma*-based fertilizer that function to enhance plant's drought tolerance by improving root development (Shukla *et al.*, 2012), activating antioxidant protection to prevent damage caused by dehydration (Brotman *et al.*, 2013), and delaying changes in stomatal opening, photosynthesis and chlorophyll content due to drought (López-Bucio *et al.*, 2015). *Trichoderma* sp. help plants better resist environmental stress such as drought via reinforcing plant growth and reprograming gene expression in roots and shoots. The tolerance to water deficit was attributed to activation of antioxidant responses and higher activity of ascorbate and glutathione-recycling enzymes (Mastouri *et al.*, 2012). The fungal mycelium secreted different compounds that increase the branching capacity of the root system, thus improving nutrient and water acquisition (López-Bucio *et al.*, 2015).

Good growth performance of chili pepper grown on soil+trichocompost+rice husk charcoal and sprayed with biostimulants was followed by good production in term of fruit number and weight. These result was the consequence of a significant interaction of the two factors. Moreover, plants grown in medium with ratio of 2:1:1 and sprayed with Citorin® produced higher total sugar and chlorophyll content and lower in proline compared to those grown on the same medium but in the absence of biostimulant, as well as plants grown on other media ratios but treated with Hantu®.

Total sugar content in chili pepper grown on soil+trichocompost+rice husk charcoal with ratio of 2:1:1 and treated with biostimulants was higher than those grown on other media but in the absence of biostimulant. The results of this study are in line with study conducted by Martim *et al.* (2009) on grapevines which showed that drought stress could increase respiration rate of plants. Increased respiration rate will lower plant carbohydrates and promote total sugar content which also function as an osmotic adjustment.

Chloroplast contains chlorophyll which is a major component involving in photosynthesis. Decrease in chlorophyll content during drought was an indication of oxidative stress caused by photo-oxidative pigment and chlorophyll degradation (Farooq *et al.*, 2009; Anjum *et al.*, 2011). The increase of chlorophyll content in chili peppers grown on different ratios of soil+trichocompost+rice husk charcoal indicates that the plants were able to survive drought stress condition. The application of biostimulants may thus improve plant physio-biochemical attributes under drought stress. This is in accordance with the results noted on *Triticum aestivum* and *Solanum lycopersicum* (Yasmeen *et al.*, 2013) and *Phaseolus vulgaris* (Rady and Mohamed, 2015; Elzaawely *et al.*, 2017). El-Mageed *et al.* (2017) claimed that the improvement of chlorophyll content due to biostimulant application under drought stress may be attributed to the protection impacts on the photosynthetic systems.

Proline is one of dissolved compounds produced

by plants in drought stress condition, which acts as an osmotic adjustment in addition to other compounds such as fructan, trehalose, polyol, polyamine and glycinbetain (Mitra, 2001). As an osmotic adjustment, proline keeps plants to continue to grow even in a low water potential condition. Low proline content in plants grown on media ratio of 2:1:1 and treated with Citorin® indicates that they do not experience stress due to drought. This is in accordance with report by Goñi *et al.* (2018) on tomato grown on limited soil water content and treated with *Ascophyllum nodosum* extract which showed a lower leaf proline content in comparison to untreated plants.

Biostimulants containing bioactive compounds are desirable in today's agriculture because of their capability to enhance nutrient uptake which positively affect overall plant vigor resulting in high quantity and quality of harvest (Parađiković *et al.*, 2017). In our study biostimulant application on chili pepper grown on soil+trichocompost+rice husk charcoal could improve growth, increase nutrient status as well as total sugar and chlorophyll contents, and reduce proline level in leaves. In addition, plant height, number of productive branches, total leaf area, and dry weight of above-ground parts were higher in biostimulant-treated plants. Biostimulant Citorin® might be used to ensure the production of chili pepper by overcoming drought stress and providing good nutrient uptake on medium consists of soil+trichocompost+rice husk charcoal with ratio of 2:1:1.

Further works would be necessary to study the application different concentrations of Citorin® on plants grown on 2:1:1 media composition to find out their effects on the yield.

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Responses of different quality parameters of Chia to arbuscular mycorrhiza and plant growth regulator

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Key words: AMF, hormones, nutritional values, *Salvia hispanica*, seed yield.

Abstract: Field experiment was conducted to evaluate the influence of arbuscular mycorrhiza fungi (AMF) and foliar spray of plant growth regulators (PGRs) and their interaction on vegetative growth, seed yield and yield attributes and some biochemical criteria of chia (*Salvia hispanica* L.), in a split plot design with three replications. Plants grown in absence or presence of AMF were sprayed every 2 weeks with benzyl adenine (BA), CPPU [N-(2-chloro-4-pyridinyl)-N'-phenylurea], common name forchlorfenuron, and Naphthalene acetic acid (NAA) at 50, 20 and 50 ppm respectively, while control plants were sprayed with tap water. The results revealed that, inoculation with AMF generally caused significant augmentation in all studied growth, yield and yield attributes, total chlorophylls and carbohydrates content in leaves, augmentation in nutritional values of seeds like carbohydrates %, macronutrient, micronutrients, proteins %, total flavonoids, oil % compared to non-inoculated plants. In absence or presence of AMF, application of PGRs generally caused significant increases in the studied parameters compared to control. The interaction between NAA and AMF was more effective since gave higher increases in the studied parameters. It can be concluded that, cultivation of chia plant in presence of mycorrhiza with foliar application of NAA at 50 ppm is recommended for enhancing growth, and nutritional values of seed yield.

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1. Introduction

Salvia hispanica, ordinarily known as chia, is an annual herbaceous plant which belongs to Lamiaceae family. It is native to southern Mexico and Northern Guatemala; the word of Chia comes from the Nahuatl word "chian" with means oily. The name *Salvia hispanica* was specified by the Swedish botanist Carl Linnaeus, who discomfited the wild-growing plant coming from the new world with a regional plant from Spain. It grows up to 1-m tall with leaves of about 4-8 cm long and 3-6 cm wide. Chia flowers are white or purple containing oval seed mottle-colored with brown, gray, black, and white with size ranging from 1 to 2 mm. it grows naturally in tropical and subtropical environments; it is optimally established from 400 to 2500 m and considered to be a short-day plant with a threshold of

12-14 h. Chia acquired acceptance owing to the high nutritional value of its seeds. The main components of seeds are polyunsaturated fatty acids Omega-3 (PUFA ω3) (58-64% of total lipids) and Omega-6 (ω6), protein with a ratio (16-24%), carbohydrates (26-41%) lipids (31-35 %), and fiber (34-56 %). in addition to some minerals, vitamins and high level of antioxidants (Baginsky *et al*., 2016; Sosa *et al*., 2016; Marcinek and Krejpcio, 2017).

Arbuscular mycorrhiza fungi (AMF) are a category of soil microorganisms which form a symbiotic association with plants. Not only it could enhance uptake of mineral elements and water by plants that promote plant growth, but also increase crop yield, quality properties and active ingredients (Dupre *et al*., 2008; Singh *et al*., 2010). The positive effect of inoculation with AMF on physiological and biochemical changes in different medicinal and aromatic plants has been reported in a number of species such as enhancing growth performance and seed yield (Gashgaril *et al*., 2020; Bilalis *et al*., 2020), increase photosynthetic pigments and carbohydrates content (Amiri *et al*., 2017), increased nutrient status in plant orangs (Chaudhary *et al*., 2008), protein content (Ouzounidou *et al*., 2015), antioxidants activity (Golubkina *et al*., 2020), fixed oil (Moghith, 2019), promoted concentration of essential oil (Chaudhary *et al*., 2008; Al-Amri *et al*., 2016), enhanced primary and secondary metabolism and bioactive compounds (Gashgaril *et al*., 2020).

Plant growth regulators (PGRs) have been defined as one of the major factors affecting plants growth and their primary and secondary metabolites, NAA is an organic compound that is synthetic plant hormone in the auxin family. it is known to enhance cell elongation, cell division, elongation of shoot, vascular tissue, photosynthesis, RNA synthesis, membrane permeability and water uptake is also involved in many physiological processes such as fruit set, delayed senescence, leaf chlorophyll content, stimulates flowering and increases yield (Davies, 1987). Foliar application of NAA on different medicinal and aromatic plants have been reported to improve growth and yield attributes, photosynthetic pigments, total carbohydrate and oil yield (Rohamare *et al*., 2013), enhance nutrient status in plant orangs, essential oil %, polyphenols and flavonoids content and antioxidant activity (Atteya and El Gendy, 2018). Moreover, it has been reported for ameliorating the harmful effects of salinity (Abou El-ghit, 2015). Cytokinins include benzyl adenine (BA) that promotes cellular elongation and division (Krug *et al*., 2006). Foliar spray of BA has been reported to increase the growth and development of medicinal and aromatic plants (Matter, 2016; Moussa, 2019). It was reported to improve photosynthetic pigments content, total carbohydrate oil percentage and oil yield (Abdel-Rahman and Abdel-Kader, 2020), contents of macronutrient and micronutrient, total phenols and total flavones (Abdel-Hamid, 2020), enhance protein concentration (Prins *et al*., 2013) and antioxidant activity (Sidkey, 2020).

Likewise, CPPU is one of PGRs. It is a cytokinin like substance that has strong cytokinin activity by stimulating fruit set and fruit quality, it plays a role in cell division and cell wall elongation (Nickell, 1985; Zhang and Whiting, 2011). It has been reported to improve growth and yield attributes or protein percentage of medicinal plants (Abbas and Zahwan, 2016).

Although the valuable roles of AMF and PGRs on medicinal and aromatic plants and their useful influence on enhancing growth and production has been formerly evaluated. However, there are not enough available data about their activity on the growth and production of Chia plants. Therefore, the objective of this study was to evaluate the impact of AMF and foliar spray of PGRs (BA, CPPU and NAA) and their interactions on vegetative growth, seed yield and yield attributes, and some chemical compositions of *Salvia hispanica* plants.

2. Materials and Methods

Open field experiment was carried out during the two successive seasons of 2018 and 2019 at Experimental area of the Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza Governorate (latitude 30°01'13.44"N, longitude 31°12'30.24"E, altitude 22 m a.s.l.). The aim of this work was to evaluate the effects of foliar application of different plant growth regulators (PGRs) such as 6-BA (6-Benzylaminopurine) benzyl adenine, CPPU [N-(2-chloro-4-pyridyl)] and NAA (Napthaleneacetic Acid) in absence or presence of arbuscular mycorrhizal fungi (AMF) on vegetative growth, seed yield and its attributes and some biochemical parameters of Chia (*Salvia hispanica* L.) plant.

Experimental procedure

Seeds of *Salvia hispanica* plants were obtained from experimental farm of Faculty of Pharmacy,

Cairo University. On 1st October and 15th October (in the two seasons, respectively), seeds were sown in the nursery at the Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza. After 45 days old from seeds sowing (on the 15th of November, and1st December, in both seasons, respectively), uniform seedlings, with an average plant height of 15-18 cm, were transplanted in the experimental open field with a distance of 70 cm among rows, 50 cm spacing between plants in plots (3.5×3.5 m).

The physical and chemical properties of the experimental soil were determined according to Jackson (1973), and the data are recorded in Table 1.

AMF inoculum contained roots, hyphae, spores and growth media from a pot culture of onion plants colonization with *Glomus mosseae* NRC31 and *Glomus fasciculatum* NRC15, and were obtained from Agricultural Microbiology Department, National Research Center. Inoculum material consisted of 275 spores g^{-1} oven dry bases in addition to the colonization roots pieces (the infectivity 10^4 propagola). AMF inoculation treatments was achieved by mixing 5 g of it with 10 g of chia seed before sowing and repeated monthly after transplanting by injection inoculum material into the soil in roots area from five sides at 5 g/seedling.

Table 1 - Some physical and chemical characteristics of experimental soil during the two seasons

Parameter	2018	2019
Soil texture	Clay	Clay
Clay[%]	40.50	41.60
Silt [%]	35.10	34.00
Fine sand[%]	21.00	20.00
Coarse sand [%]	3.40	4.40
Field capacity [% V]	67.30	69.37
pH	7.12	7.19
EC [dS/m]	1.67	1.55
CEC [meq/100g]	39.40	35.72
Organic matter [%]	1.60	1.75
$CaCO_3$ [%]	1.65	1.75
K^+ [ppm]	65.85	67.95
Mg^{++}[ppm]	39.83	40.94
Available N [ppm]	93.35	98.75
Available p [ppm]	20.25	22.35
Available Fe [ppm]	2.11	2.19
Available Mn [ppm]	3.12	3.99
Available Zn [ppm]	1.59	1.53

pH= soil acidity, EC= Electrical conductivity, CEC= cation exchange capacity, $CaCO_3$= calcium carbonate.

PGRs treatments were initiated after 15 days from the transplanting, by foliar spraying the plants every 2 weeks with Sytonine 4%® [commercial name, consists of 6- BA (6-Benzylaminopurine) benzyl adenine 4 %], Cytovac® (CPPU) and Fast tonic® [commercial name, consists of Napthaleneacetic Acid 25% + Sodium nitrophenolate 0.6%]. The three commercial products were obtained from Bio Green Company, the regional representative of the Jordanian company, Elite Company for the manufacture of agricultural fertilizers development, Egypt. The concentrations of BA and NAA were 50 ppm for each one, while CPPU was applied at 20 ppm and the untreated control plants were sprayed with tap water. Freshly prepared solutions of PGRs (50 mL containing Tween 20 at 1 mL/L (0.1%) as surfactant agent) were sprayed using plastic automizer until run off point. The common horticulture practices (such as irrigation, manual weeds control, fertilization) were carried out when needed.

The experimental design was split plot design with eight treatments [2 AMF (absence or presence) x 4 PGR_S concentrations (including the control)] with 3 replicates, each consisting of 16 plants (2 plants from each treatment). AMF assigned to the main plots in a randomized complete blocks design and PGRs concentrations were allocated at random in sub-plots.

Measurement of vegetative growth and yield parameters

Vegetative growth parameters were recorded after 90 days from transplanting (On 15th of February to 1st March). In both seasons plant samples were taken for measurements growth characters in terms of plant height (cm), number of leaves/plant, stem diameter (cm, at 5 cm above the soil surface), number of branches/plant, fresh and dry weights of the herb (g/plant), root length (cm), number of root/plant and fresh and dry weights of the roots (g/plant). Whereas, at harvesting stage yield and yield attributes including number of inflorescence/ plant, number of seeds/plant, weight of seeds (g /plant), weight of 1000 seeds (g), and seeds yield (Kg/Fed.) were recorded.

Seeds yield per feddan was obtained according to the equation:

[Weight of seeds (g/plant) × number of plants/fed]/1000

Number of plants/fed= (100×100×4200)/ (50×70) = 12000 plant/fed.

Some chemical parameters were determined in the seeds as carbohydrates %, some macro & micro elements, proteins %, phenols, flavonoids content

and antioxidant activity.

Chemical analysis

Total chlorophylls in fresh leaf samples were determined by using chlorophyll meter (Model SPAD 502 Minolta Co. Japan) as described by Netto *et al*. (2005). The total carbohydrates content in leaves and seeds (% 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 completed 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. Dried seeds samples were digested to extract nutrients and the extract was analyzed to determine concentrations of N, P, K, Ca, Mg (% of dry seeds), Fe and Zn (ppm) 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). The concentrations of Ca, Mg, Fe and Zn were determined using atomic absorption spectrophotometer with air-acetylene and fuel (Pye Unicam, model SP-1900, US). Protein content in seeds was estimated by multiplying N values by 5.71 (conversion factors). Total phenolics content was determined by using the Folin Ciocalteau's reagent colorimetric method while total flavonoids content was estimated by the aluminum chloride colorimetric method and results are expressed as milligram Catechin equivalent per gram of seeds dry weight extract (mg CE/g DW) (John *et al*., 2014). The antioxidant activity of seeds extract and standard antioxidant was assessed on the basis of the radical scavenging effect on DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radicals (Brand-Williams *et al*., 1995).

Fixed oil %: the clean air dried seeds of chia 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. Fixed oil % was calculated according to the equation:

Fixed oil % = Extracted fixed oil weight (g)/seeds sample weight (g)/× 100.

Statistical analysis

The means of all obtained data were subjected to statistical analysis of variance (ANOVA) in split plot 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

Vegetative growth parameters

It is evident from data in Table 2 and 3 that under the same treatments of PGRs, chia plants grown in presence of AMF had significant increase in studied vegetative growth parameters (*viz*., plant height, number of leaves, stem diameter, number of branches/plant, fresh and dry weights of herb, root length, number of roots, root fresh and dry weights) compared to those grown in absence of AMF. The obtained increases in vegetative growth attributes due to AMF inoculation are in agreement with reports of several researches on medicinal and aromatic plants including *Artemisia annua* (Chaudhary *et al*., 2008), *Salvia officinalis* (Geneva *et al*., 2010; AbdelKader *et al*., 2014), *Corianderum sativum* (Al-Amri *et al*., 2016; Oliveira *et al*., 2016), *Origanum majorana* (Engel *et al*., 2016), *Pelargonium graveolens* (Amiri *et al*., 2017), *Salvia miltiorrhiza* (Yang *et al*., 2017), *Salvia hispanica* (Moghith, 2019), *Artemisia dracunculus* and *Hyssopus officinalis* (Golubkina *et al*., 2020) and *Foeniculum vulgare* (Mohamed, 2020).

The stimulatory influence of AMF on vegetative growth traits could be explained by AMF form symbiotic relationship with the host increase of root surface, which led to promote root uptake of nutrients. Thus, it could significantly augment growth parameters of tested plant (Jian-heng *et al*., 2016). Furthermore it was indicated that the ability of AMF to enhance the availability of essential elements macro- and micronutrients in the rhizospheric soil that induce its uptake and the accumulation in plant (Gashgaril *et al*., 2020).

Results in Table 2 and 3 also indicate that, in absence or presence of AMF, treating plants with different concentrations of PGRs resulted in significant increase in tested vegetative growth parameters compared to control. Among PGRs, application of NAA (at 50 ppm) appeared to be the most effective one particularly in presence of AMF since recorded the highest values in most cases. The results of the pronounced increase in growth parameters due to application of NAA are in accordance with findings of previous studies on medicinal plants (Alam *et al*., 2012; Rohamare *et al*., 2013; Danesh-Talab *et al*., 2014; Abou El-ghit, 2015; Rostami and Movahedi, 2016; Dheeraj and Saravanan, 2018). Moreover, numerous studies reported increase in growth attributes of medicinal plants owing to either BA (Matter, 2016; Moussa, 2019; Abdel-Rahman and Abdel-Kader, 2020 and Abdel-Hamid, 2020) or CPPU treatments (Abbas and Zahwan, 2016).

The stimulation effect of NAA on morphological

Table 2 - Mean square for the effect of Arbuscular mycorrhiza fungi (AMF), plant growth regulators (PGRs) and their interaction on vegetative growth, yield and yield parameters of *Salvia hispanica*

Traits	Source of variation						
	Treatment			Error		Cv	
	AMF (A)	PGRs (B)	A × B	A	B	A	B
Plant height (cm)	233.750 **	514.534 ***	24.278 ***	1.182	1.037	1.322	1.239
No. of leaves	213.010 **	194.372 ***	5.844 *	2.042	2.222	4.527	4.723
Stem diameter (cm)	0.375 **	0.124 ***	0.011 *	0.004	0.005	7.036	8.386
No. of branches/plant	37.500 ***	36.301 ***	1.701 **	0.035	0.189	1.685	3.921
Herb fresh weight (g)	27.307 ***	18.106 ***	2.681 ***	0.003	0.090	0.433	2.412
Herb dry weight (g)	25.627 ***	21.694 ***	1.288 ***	0.002	0.072	0.609	3.991
Root length (cm)	145.042 ***	96.486 ***	12.042 ***	0.087	0.746	1.979	5.808
No. of roots/plant	32.667 ***	34.375 ***	1.167 *	0.135	0.448	4.576	8.322
Root fresh weight (g)	38.760 ***	20.386 ***	1.412 ***	0.020	0.030	2.531	3.068
Root dry weight (g)	4.770 **	5.794 ***	0.228 **	0.050	0.035	7.233	6.039
No. of inflorescence /plant	145.042 *	70.972 ***	7.903 *	0.510	1.313	4.844	7.767
No. of seeds / plant	18897.29 ***	57551.63 ***	283.104 ***	1.439	5.467	0.219	0.426
Weight of 1000 seeds (g)	1.321 ***	0.371 **	0.001 *	0.145	0.061	20.73	13.45
Weight of seeds (g/plant)	13.024 **	40.227 ***	0.556 *	0.290	0.367	7.02	7.89
Seeds yield (Kg/Fed.)	1875.494 **	5792.741 ***	80.027 *	5.041	21.110	2.44	4.99

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

Table 3 - Vegetative growth parameters of *Salvia hispanica* as affected by the interaction between plant growth regulators (PGRs) in absence (-) or presence (+) of Arbuscular mycorrhiza fungi (AMF) (mean of two seasons)

Treatment		Plant height (cm)	No. of leaves	Stem diameter (cm)	No. of branches/ plant	Herb fresh weight (g)	Herb dry weight (g)	Root length (cm)	No. of roots/ plant	Root fresh weight (g)	Root dry weight (g)
AMF	PGRs										
-	0	67.53±0.44 h	23.67±0.60 d	0.57±0.07 d	7.00±0.29 f	9.77±0.03 f	3.53±0.15 g	8.50±0.060 f	4.00±0.58 e	2.67±0.07 g	1.57±0.09 f
	BA at 50 ppm	78.50±0.50 f	27.17±0.33 c	0.77±0.03 c	9.93±0.35 d	11.83±0.07 cd	5.87±0.12 e	12.33±0.67 d	7.50±0.29 c	4.07±0.03 f	2.30±0.06 e
	CPPU at 20 ppm	81.83±0.67 e	28.00±0.58 c	0.87±0.03 c	11.10± 0.06 c	11.67±0.23 d	6.63±0.28 d	14.00±0.50 c	7.50±0.29 c	4.83±0.15 e	3.27±0.09 d
	NAA at 50 ppm	88.50±1.15 c	35.50±0.29 b	0.83±0.03 c	11.37±0.105 c	12.33±0.19 c	6.67±0.13 d	14.83±0.73 c	8.50±0.50 c	5.93±0.03 d	3.50±0.06 cd
+	0	70.83±0.68 g	26.67±0.60 c	0.80±0.02 c	8.00±0.29 e	10.4 3±0.09 e	4.37±0.07 f	10.67±0.44 e	5.17±0.17 d	3.87±0.15 f	2.03±0.2 e
	BA at 50 ppm	90.67±0.60 b	34.00±0.58 b	1.00±0.06 b	13.30±0.15 b	13.67±0.12 b	8.17±0.15 c	17.17±0.17 b	9.67±0.44 b	6.53±0.13 c	3.60±0.1 c
	CPPU at 20 ppm	86.17±0.33 d	35.00±1.00 b	1.03±0.03 b	13.60±0.20 b	13.80±0.10 b	8.70±0.10 b	17.83±0.17 b	10.33±0.73 b	8.00±0.06 b	3.93±0.12 b
	NAA at 50 ppm	93.67±0.73 a	42.50±0.29 a	1.20±0.06 a	14.50±0.15 a	16.23±0.32 a	9.73±0.09 a	23.67±0.33 a	11.67±0.44 a	9.27±0.12 a	4.63±0.03 a

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.

attributes may be due its ability to increase membrane permeability and water uptake which accompanied by elements absorption, synthesis of chlorophyll and carbohydrates contents that achieving the highest dry weight of plants (Atteya and El Gendy, 2018).

Yield and yield attributes

Data in Table 4 revealed that under the same treatments of PGRs, the values of yield and yield attributes (*viz*., number of inflorescence /plant, number of seeds/plant, weight of seeds, weight of 1000 seeds, and seeds yield) were significantly higher in plants grown in the presence of AMF than corresponding values in the absence of AMF. The present increase in number of inflorescence/plant due to AMF inoculation is supported by the results of previous report (Engel *et al*., 2016). Moreover, recent study (Mohamed, 2020) on *Foeniculum vulgare* revealed that AMF inoculation significantly increased seeds yield parameters.

Exogenous application of PGRs in absence or presence of AMF resulted in significant increase in yield and yield parameters compared to control. Among PGRs, NAA was the most effective one particularly when interacted with presence of AMF. In this concern, application of NAA at 50 ppm has been reported to increase yield attributes (Rohamare *et al*., 2013; Danesh-Talab *et al*., 2014; Khudus *et al*., 2017; Venkatesan and Shakila, 2017). Other study (Kassem *et al*., 2011) found that, foliar spray of NAA at 75 mg/l or CPPU at 10 mg/l caused significant increase in yield and yield attributes of *Ziziphus jujuba* compared with the control. Moreover, increases in number of inflorescence /plant owing to application of NAA are in accordance with findings of earlier studies (Atteya and El Gendy, 2018; Dheeraj and Saravanan, 2018). While increasing yield and its attributes due to either BA application are in harmony with the findings of various studies (Mousa *et al*., 2001; Matter, 2016; Abdel-Rahman and Abdel-Kader, 2020) or CPPU (Abbas and Zahwan, 2016).

The augmentation in yield and its attributes due to PGRs treatments may be due to its role in enhanced absorption of nutrients which promoted photosynthesis rate and translocation of photosynthates and other metabolites to the sinks that leading to increase yield and its attributes in present study (Alam *et al*., 2012).

Total chlorophylls and total carbohydrates

Data in figure 1 indicate that under the same treatments of PGRs, plants grown in the presence of AMF had significantly higher values of total chlorophylls in leaves and total carbohydrates in leaves and seeds compared to corresponding values in the absence of AMF in most cases. These results run parallel with those obtained by earlier reports on medicinal plants that reported increase in chlorophyll and carbohydrates content in plants inoculated with AMF compared to controls (Amiri *et al*., 2017; Gashgaril *et al*., 2020; Moghith, 2019; Mohamed, 2020).

Results also show that, the recorded values were significantly increased in plants grown in absence or presence of AMF as a result of spraying tested PGRs compared to control. In most cases, plants sprayed with NAA in the presence of AMF had the highest values of the tested components. The results of increase

Table 4 - Yield and yield attributes of *Salvia hispanica* as affected by the interaction between plant growth regulators (PGRs) in absence (-) or presence (+) of Arbuscular mycorrhiza fungi (AMF) (mean of two seasons)

Treatments		No. of inflorescence/ plant	No. of seeds/ plant	Weight of seeds (g/plant)	Weight of 1000 seeds (g)	Seeds yield (Kg/Fed.)
AMF	PGRs					
-	0	9.17±0.44 f	409.17±0.58 h	3.43±0.20 f	1.23±0.03 e	41.20±2.45 f
	BA at 50 ppm	12.67±0.88 e	477.17±1.11 f	7.47±0.01 d	1.69±0.09 d	89.64±0.14 d
	CPPU at 20 ppm	12.33±0.33 e	573.67±1.48 d	7.32±0.38 d	1.73±0.15 cd	87.88±4.52 d
	NAA at 50 ppm	15.00±0.58 d	622.17±1.21 c	9.53±0.15 bc	1.76±0.18 bcd	114.40±1.79 b
+	0	11.00±0.58 e	448.50±0.76 g	4.64±0.03 e	1.70±0.21 d	55.68±0.36 e
	BA at 50 ppm	17.17±0.6 c	546.83±1.72 e	8.57±0.13 c	2.16±0.17 abc	102.84±1.54 c
	CPPU at 20 ppm	19.00±0.58 b	638.33±1.15 b	9.71±0.26 b	2.19±0.11 ab	116.48±3.17 b
	NAA at 50 ppm	21.67±0.67 a	673.00±1.15 a	10.74±0.18 a	2.23±0.192 a	128.84±2.22 a

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.

of total chlorophylls or total carbohydrates due to application of NAA are similar to those obtained by previous studies (Alam *et al*., 2012; Rohamare *et al*., 2013; Rostami and Movahedi, 2016; Venkatesan and Shakila, 2017; Atteya and El Gendy, 2018), whereas the obtained increase due to either BA treatments are in close conformity with the findings of previous reports (Matter, 2016; Moussa, 2019; Abdel-Hamid, 2020; Abdel-Rahman and Abdel-Kader, 2020) or CPPU treatments (Kassem *et al*., 2011; Abbas and Zahwan, 2016).

Content of macronutrients in seeds

Results of chemical analysis of dried seeds of *Salvia hispanica* plants (Table 5 and 6) disclosed that, under the same treatments of PGRs the uptake and accumulation of macronutrients in seeds of plants inoculated with of AMF were significantly higher in most cases compared to non-inoculated plants. The only exception to this general trend was observed in the case of P and K% as in the presence of AMF with control treatments they recorded insignificantly higher values compared to corresponding values in the absence of AFM. The obtained results are in agreement with those obtained by various researchers that reported the potential effects of AMF on the accumulation of macronutrients in medicinal plant orangs (Chen and Zhao, 2009; Karagiannidis *et al*., 2011; AbdelKader *et al*., 2014; Vafadar *et al*., 2014; Oliveira *et al*., 2016; Yang *et al*., 2017; Moghith, 2019; Mohamed, 2020).

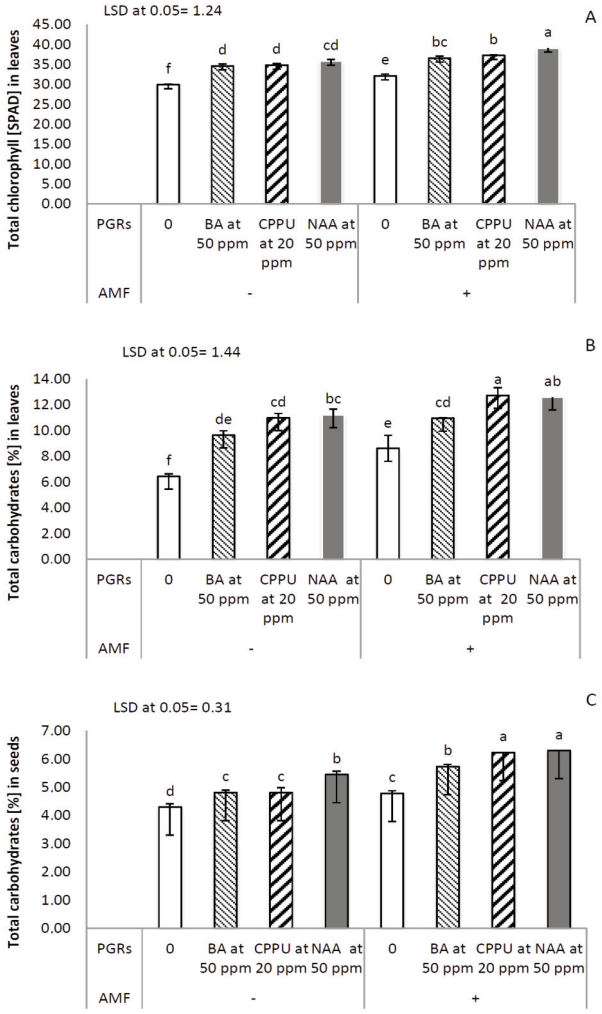

Fig. 1 - Total chlorophyll in leaves (A), total carbohydrates in leaves (B), and total carbohydrates in seeds (C) of Salvia hispanica as affected by the interaction between plant growth regulators (PGRs) in absence (-) or presence (+) of Arbuscular mycorrhiza fungi (AMF) (mean of two season). Column with different letters indicate a significant difference at 5% level. Vertical bars indicate to standard error (SE) of three replicates.

The enhanced mineral absorption by AMF inoculated plants could be elucidated by the efficiency of AMF to boost mineral affinities, reduce the critical concentration of elements absorption, augment the area of uptake and decrease the area of diffusion (Gashgaril *et al*., 2020).

Data in Table 6 also indicate that, in absence or presence of AMF foliar spraying with any concentrations of PGRs resulted in significant increase in macronutrient in seeds in most cases, compared to control. With some exceptions recorded in absence of AMF as foliar application of CPPU resulted in insignificantly higher values of P% than the control, also in absence of AMF, foliar application of three tested PGRs resulted in insignificantly higher values of Ca % compared to control. Among PGRs, application of NAA was the most effective treatment especially in presence of AMF. The results of increasing macronutrients accumulation due to NAA treatments are accordance with findings of previous studies (Alam *et al*., 2012; Atteya and El Gendy, 2018), while the obtained increase in macronutrients due to BA is similar to those described in numerous reports (Matter, 2016; Moussa, 2019; Abdel-Hamid, 2020; Abdel-Rahman and Abdel-Kader, 2020).

Content of micronutrients in seeds

As shown in figure 2 that under the same treatments of PGRs plants inoculated with AMF had significantly higher values of Fe and Zn in their seeds than those grown in the absence of AMF. These results are in the same line of the findings of earlier authors (Chaudhary *et al*., 2008; Golubkina *et al*, 2020).

Data in figure 2 also showed that, in the absence or presence of AMF foliar application of any concen-

trations of PGRs resulted in significant increase in tested micronutrients (Fe and Zn) in seeds compared to control. The increases in the recorded values were more evident in the presence of AMF mostly with application of NAA. Such increase in Fe and Zn content due to BA treatments is in good agreement with those elicited by prior works (Baydar and Erdal, 2004; Abdel-Hamid, 2020).

Total protein

Data in Table 7 displayed that under the same PGRs treatments total protein percentage was significantly higher in plants grown in the presence of AMF than those grown in the absence of AMF. These results are in conformity with that recorded by previous studies (Ouzounidou *et al*., 2015; Al-Amri *et al*., 2016). Increasing protein due to AMF may be due to

Table 5 - Mean square for the effect of Arbuscular mycorrhiza fungi (AMF), plant growth regulators (PGRs) and their interaction on some chemical constituents of *Salvia hispanica*

Traits	Source of variation						
	Treatment			Error		Cv	
	AMF (A)	PGRs (B)	A × B	A	B	A	B
Total chlorophylls in leaves (SPAD)	36.630 **	46.448 ***	0.517 *	0.782	0.428	2.53	1.87
Total carbohydrates in leaves (%)	16.368 **	25.279 ***	0.242 *	0.167	0.878	3.94	9.02
Total carbohydrates in seeds (%)	5.042 ***	1.914 **	0.221 *	0.007	0.037	1.58	3.61
N (%)	0.855 ***	0.824 ***	0.003 *	0.001	0.004	1.21	2.39
P (%)	0.014 **	0.008 ***	0.001 *	0.00	0.00	3.14	4.01
K (%)	0.005 *	0.006 **	0.001 *	0.000	0.001	8.20	11.28
Mg (%)	0.001 NS	0.002 *	0.000 *	0.001	0.001	13.82	9.71
Ca (%)	0.013 *	0.001 *	0.001 *	0.001	0.000	6.49	3.20
Fe (ppm)	62.210 *	32.204 ***	2.316 *	1.148	1.473	1.66	1.88
Zn (ppm)	29.704 *	67.935 ***	0.778 *	2.251	0.445	3.34	1.48
Total protein (%)	27.907 ***	26.881 ***	0.083 *	0.038	0.145	1.22	2.39
Total phenol (µg CE/g)	0.917 *	1.774 **	0.119 *	0.158	0.204	9.36	10.65
Total flavonoid (µg CE/g)	60.770 *	63.393 ***	2.161 *	0.621	0.473	1.83	1.60
Antioxidant (DPPH IC50 (µg/ml)	8.143 **	23.270 ***	1.989 *	0.619	0.843	1.19	1.38
Fixed oil %	93.102 *	139.584 ***	0.509 *	2.833	2.978	6.39	6.55

*, **, *** significant at P≤0.05, P≤0.01, P≤0.001, respectively; NS= Not significant at p=0.05.

Table 6 - Macronutrients in seeds of *Salvia hispanica* as affected by the interaction between plant growth regulators (PGRs) in absence (-) or presence (+) of Arbuscular mycorrhiza fungi (AMF) (mean of two seasons)

Treatment		N (%)	P (%)	K (%)	Mg (%)	Ca (%)
AMF	PGRs					
-	0	2.21±0.03 f	0.33±0.01 d	0.19±0.02 f	0.21±0.01 d	0.40±0.01 c
	BA at 50 ppm	2.40±0.02 e	0.37±0.01 c	0.23±0.02 d	0.25±0.02 b	0.43±0.02 bc
	CPPU at 20 ppm	2.73±0.08 c	0.35±0.00 cd	0.21±0.01 e	0.24±0.00 c	0.43±0.01 bc
	NAA at 50 ppm	3.080±0.01 b	0.41±0.01 b	0.25±0.02 c	0.26±0.03 b	0.42±0.00 bc
+	0	2.60±0.02 d	0.36±0.00 cd	0.20±0.01 f	0.23±0.01 c	0.45±0.01 ab
	BA at 50 ppm	2.77±0.03 c	0.41±0.01 b	0.27±0.02 ab	0.27±0.01 a	0.47±0.01 a
	CPPU at 20 ppm	3.15±0.02 b	0.43±0.00 b	0.25±0.01 c	0.25±0.01 b	0.47±0.01 a
	NAA at 50 ppm	3.40±0.01 a	0.46±0.01 a	0.28±0.02 a	0.27±0.01 a	0.48±0.01 a

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.

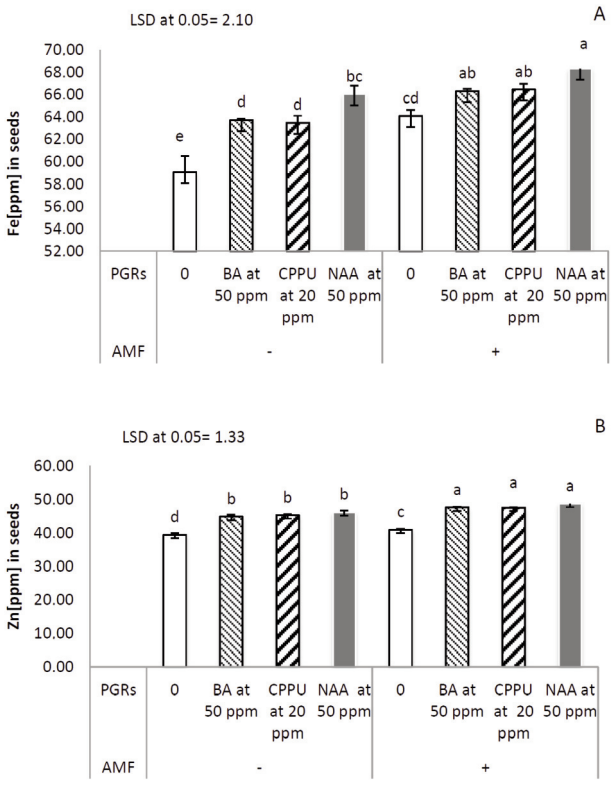

Fig. 2 - Fe (A) and Zn (B) in seeds of *Salvia hispanica* as affected by the interaction between plant growth regulators (PGRs) in absence (-) or presence (+) of Arbuscular mycorrhiza fungi (AMF) (mean of two season), column with different letters indicate a significant difference at 5% level. Vertical bars indicate to standard error (SE) of three replicates.

its role on inducing NH_4^+ and NO_3^- absorption, and assimilation of these molecules into free amino acids that are involved in protein synthesis (Gashgaril *et al*., 2020).

The data in Table 7 also exhibited that in the absence or presence of AMF, spraying of plants with PGRs resulted in significant increase in total protein content compared to control. Among the tested PGRs NAA was superior in its effect predominately in the presence of AMF. The results of increasing protein content due to application of BA are in good agreement with those elicited by Prins *et al*., 2013, while such increase owing to CPPU treatments is coincided with those obtained by Abbas and Zahwan, 2016.

Total phenol content (TPC), Total flavonoid content (TFC) in in seeds

The data presented in Tables 7 showed that under the same treatments of PGRs TPC and TFC in seeds were higher in plants inoculated with AMF compared to non-inoculated plants; however such increase was statically insignificant in the case of TPC compared to corresponding values in absence of AMF. The results of increasing TPC or TFC due to inoculation with AMF are analogy with that recorded by earlier workers (Amiri *et al*., 2017; Gashgaril *et al*., 2020).

Data outlined in Table 7 also indicate that in the absence or presence of AMF TPC and TFC in seeds were significantly higher in seeds of plants foliar sprayed with any concentrations of PGRs compared to control, with superiority of BA for increasing TPC and NAA for TFC especially in the presence of AMF. The present augmentations in TPC or TFC owing to NAA are in harmony with those obtained by previous author (Atteya and El Gendy, 2018), whereas increasing in tested components due to BA are in the same line with the findings of earlier study (Abdel-Hamid,

Table 7 - Total protein, total phenol, total flavonoid and antioxidants in seeds as affected by the interaction between plant growth regulators (PGRs) in absence (-) or presence (+) of Arbuscular mycorrhiza fungi (AMF), (mean of two seasons)

Treatments		Total protein (%)	Total phenol (µg CE/g)	Total flavonoid (µg CE/g)	Antioxidant (DPPH IC50 (µg/ml)
AMF	PGRs				
-	0	12.62±0.18 f	3.18±0.12 c	37.56±0.06 f	63.14±0.60 d
	BA at 50 ppm	13.68±0.13 e	4.15±0.49 ab	40.74±0.13 d	66.18±0.58 c
	CPPU at 20 ppm	15.57±0.45 c	4.55±0.03 a	43.18±0.05 c	67.54±0.02 bc
	NAA at 50 ppm	17.57±0.06 b	4.31±0.04 ab	44.22±0.07 bc	66.34±0.6 c
+	0	14.87±0.10 d	3.68±0.34 bc	39.3±1.15 e	64.18±0.58 d
	BA at 50 ppm	15.82±0.17 c	4.83±0.09 a	45.33±0.13 b	66.25±0.43 c
	CPPU at 20 ppm	17.97±0.12 b	4.56±0.19 a	46.74±0.08 a	68.31±0.17 ab
	NAA at 50 ppm	19.41±0.07 a	4.70±0.32 a	47.06±0.01 a	69.12±0.57 a

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; Sidkey, 2020).

Antioxidant activity in seeds

As shown from data listed in Table 7 that under the same treatments of PGRs antioxidant activity in seeds were higher in plants inoculated with AMF compared to corresponding values of non-inoculated plants, however such increase was insignificant in most cases. The results of increasing antioxidant activity due to mycorrhizal treatments is supported by the results of other authors (Geneva *et al*., 2010; Amiri *et al*., 2017; Golubkina *et al*., 2020; Gashgaril *et al*., 2020).

Data recorded in Table 7 also indicate that in the absence or presence of AMF antioxidant activity was significantly higher in seeds of plants sprayed with PGRs concentrations compared to control, with superiority of NAA especially in the presence of AMF, since recorded the highest values compared to control. The obtained increase in antioxidant activity due to NAA are in harmony with those obtained by previous author (Atteya and El Gendy, 2018), while the augmentation due to BA are in harmony with the finding of recent study (Sidkey, 2020).

Fixed oil percentage

It is obvious from data listed in figure 3 that under the same treatments of PGRs, the values of fixed oil % for plants grown in presence of AMF was significantly higher than corresponding values in the absence of AMF. The results of increasing fixed oil % owing to inoculation with AMF are in sequence with the findings of earlier author (Moghith, 2019).

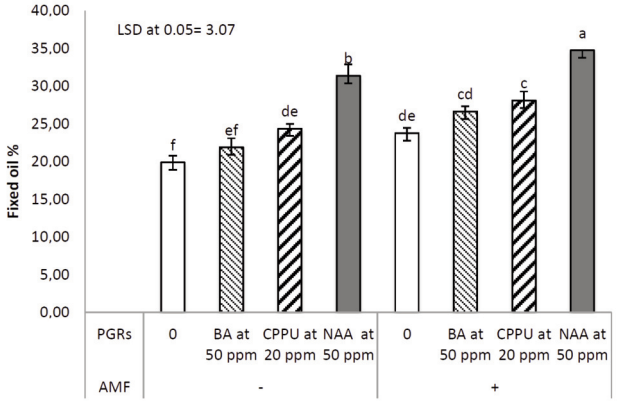

Fig. 3 - Fixed oil % of *Salvia hispanica* as affected by the interaction between plant growth regulators (PGRs) in absence (-) or presence (+) of Arbuscular mycorrhiza fungi (AMF) (mean of two season), column with different letters indicate a significant difference at 5% level. Vertical bars indicate to standard error (SE) of three replicates.

Data in figure 3 also showed that, in the absence or presence of AMF in general application of PGRs resulted in significant increase in fixed oil % compared to control. These increases in the recorded values were more evident in the presence of AMF particularly with application of NAA. Such augmentation in fixed oil due to application of BA is in agreement with those reported by previous study (Mousa *et al*., 2001).

4. Conclusions

Based on the outcome of the present research it could be concluded that, cultivation of chia plants in presence of mycorrhiza with foliar application of NAA at 50 ppm is recommended for enhancing growth, and nutritional values of seed yield.

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Single and combined effects of *Bacillus* spp. and brown seaweed (*Sargassum vulgare*) extracts as bio-stimulants of eggplant (*Solanum melongena* L.) growth

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Key words: Bacillus spp., bio-stimulant, compost tea, eggplant growth, extracts, *Sargassum vulgare*.

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Abstract: *Bacillus subtilis* SV41, *B. amyloliquefaciens* subsp. *plantarum* SV65 and *Sargassum vulgare* extracts were evaluated for their plant growth-promoting potential on eggplant (*Solanum melongena* L.) plants. Bio-treatments applied singly and/or in combination were further compared to a compost tea and to a commercial bio-fertilizer (Acadian™). Results clearly showed that the combined treatments based on the two *Bacillus* spp. strains and the aqueous algal extract and the last one mixed with *B. amyloliquefaciens* subsp. *plantarum* SV65 induced the highest enhancements in the plant height and the maximum root length which were estimated at 32.4-33.9%, 23.9-25.5% and 23.4-25% and at 36.8-41%, 32.9-37.4% and 36.3-40.5% compared to water, compost tea and Acadian™ based treatments, respectively. Furthermore, the combined treatment based on the aqueous algal extract and *B. amyloliquefaciens* subsp. *plantarum* SV65 had significantly improved eggplant growth where the recorded increments in the stem diameter, the aerial part fresh weight, and the root fresh weight varied from 17.5 to 24.6%, 38.4 to 46.1%, and 32.3 to 50% as compared to the three controls, respectively. As for single treatments tested, the aqueous extract had induced a significant improvement in the major growth parameters measured. Developed bio-stimulant was found to be more effective than compost tea and commercial bio-fertilizer based treatments.

1. Introduction

The eggplant (*Solanum melongena* L.) contributes to the diversification of market gardening products and constitutes a new product requested by foreign markets. In Tunisia, the exported quantities over the past five

years were estimated at 187 tons. The export rate remains low compared to 56 thousand tons recorded in 2013/2014 agricultural campaign concentrated in tomato, watermelon, potato, and salad crops. To meet the requirements of consumers and increase the competitiveness of our exports at the international markets level, significant efforts have been made in terms of improving quality and productivity of this crop (GIL, 2020).

The increasing demand for eggplants has gone along with the rapid population growth (Maghfoer *et al*., 2014). Eggplants contain low calories and high nutrient potential (Sowinska and Krygier, 2013). According to Gandhi and Sundari (2012), eggplant is widely used for medicinal features to reduce blood cholesterol and to regulate hypertension. Thus, due to these benefits, the demand of eggplant and its production is expected to increase (Sowinska and Krygier, 2013).

Long term use of inorganic fertilizer has altered soil fertility leading to decreased efficiency of nutrient absorption and productivity and adverse effects on environment and human health (Jagatheeswari, 2013; Waseem *et al*., 2013). Therefore, research efforts are concentrated on alternative nutrients to improve soil physical, chemical, and biological traits through the application of chimerical organic fertilizers (Maghfoer *et al*., 2014) and/or various organic soil amendments such as compost (D'Hose *et al*., 2012), plant extracts (Bijarniya, 2011), algae (Eyras *et al*., 2008), and microbial inoculants (Arora *et al*., 2020). Application of chemical fertilizers with inoculants has been also explored (Carvajal-Muñoz and Carmona-Garcia, 2012). Application of microbial inoculants has gained an increased interest in the last three decades (Babalola and Glick, 2012).

Microbial inoculants are the formulations of beneficial living microorganisms that, when added to the soil, they can improve the availability of nutrients to host plant either directly or indirectly, thereby leading to improved plant growth (Gaind, 2011). Various microorganisms are explored for the production of microbial inoculants such as *Azotobacter*, *Azospirillum*, *Bradyrhizobium*, mycorrhizae, phosphorus solubilizing bacteria, and *Rhizobium*. These bioinoculants can colonize the soil and perform various biophysical and biochemical soil activities that facilitate the availability and the uptake of nutrients to plants (Alori *et al*., 2017). Microbial inoculants could be grouped into nitrogen fixers i.e. *Rhizobium* and *Bradyrhizobium*, phosphate solubilizers i.e. *Pseudomonas*, *Bacillus*, *Aspergillus* etc., cellulose degraders such as *Cytophaga*, and phosphate mobilizers such as mycorrhizae.

Recent demands of organic farming enhanced the application of organic treatments such as seaweed extracts in agriculture. Seaweeds are aquatic plants belonging to the plant kingdom of *Thallophyta* (Arioli *et al*., 2015). At least 59 species of seaweeds can stimulate germination, growth, and yields of some horticultural plants (Sunarpi *et al*., 2010). Seaweed application in the agricultural field has numerous benefits such as stimulation of seed germination, promoting plant growth, improvement of water and nutrient uptake, enhancement of frost and saline resistance, biocontrol and resistance towards phytopathogenic agents, and remediation of pollutants of contaminated soil (Nabti *et al*., 2016). Fresh and dry seaweed or its derived products i.e extracts, composts, and soil conditioners, have been long used in agriculture to enhance plant growth and productivity (Eyras *et al*., 2008). Seaweeds applied, singly or in combination with other macroalgae and/or bacteria, enhance crop productivity. Sridhar and Rengasamy (2010) successfully applied a brown marine alga *S. wightii* combined with a green seaweed *Ulva lactuca* to enhance peanut growth. Additionally, a mixture of two bacteria *Azotobacter chrocoocum* and *Bacillus megaterium* var. *phosphaticum* combined with seaweed extracts increased growth of bitter orange plants (Ismail *et al*., 2011).

In view of previous studies, aqueous and methanolic extracts from a brown macroalgae (*S. vulgare*) were assessed singly and in combination with two endophytic bacteria i.e *B. subtlis* SV14 and *B. amyloliquefaciens* subsp. *plantarum* SV65 for eggplant growth. Both *Bacillus* spp. used in this study showed growth and health bio-stimulating effects on tomato plants through their capacity to produce indole-3-acetic acid, organic acids siderophore and their ability to solubilize phosphate and to biocontrol Fusarium wilt disease in tomato (Aydi Ben Abdallah *et al*., 2017, 2018). Furthermore, Ammar *et al*. (2017) demonstrated *S. vulgare* aqueous and methanolic extracts' ability to efficiently control Fusarium dry rot disease in potato. Phenolic acids and flavonoids are the major components in the methanolic extract of *S. vulgare* using HPLC-DAD analysis (Ammar *et al*., 2017).

The main objective of this study was to evaluate the ability of two *Bacillus* spp. strains applied singly and/or combined with *S. vulgare* aqueous or

methanolic extracts on eggplant growth and productivity.

2. Materials and Methods

Bacillus spp. Culture

B. subtilis SV41 (Accession number KR818071) and *B. amylolequifaciens* subsp. *plantarum* SV65 (Accession number KR818073) isolated from two wild *Solanaceous* species *Datura metel* and *Solanum nigrum*, respectively, were used in this study. Their isolation protocol, characterization and identification analysis were mentionned in Aydi Ben Abdallah *et al*., (2017) and (2018) studies. They were previously selected based on their growth bio-stimulating effect and ability to control tomato Fusarium wilt disease when tested in pot experiment or under field conditions (Aydi Ben Abdallah *et al*., 2017; 2019). The plant growth-promoting traits of both *Bacillus* strains are detailed in Table 1.

Stock cultures of both bacterial strains, were conserved at -20°C in Nutrient Broth (NB) medium amended with 40% glycerol. *Bacillus* spp. colonies of a 1-day-old culture on Nutrient Agar (NA) medium were transferred to Luria-Bertani broth (LB) and incubated at 28 ± 2°C for 48 h and under continuous shaking at 150 rpm. The bacterial strains were tested at the exponential stage of growth (data unpublished). The concentration of *Bacillus* spp. was adjusted at 10^8 cells/ml using spectrophotometre at DO 600 nm.

Table 1 - Plant growth-promoting (PGP) traits of *Bacillus subtilis* SV41 and Bacillus amyloliquefaciens subsp. plantarum SV65 used in the current investigation (Aydi Ben Abdallah *et al*., 2017, 2019)

PGP traits	Bacterial strain	
	B. subtilis SV41	*B. amyloliquefaciens* subsp. *plantarum* SV65
IAA production [z]	+	+
Phosphatase activity [y]	-	+
Organic acids [x]	+	+
Siderophore production [w]	+	+

[z] IAA = Indole-3-acetic acid; +: Production of IAA.
[y] + = Presence of phosphatase activity; -: Absence of phosphatase activity.
[x] = Organic acids; +: Production of oxalic and malic acids.
[w] + = Presence of siderophore activity.

Preparation of aqueous and methanolic extracts from Sargassum vulgare

Brown seaweed was sampled during February 2014 from Monastir, Tunisia (N 35°46'47.754"; E 10°47'9.312"). The alga sampling and processing are detailed in a previous study (Ammar *et al*., 2017). Grounded samples were packed and stored at 4°C until use.

For aqueous extraction, 1 kg of powder sample of *S. vulgare* was soaked in 20 l of sterile distilled water (SDW) and boiled at 100 ± 2°C for 1 h. After cooling, extracts were filtered twice through Whatman N°1 sterile filter paper and further sterilized by filtration through sterile microfilter (0.22 µm pore size). The collected aqueous extracts, prepared at the concentration 50 g/l, were stored at 4°C until further use within a week to avoid any chemical alteration (Ammar *et al*., 2017).

For methanolic extraction, samples of the brown seaweed (1 kg each) were subjected to a series of maceration in methanol (3 l) for three days under ambient room conditions. After filtration, the solvent was evaporated using a rotary evaporator under reduced pressure (at 60°C). One gram of the methanolic dry residue was separately dissolved into 10 ml of methanol. Methanolic extracts used at the concentration 1 g/l were stored at 4°C until further use (Ammar *et al*., 2017).

Eggplant seedling preparation

The cultivar Bonica, the most used by agricultures in the Tunisian Centre-East regions, was used in this study.

Eggplant cv. Bonica seeds were disinfected by immersion into 0.2% sodium hypochlorite for 3 min. They were washed several times with SDW. Disinfected seeds were subsequently treated with bacterial suspensions (~10^8 cells/ml) and/or aqueous and methanolic *S. vulgare* extracts using 20 µl per seed for 1 h. The same volume of SDW was used for treatment of control seeds.

Eggplant treated and untreated seeds were sown in alveolar plastic trays (7×7 cm) filled with sterilized peat™ (Floragard VertriebsGmbH für gartenbau, Oldenburg). Seeds were further treated at trays with 5 ml of bacterial suspensions (~10^8 cells/ml) and/or aqueous and methanolic extracts from the brown seaweed. Control seeds were treated with the same volume of SDW. During all the growing period, trays were watered regularly to avoid drought stress and seedlings were kept under greenhouse conditions (20-30°C with a 16 h light and 8 h dark cycle, and 60-

70% relative humidity) until reaching the two-true leaf growth stage.

Screening of the effects of Bacillus spp. and Sargassum vulgare extracts on eggplant growth
Effect of single bio-treatments

Each *Bacillus* spp. strain (*B. subtilis* SV41 or *B. amyloliquefaciens* subsp. *plantarum* SV65) was singly inoculated to eggplant seedlings by dipping roots for 30 min in a bacterial suspension (10^8 cells/ml) prepared as described above (Aydi Ben Abdallah *et al*., 2017). Control seedlings were dipped in SDW only and LB medium. Treated and control seedlings were transplanted into individual pots (12.5 cm × 14.5 cm) containing sterilized peat. Treated seedlings were re-treated as substrate drenching with 50 ml of each bacterial cell suspension or with 50 ml of *S. vulgare* aqueous and methanolic extracts prepared as described above. Four weeks after transplanting, eggplant seedlings were re-treated with 50 ml of each bacterial suspension and/or tested aqueous and methanolic extracts.

Seven replicates of one seedling each were used for each individual treatment and the whole experiment was conducted twice. Treated and control seedlings were grown for 60 days under greenhouse conditions as described above (Botta *et al*., 2013). After 60 days of growth, the plant height, the stem diameter, the aerial part fresh and dry weights, the maximum root length, the root fresh and dry weights, the flower number, the fruit number, and the fruit fresh and dry weights were noted.

Effect of combined bio-treatments

For combined bio-treatments, equal volumes of cell suspensions of each bacterial strain from 2 d-old LB cultures were mixed and adjusted to 10^8 cells/ml with SDW. Equal volumes of each aqueous and/or methanolic extract from the brown seaweed were mixed with an equal volume of bacterial suspension of *B. subtilis* SV41and *B. amyloliquefaciens* subsp. *plantarum* SV65 adjusted at 10^8 cells/ml or their combination. Seven combined bio-treatments were tested and detailed in Table 2.

Eggplant cv. Bonica seedlings were treated by dipping roots for 30 min in each combined bio-treatment prepared as described above. Control seedlings were dipped in SDW only and LB medium. Treated and control seedlings were potted in commercialized sterile peat. Treated seedlings were re-treated as substrate drenching with 50 ml of each tested combined bio-treatment. Four weeks after transplanting, eggplant seedlings were re-treated with 50 ml of each combined bio-treatment as described above.

Seven replicates of one seedling each were used for each individual treatment and the whole experiment was conducted twice. After 60 days of growth under the same greenhouse conditions, the same growth parameters detailed above were measured.

Comparative efficacy of tested bio-treatments (Bacillus spp. and Sargassum vulgare extracts) and organic amendments

Bacillus spp. strains and *S. vulgare* extracts even applied singly or in combination were compared to a compost tea and to a commercial bio-fertilizer for their growth-promoting potential on eggplant seedlings.

Comparative efficacy of tested bio-treatments and a compost tea

The compost used in this study contained 70% of bovine manure, 25% of sheep manure and 5% of olive-mill solid waste. The characterization of compost and the preparation procedure of compost tea (1:5 w/v) were described in a previous study (Ayed *et al*., 2018). The physico-chemical and microbial characterization of compost are listed in Table 3. The compost used in this study had significantly improved the plant height, the leaf number, the aerial part dry

Table 2 - The seven combined bio-treatments tested

Bio-treatment	Code of bio-treatment
Bacillus subtilis SV41 + *Bacillus amyloliquefaciens* subsp. *plantarum* SV65	B1 + B2
Sargassum vulgare aqueous extract + *B. subtilis* SV41	E Aq + B1
S. vulgare aqueous extract + *B. amyloliquefaciens* subsp. *plantarum* SV65	E Aq + B2
S. vulgare aqueous extract + *B. subtilis* SV41 + *B. amyloliquefaciens* subsp. *plantarum* SV65	E Aq + B1 + B2
S. vulgare methanolic extract + *B. subtilis* SV41	E Meth + B1
S. vulgare methanolic extract + *B. amyloliquefaciens* subsp. *plantarum* SV65	E Meth + B2
S. vulgare methanolic extract + *B. subtilis* SV41 + *B. amyloliquefaciens* subsp. *plantarum* SV65	E Meth + B1 + B2

weight of tomato plants compared to the untreated control plant (Ayed *et al*. 2018).

Eggplant seedlings were treated by dipping roots for 30 min in compost tea (CT). Control seedlings were dipped in SDW only. Treated and control seedlings were transplanted into individual pots (12.5 × 14.5 cm) containing sterilized peat. Treated seedlings were re-treated as substrate drenching with 50 ml of compost tea. Four weeks after transplanting, eggplant seedlings were re-treated with 50 ml of compost tea.

After 60 days of growth under greenhouse conditions, the growth parameters were measured as described above.

Comparative efficacy of tested bio-treatments and a commercial bio-fertilizer

The commercial bio-fertilizer used in this study was the Acadian™ seaweed extract powder used at 2 g/l. The procedure of seedling treatment, the greenhouse conditions and the noted growth parameters were the same as described above.

Statistical analysis

A one-way analysis of variance (ANOVA) was used for data analysis. The software used is the Statistical Package for the Social Sciences (SPSS) for Windows version 16.0. Each Experiment was conducted twice yielding similar results. No significant interactions between treatment and experiment were noted. Therefore, one representative trial of each experiment is reported. Experiments were undertaken according to a completely randomized design. Means were compared using Multiple Range Duncan test at P≤0.05.

Table 3 - The physico-chemical and microbial characterization

Physico-chemical characterization	
Total organic carbon (%)	25
Organic matter (%)	43
Water retention (%)	33
Total porosity (%)	50
Bulk density (g/cm^3)	0.55
Dry matter (%)	70
Electrical conductivity (mS/cm)	3.6
Potential of Hydrogen (PH)	7.3
Ambient temperature (°C)	~ 34
Nutrient content (% of Dry matter)	
Nitrogen (N)	1.82
Phosphorus (P)	0.07
Potassium (K)	1.2
Calcium (Ca)	3.39
Sodium (Na)	0.59
Microbial characterization	
Bacterial count [z] (10^5 CFU/g of compost)	3.92
Fungal count [y] (10^4 CFU/g of compost)	6.6

[z, y] Bacteria and fungal counts from compost during the maturation phase of composting after 72 h of incubation at 35°C onto PCA and PDA, respectively.

3. Results

Growth-promoting potential of tested single bio-treatments

B. subtilis SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 based treatments and the aqueous and methanolic *S. vulgare* extracts were screened singly for their plant growth-promoting (PGP) ability on eggplant plants. As shown in Figs. 1, 2 and 3, the plant growth parameters (plant height, stem diameter, fresh and dry weight of the aerial part, maximum root length, root fresh weight, flower and fruit number, and fruit fresh weight), noted 60 days post-treatment, varied significantly (at P≤0.05) depending on tested bacterial and/or algal extracts.

Plants treated separately with the whole bacterial cells of both *Bacillus* strains and the aqueous extracts from *S. vulgare* were significantly 19 to 29.2% taller than the untreated control plants (Fig. 1a). The treatments based on *B. amyloliquefaciens* subsp. *plantarum* SV65 cells, the aqueous and the methanolic extracts from the brown seaweed led to a significant increase by 14.3 to 20.9% in the stem diameter as compared to water control (Fig. 1b). Treatments with the methanolic and the aqueous algal extracts had stimulated by 33.8 and 43.4% the aerial part fresh over the untreated control (Fig. 1c). Only the aqueous extract had significantly enhanced the aerial part dry weight by 32.3% compared to control (Fig. 1d). It should be highlighted that eggplant aerial part development was similar for LB medium- treated plants and water control ones (Figs. 1a, 1b, 1c, 1d).

As for their effects on the root development, all tested bio-treatments induced a significant (at P ≤ 0.05) increment in the maximum root length and the root fresh weight when compared to control (Figs. 2a, 2b). The maximum root length was significantly increased by 16.6 to 27.7% with both *Bacillus* spp. strains and *S. vulgare* aqueous extract when applied separately as compared to control (Fig. 2a). The root

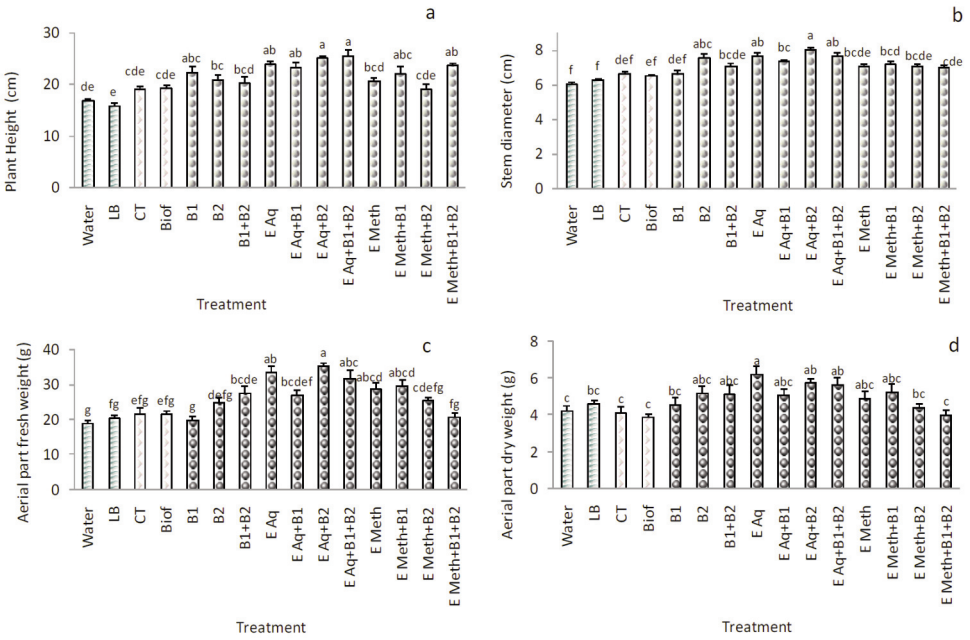

Fig. 1 - Comparative efficacy of single and combined bio-treatments with *Sargassum vulgare* extracts and selected *Bacillus* spp. strains on the aerial part development of eggplant plants compared to the untreated control and to two organic amendments. Water= Plants treated with water; LB= Plants treated with Luria-Bertani broth medium; CT= Plants treated with a compost tea; Biof= Plants treated with a commercial bio-fertilizer (Acadian™); B1= Single treatment with B. subtilis SV41; B2= Single treatment with *B. amyloliquefaciens* subsp. *plantarum* SV65; B1+B2= Combined treatment with B. subtilis SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65; E Aq: Single treatment with aqueous *S. vulgare* extract; E Aq+B1= Combined treatment with *S. vulgare* aqueous extract and *B. subtilis* SV41; E Aq+B2= Combined treatment with aqueous *S. vulgare* extract and *B. amyloliquefaciens* subsp. *plantarum* SV65; E Aq+B1+B2= Combined treatment with aqueous *S. vulgare* extract and both *Bacillus* spp. strains. E Meth= Single treatment with methanolic *S. vulgare* extract. E Meth+B1= Combined treatment with methanolic *S. vulgare* extract and *B. subtilis* SV41; E Meth+B2: Combined treatment with methanolic *S. vulgare* extract and *B. amyloliquefaciens* subsp. *plantarum* SV65; E Meth+B1+B2: Combined treatment with methanolic *S. vulgare* extract and both *Bacillus* spp. strains. Bars sharing the same letters are not significantly different according to Multiple Range Duncan test at 5%. (a) Comparative efficacy of tested bio-treatments on eggplant height; (b) Comparative efficacy of tested bio-treatments on eggplant stem diameter; (c) Comparative efficacy of tested bio-treatments on eggplant aerial part fresh weight; (d) Comparative efficacy of tested bio-treatments on eggplant aerial part dry weight.

fresh weight was enhanced by 29.9 and 38.2% over control following treatments with *B. amyloliquefaciens* subsp. *plantarum* SV65 (B2) and *S. vulgare* methanolic extract, respectively (Fig. 2b). It should be highlighted that eggplant root development parameters were comparable on plants treated with LB medium as well as water control plants (Figs. 2a, 2b, 2c).

Data illustrated in figure 3a indicated a significant increase by 65.5 to 78.7% over the untreated control in the flower number following the individual application of all tested bio-treatments where the highest increment, of about 78.7% over control, was achieved using the algal aqueous extract. The fruit number was 30% higher than control in plants treated with the aqueous extract (Fig. 3b). Eggplant plants treated separately with *S. vulgare* extracts and the whole cell suspensions of *B. subtilis* SV41 showed 25.7-28.7% higher fruit fresh weight relative to control (Fig. 3c). It should be highlighted that plants treated with LB medium behaved similar than water control plants for eggplant flower and fruit production (Figs. 3a, 3b, 3c, 3d).

Growth-promoting potential of tested combined bio-treatments

Seven combinations of the tested bio-treatments were evaluated for their effect on eggplant growth. Analysis of variance revealed a significant (at $P \leq 0.05$) variation in the plant height, the stem diameter, the fresh and dry weights of the aerial part, the maximum root length, the root fresh weight, the flower number, and the fruit fresh weight, depending on tested treatments. As shown in figure 1a, a significant increment in plant height, by 23.7 to 33.9% compared to control, was noted on eggplant plants treated with *S. vulgare* aqueous extract combined with each of *Bacillus* strains (EAq+B1 and EAq+B2),

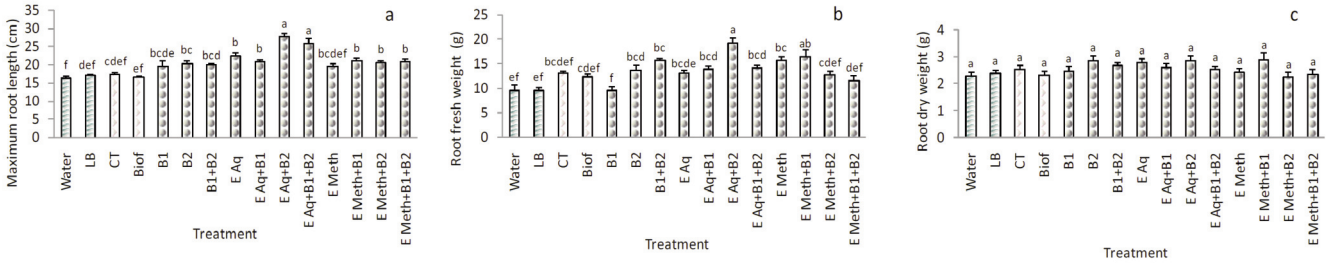

Fig. 2 - Comparative efficacy of single and combined bio-treatments with *Sargassum vulgare* extracts and selected *Bacillus* spp. strains on the root development of eggplant plants compared to the untreated control and to two organic amendments. Water= Plants treated with water; LB= Plants treated with Luria-Bertani broth medium; CT= Plants treated with a compost tea; Biof= Plants treated with a commercial bio-fertilizer (Acadian™); B1= Single treatment with *B. subtilis* SV41; B2= Single treatment with *B. amyloliquefaciens* subsp. *plantarum* SV65; B1+B2= Combined treatment with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65; E Aq= Single treatment with aqueous *S. vulgare* extract; E Aq+B1= Combined treatment with *S. vulgare* aqueous extract and *B. subtilis* SV41; E Aq+B2= Combined treatment with aqueous *S. vulgare* extract and *B. amyloliquefaciens* subsp. *plantarum* SV65; E Aq+B1+B2= Combined treatment with aqueous *S. vulgare* extract and both *Bacillus* spp. strains. E Meth= Single treatment with methanolic *S. vulgare* extract. E Meth+B1= Combined treatment with methanolic *S. vulgare* extract and *B. subtilis* SV41; E Meth+B2= Combined treatment with methanolic *S. vulgare* extract and *B. amyloliquefaciens* subsp. *plantarum* SV65; E Meth+B1+B2= Combined treatment with methanolic *S. vulgare* extract and both *Bacillus* spp. strains. Bars sharing the same letters are not significantly different according to Multiple Range Duncan test at 5%. (a) Comparative efficacy of tested bio-treatments on eggplant maximum root length; (b) Comparative efficacy of tested bio-treatments on eggplant root fresh weight; (c) Comparative efficacy of tested bio-treatments on eggplant root dry weight.

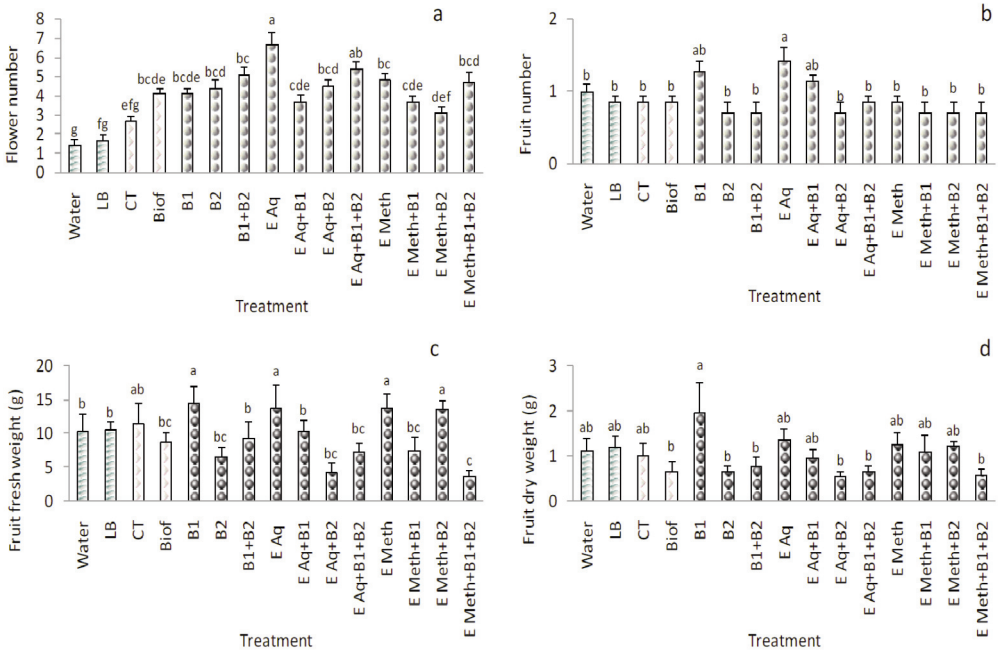

Fig. 3 - Comparative efficacy of single and combined bio-treatments with *Sargassum vulgare* extracts and selected *Bacillus* spp. strains on the eggplant flowers and fruit production compared to the untreated control and to two organic amendments. Water= Plants treated with water; LB= Plants treated with Luria-Bertani broth medium; CT= Plants treated with a compost tea; Biof= Plants treated with a commercial bio-fertilizer (Acadian™); B1= Single treatment with *B. subtilis* SV41; B2= Single treatment with *B. amyloliquefaciens* subsp. *plantarum* SV65; B1+B2= Combined treatment with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65; E Aq= Single treatment with aqueous *S. vulgare* extract; E Aq+B1= Combined treatment with *S. vulgare* aqueous extract and *B. subtilis* SV41; E Aq+B2= Combined treatment with aqueous *S. vulgare* extract and *B. amyloliquefaciens* subsp. *plantarum* SV65; E Aq+B1+B2= Combined treatment with aqueous *S. vulgare* extract and both *Bacillus* spp. strains. E Meth= Single treatment with methanolic *S. vulgare* extract. E Meth+B1= Combined treatment with methanolic *S. vulgare* extract and *B. subtilis* SV41; E Meth+B2= Combined treatment with methanolic *S. vulgare* extract and *B. amyloliquefaciens* subsp. *plantarum* SV65; E Meth+B1+B2= Combined treatment with methanolic *S. vulgare* extract and both *Bacillus* spp. strains. Bars sharing the same letters are not significantly different according to Multiple Range Duncan test at 5%. (a) Comparative efficacy of tested bio-treatments on eggplant flower number; (b) Comparative efficacy of tested bio-treatments on eggplant fruit number; (c) Comparative efficacy of tested bio-treatments on eggplant fruit fresh weight; d: Comparative efficacy of tested bio-treatments on eggplant fruit dry weight.

the aqueous and the methanolic algal extracts combined each one with both bacterial strains (EAq+B1+B2 and EMeth+B1+B2) and the methanolic extract mixed with *B. subtilis* SV41 (EMeth+B1). The highest increase of this parameter, of about 32.4-33.9% over control, was recorded following treatments with *S. vulagre* aqueous extract combined with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2) and the algal aqueous extract mixed with the two *Bacillus* strains (EAq+B1+B2).

A significant enhancement of the stem diameter of the treated plants, estimated at 13.6 to 24.7% over control, was also noted following the seven tested combined bio-treatments. The highest increment (24.7%) was induced by the aqueous extract combined with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2) and at a lesser extent this extract when mixed with both *Bacillus* strains (EAq+B1+B2) (20.9%) (Fig. 1b).

The aerial part fresh weight was also significantly increased by 29.6 to 46.1% over control following treatments with the two bacterial strains (B1+B2), the aqueous extract combined with each bacterial strain separately (namely EAq+B1 and EAq+B2) or in combination (EAq+B1+B2) and the methanolic extract mixed with *B. subtilis* SV41 (EMeth+B1) (Fig. 1c). The highest increment (by 46.1% relative to the control) was induced by the aqueous extract combined with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2). Eggplant plants treated with the algal aqueous extract combined with *B. amyloliquefaciens* subsp. *plantarum* SV65 cells (EAq+B2) or mixed with both *Bacillus* strains (EAq+B1+B2) showed a significant enhancement in their aerial part dry weight by 25.4-26.5% compared to control (Fig. 1d).

As shown in figure 2a, the maximum root length increase over control ranged between and 18.4 to 41% following all tested combined bio-treatments and the highest improvement, of about 36.8-41% over control, was induced by *S. vulgare* aqueous extract mixed with both *Bacillus* strains (EAq+B1+B2) or with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2). All tested bio-treatments combined with the algal aqueous extract induced a significant enhancement in the root fresh weight of about 30.6-50% as compared to control (Fig. 2b). Furthermore, the combined treatment based on *S. vulagre* methanolic extract and *B. subtilis* SV41 (EMeth+B1) had also significantly improved this parameter by 41.4% over control. The highest increment in the root fresh weight, of about 50% relative to control, was noted on plants treated with the combined treatment composed of the aqueous extract and *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2).

When screened for their effects on the flower number, the seven combined bio-treatments led to 54.5-73.7% increment in this parameter compared to control (Fig. 3a). Also, the fruit fresh weight was significantly increased by 24.2% over control on eggplant plants treated with *S. vulgare* methanolic extract mixed with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EMeth+B2) (Fig. 3c).

Comparative efficacy of tested bio-treatments with a compost tea and a commercial bio-fertilizer

Eleven tested bio-treatments, applied singly or in combination, were evaluated for their growth-promoting potential on eggplant seedlings as compared to a compost tea and to a commercial bio-fertilizer (Acadian™).

Aerial part development

Analyses of variance of all growth parameters measured (plant height, stem diameter, aerial part fresh and dry weights) showed a significant variation (at $P \leq 0.05$) between tested bio-treatments as compared to compost tea- and Acadian™ based treatments.

Data showed a significant enhancement by 18.3 to 25.5% over compost tea based treatment in plant height of eggplant plants treated with *S. vulgare* aqueous extract applied either singly or in combination with both *Bacillus* spp. strains and/or singly with each tested bacterial strain (Fig. 1a). Furthermore, plants treated with the methanolic extract combined with *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65 (EMeth+B1+B2) were 19.3% taller than those treated with the compost tea. Similarly, compared with the tested commercial bio-fertilizer, the recorded increment varied from 17.7 to 25% depending on treatments. The highest increase of plant height, of about 23.4-25.5% and 23.3-25% over the commercial bio-fertilizer (i.e. Acadian™ treatment), were induced by the aqueous extract mixed with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2) or with both bacterial strains (EAq+B1+B2), respectively.

All treatments with *S. vulgare* aqueous extract, applied either singly or in combination with each *Bacillus* strains separately or both strains combined, showed significant improvement in eggplant stem diameter by 9.9-17.5% and 11.2-18.7% over compost

tea and the commercial bio-fertilizer treatments, respectively (Fig. 1b). *B. amyloliquefaciens* subsp. *plantarum* SV65 applied singly (B2) induced a significant increment in this parameter by 12.2% versus compost tea-based treatment. *S. vulgare* methanolic extract mixed with *B. subtilis* SV41 (EMeth+B1) led to a significant improvement in the stem diameter by 9.2% when compared to Acadian™ based treatment (Fig. 1b). As compared to both tested organic amendments, eggplant plants treated with *S. vulgare* aqueous extract combined with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2) showed an increment by 17.5-18.7% in this growth parameter.

As shown in Fig. 1c, the aerial part fresh weight was significantly improved by 24.4 to 38.5% over the compost tea treatment in plants treated separately with the aqueous (EAq) and the methanolic (EMeth) *S. vulgare* extracts, the aqueous one combined with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2) or mixed with both *Bacillus* strains (EAq+B1+B2), and the methanolic extract associated with *B. subtilis* SV41 cells (EMeth+B1). Compared to the commercial bio-fertilizer, tested bio-treatments had also significantly improved this parameter by 24.9 to 38.9%. The highest increments in the fresh weight of the aerial part, of about 38.5 and 38.9% compared to compost tea- and Acadian™ based treatments, were induced by the aqueous extract combined with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2), respectively.

The aerial part dry weight was enhanced by 26.7-33.4% and 31.2-37.5% over the compost tea and the Acadian™ controls, using *S. vulgare* aqueous extracts either singly or in combination with *B. amyloliquefaciens* subsp. *plantarum* SV65 and both selected bacterial strains (Fig. 1d). Plants treated singly with the aqueous extract showed the highest increment of this parameter of about 33.4 and 37.5% compared to those amended with compost tea and Acadian™, respectively.

Root development

The maximum root length and the root fresh weight varied significantly (at $P \leq 0.05$) depending on tested bio-treatments. Plants treated with seven bio-treatments (the aqueous and methanolic extracts combined with each bacterial strain and/or with both strains and the aqueous extract applied singly) showed significant improvement in the maximum root length of about 15.9 to 37.4% compared to ones treated with the compost tea (Fig. 2a). As compared to Acadian™ treatment, nine bio-treatments (same as previously, combined *Bacillus* spp. strains and *B. amyloliquefaciens* subsp. *plantarum* SV65 applied singly) induced a significant enhancement by 17.7 to 40.5% in this parameter. The highest increments of the maximum root length, of about 37.4 and 40.5% over the compost tea and the commercial bio-fertilizer controls, were induced by *S. vulgare* aqueous extract combined with *B. amyloliquefaciens* subsp. *plantarum* SV65 (EAq+B2) or with both *Bacillus* strains (EAq+B1+B2), respectively, (Fig. 2a). The root fresh weight was significantly improved by 32.3% compared to compost tea using the aqueous extract mixed with *B. amyloliquefaciens* subsp. *plantarum* SV65 and by 36.1 and 25% versus Acadian™ treatment using the last bio-treatment and the methanolic extract combined with *B. subtilis* SV41, respectively (Fig. 2b). The highest increments on this parameter, by 32.3 and 36.1% compared to compost tea- and Acadian™ based treatments, were induced by the aqueous extract from *S. vulgare* mixed with *B. amyloliquefaciens* subsp. *plantarum* SV65, respectively (Fig. 2b).

Fruit production

ANOVA analyses performed for the flower number, fruit number and fruit fresh weight showed a significant variation (at $P \leq 0.05$) between the eleven bio-treatments tested and compost tea and Acadian™ based treatments.

As compared to compost tea control, the seven tested bio-treatments had significantly improved the flower number by 40.6 to 59.6%. The highest increase (59.6%) was noted on plants treated singly with the aqueous *S. vulgare* extract. Compared to the commercial bio-fertilizer (Acadian™), only the treatment with the algal aqueous extract had significantly enhanced this parameter by 38.3% (Fig. 3a). This aqueous extract when applied singly had also induced a significant improvement of the fruit number by 40% compared to compost tea and Acadian™ based treatments (Fig. 3b). As shown in Fig. 3c, the average fruit fresh weight was significantly enhanced by 15.8 to 20.8% over the compost tea control using separately *B. subtilis* SV41, the aqueous and the methanlic *S. vulgare* extracts and the last one combined with *B. amyloliquefaciens* subsp. *plantarum* SV65. These bio-treatments had significantly improved this production parameter by 35.8 to

39.6% relative to the commercial bio-fertilizer.

4. Discussion and Conclusions

The use of eco-friendly resources has been a major focus of attention in the past three decades. Although reports on the benefits of using microbial inoculants for the promotion of plant growth and health in agricultural soil have been inconsistent, there is a promising trend for microbial inoculants to meet the sustainable agricultural production needs (Alori *et al*., 2017). The use of seaweeds as bio-fertilizers in horticulture and agriculture has increased in the recent years Basmal *et al*. (2019).

This study was aimed to evaluate the efficacy of combining *Bacillus* spp. strains and *S. vulagre* extracts (aqueous and methanolic extracts) in order to select the best combination for the bio-stimulation of eggplant growth. Furthermore, bio-treatments (bacteria and algae extracts) tested singly and/or in combination were compared against two organic amendments i.e compost tea and Acadian™ (a commercial bio-fertilizer) to select the most effective bio-stimulant among the tested treatments.

Bio-treatments (bacteria and/or algae extract) could be applied either singly as seed priming prior sowing, seedlings root dipping prior transplanting, soil drenching and foliar spraying or combination of two or more methods of application (Papenfus *et al*., 2013). In this study, bio-treatments either used singly or in combination were applied as seed priming, then seedlings root dipping and finally as substrate drenching. The recommended method, timing and the rate of applications were greatly different according to plant variety and growth stages (Lola-Luz *et al*., 2013). According to Matysiak *et al*. (2011) study, the stimulatory potential is more efficient at the early stage of plant growth. In this study, all bio-treatments were applied early at pre-sowing, the first application occurred at the two-true leaf stage and the second one four weeks post-planting.

As single application, the aqueous extract from *S. vulgare* used at 50 g/l showed higher growth-promoting potential based on major growth parameters of eggplant than *B. subtilis* SV41, *B. amyloliquefaciens* subsp. *plantarum* SV65 and the methanolic extract compared to the untreated control, and to compost and Acadian™ based treatments. As demonstrated by Michalak and Chojnacka (2015), water extraction was found the most effective for better release of micro- and macro-elements from seaweed biomass even used as fertilizer and bio-stimulant. The application of seaweed extracts exhibit stimulating activities of plant growth, yield and fruit quality in a variety of horticultural crops (Battacharyya *et al*., 2015; Kocira *et al*., 2018; Mahmoud *et al*., 2019). Indeed, the use of water extract from algae as plant growth bio-stimulant was described in several crops such as wheat, tomato, *Arabidopsis*, spinach, and *Vigna sinensis* and this under normal and stressed environments (Nabti *et al*., 2010; Craigie, 2011; Kavipriya *et al*., 2011). In this study, the boiling aqueous extract from *S. vulgare* at 100°C for 1 h did not affect its growth-promoting potential and the contents of polyphenol and flavonoids (Ammar *et al*. 2017). Water extracts prepared by autoclaving or heating previously washed marine alga in distilled water are found to have beneficial growth stimulating effects in cereal and flowering plants (Nabti *et al*., 2010; Craigie, 2011).

The aqueous *S. vulgare* extract applied singly had significantly improved the majority of growth parameters as compared to the untreated control and to the two tested organic amendments. Some seaweeds have been successfully used as soil conditioners and fertilizers in agriculture (Duarte *et al*., 2018). Commercially, extracts from brown algae such as Acadian are good sources of fertilizer (Hurtado *et al*., 2008). Fertilizers derived from seaweeds such as *Fucus*, *Laminaria*, *Ascophyllum*, *Sargassum* etc. are known to be biodegradable, non-polluting and non-hazardous to human and environment (Dhargalkar and Pereira, 2005). Mathur *et al*. (2015) study demonstrated the benefical effects of seaweed liquid fertilizer from *Sargassum wightii*, *Ulva lactuca* and *Enteromorpha intestinalis* on stimulation of seed germination and growth, and enhancement of biochemical traits of *Glycine max* plants. Seaweeds extracts were found to be more active than chemical fertilizers in enhancing seed germination and growth parameters (Godlewska *et al*., 2016). Vasantharaja *et al*. (2019) found that foliar spraying of cowpea plants with the brown seaweed extract at 3% significantly improves the shoot length, the number of leaves per plant, yield, the total phenolic and flavonoid contents and the antioxidant activity as compared to control plants. Foliar spray of liquid fertilizer based on *S wightii* extract has successfully enhanced the chlorophyll content, the internodes and the shoot length of tomato and chilli pepper plants compared to seed soaking (Murugan *et al*., 2020). The mechanisms of

stimulation of plant growth by the marine algal extracts may be due to the diverse compounds observed in their extracts. Indeed, macronutrients, organic substances such as amino acids and plant growth regulators substances are presents in the seaweed liquid fertilizer of *Sargassum* species (Zodape *et al*., 2008; Nabti *et al*., 2016; Murugan *et al*., 2020). Furthermore, seaweed based treatments are able to increase the level of nutrient in soil such as nitrogen, phosphorus and potassium and other compounds as polysaccharides wich are necessary for plant growth that are highly diverse and constitute the major compounds of algae cell walls (Heltan *et al*., 2015; Mirparsa *et al*., 2016; Nabti *et al*., 2016).

To improve the plant growth-promoting ability of both selected *Bacillus* spp. used in the current study, they were combined either single or in combination with the aqueous and/or the methanolic *S. vulgare* extracts. Microbial inoculants, applied singly or in combination, are able to improve nutrient availability and uptake, and to strengthen plant health (Alori *et al*., 2017).

As compared to untreated control and to the two tested organic amendments (compost tea and Acadian™), eggplants treated with combined formulations of *B. amyloliquefaciens* subsp. *plantarum* SV65 and aqueous *S. vulgare* extract showed the highest enhancements in plant height, stem diameter, aerial part fresh weight, maximum root length, and root fresh weight. Furthermore, the combination of *B. subtilis* SV41, *B. amyloliquefaciens* subsp. *plantarum* SV65 and the aqueous extract had significantly increased the plant height, the stem diameter and the maximum root length as compared to water, compost tea and Acadian™ based treatments. When applied on seeds, plant surfaces or soil, microbial inoculants are shown able to enhance root exudation, increase the availability and supply essential nutrients to host plants, and thereby promoting their growth (Trabelsi and Mhamdi, 2013). The phytohormones synthetized by microbial inoculants can result in development of the root system, expansion and elongation of the root hairs and lateral roots, leading to improved uptake of water and nutrients (Halpern *et al*. 2015). Fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus by microbial inoculants are also involved in plant growth promotion (Babalola, 2010). Indirectly, microbial inoculants also affect the status of plants by eliciting the induced systemic resistance (ISR) or the systemic acquired resistance (SAR) thus improving their health. These acts prevent soil-borne pathogens from inhibiting plant growth (Yang *et al*., 2009). The ability to trigger a salicylic acid (SA)-independent pathway controlling systemic resistance is a common trait of ISR-inducing bio-control bacteria. Both *Bacillus* spp. used in this study, have been demonstrated as promising bio-stimulants when challenged to tomato plants and their ability to produce the indole-3-acetic acid, organic acids and/or siderophores, to solubilize phosphate, and to control Fusarium wilt disease was evidenced (Aydi Ben Abdallah *et al*., 2017, 2018).

Plant growth-promoting rhizobacteria (PGPR) applied singly and/or in combination reduced application rates of chemical fertilizers. As demonstrated by Adesemoye *et al*. (2009), a mixture of PGPR strains *B. amyloliquefaciens* IN937a and *Bacillus pumilus* T4, and the arbuscular mycorrhizae (AM) *Glomus intraradices* added to 75% fertilizer successfully enhance growth, yield, and nutrient (nitrogen and phosphorus) uptake of tomato plants compared to the 100% fertilizer control. In the same way, three bio-stimulants consisting of a mix of rhizospheric microorganisms i.e. *Pseudomonas* sp. 19Fv1T, *P. fluorescens* C7 and AM fungi, tested in conditions of reduced fertilization, induced an increment in the yield, the fruit quality and the nutritional value of tomato fruits (Bona *et al*., 2018). El-Yazeid *et al*. (2007) demonstrated that the double inoculation with *Paenibacillus polymyxa* and *Bacillus megaterium* associated with a foliar spray of boron led to an enhancement of growth-promoting hormone levels including gibberellic acid, 3-indole acetic acid and cytokinines associated with a decrease in the abscisic acid inhibitor. Double inoculation especially with the mycorrhizal fungus *G. intraradices* and boron spray improved sex ratio and early production of fruits accompanied with high yield of squash.

Several investigations support different aspects of potential macro algal applications in agriculture. Currently, seaweed extracts are the new type of products used in plant cultivation (Elsharkawy *et al*., 2019). It should be highlighted that the improvement of growth parameters in eggplant plants treated with combined *Bacillus* spp. and aqueous extract from *S. vulgare*, recorded in the current study, is higher than that induced following the single application of aqueous extract. Hence, the combinations of bio-treatments enhance either the efficacy of bacteria and algal aqueous extract more than when applied singly. However, the combinations of the methanolic extract with tested *Bacillus* spp. strains did not induce signifi-

cant increments in the major growth parameters. The synergism occurring between both tested bacterial strains and the aqueous *S. vulgare* extract was confirmed based on various growth parameters. Crocker (2018) investigation clearly demonstrated the *in vitro* ability of seaweed extract to enhance PGPR growth which may explain the synergism noted. Also, Basmal *et al*. (2019) found that the biological fertilizer formulation based on *Sargassum* sp. extract enhance the growth rate of beneficial *Pseudomonas fluorescens*. Through the *in planta* experiments, combined PGPR inocula and seaweed extract enhanced significantly the root growth parameters of treated soybean plants compared to the untreated ones (Crocker, 2018). The addition of bio-fertilizer containing multi-strains of *Bacillus* acting as phosphorus-fixing agents and *Azotobacter*, *Azospirllum* and *Rhizobium* as nitrogen-fixing inoculants combined with a foliar spay with mixed seaweed extracts from *Ulva lactuca*, *Ulva faciata* and *Peterocladia caplicia* at 10 ml/l led to increment of growth characters and to enhancement of the total yield of pea plants (Elsharkawy *et al*., 2019).

As conclusions, the use of plant-growth promoting bacteria especially *Bacillus* strains and the brown seaweed extracts (aqueous and methanolic extracts) as bio-stimulants on eggplant plants was emphasized as compared to untreated ones. The combined treatment based on *Bacillus* spp. strains and the aqueous *S. vulgare* extract was found to be the most efficient bio-stimulant as compared to a compost tea and a commercial bio-fertilizer tested i.e. Acadian™. The beneficial roles of the above combined bio-treatments on growth parameters were higher than their single applications. The influence of the combined bio-stimulant developed based on the two tested *Bacillus* spp. strains and the brown seaweed aqueous extract on the soil microbial community need to be explored in the future to find out ways to more effectively apply this combined bio-treatment and to elucidate its effects on soil microbiome including phytopathogenic and beneficial microorganisms.

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AHS
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Growth response nitrogen metabolism of grafted cucumber fertilized with different ratios of nitrate: ammonium fertilizer

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Abstract: The use of an endemic plant as rootstock has many merits but its application for cucurbits production has not been extensively investigated. The present study determined the growth responses of grafted cucumber using two endemic rootstocks from *Cucurbita pepo* L. fertilized with different ratios of nitrate (NO_3^-): ammonium (NH_4^+) fertilizer. A greenhouse study was carried out using cucumber (*C. sativus* 'Dominos') grafted on two accessions of *Cucurbita pepo* L. collected from Babol and Isfahan with the control being ungrafted *C. sativus* 'Dominos'. Different ratios of NO_3^-/NH_4^+ as follows: 100:0 (NO_3^- alone), 25/75, 50/50, 75/25 and 0/100 (NH_4^+ alone) were applied. It was found that different rootstock has the same physiology but different growth attributes. The growth of the ungrafted cucumber was lower than the grafted ones, and Babol showed better or equal growth compared to the Isfahan rootstock. The NO_3^-/NH_4^+ effect on growth of the cucumber shoot and root fresh and dry weights, root and shoot lengths, nodes, and number of leaves were increased in the 75/25 ratio compared to the other treatments. Grafting on the Isfahan and Babol showed the same effect of N metabolism i.e., grafting increased nitrate reductase activity and NO_3^- concentration in the 75/25 and the 100/0. Protein content and amino acids content of leaves increased in the grafted cucumber treated with 50/50 NO_3^-/NH_4^+. The same response of photosynthesis parameters was observed in the different rootstocks. In conclusion, the result suggested that the grafted 'Dominos' on Babol endemic rootstock at 50/50 NO_3^-/NH_4^+ ratio gave the high growth.

1. Introduction

Grafting is a horticultural technique performed by joining two plants i.e., a scion and a rootstock. Grafting of plants can minimize the detrimental effects of biotic and abiotic stress if the proper rootstock is used (Lee

and Oda, 2003; Rouphael *et al*., 2008; Keshavarzi and Shekafandeh, 2019). Many studies have shown that grafting promotes root and shoot growth, increase plant resistance against diseases, increase plant tolerance to temperature extremities and soil salinity, increase nutrient and water absorption, and plant productivity (Colla *et al*., 2010; Lee *et al*., 2010). Nutrient uptake like nitrogen (N) and phosphorus (P) absorptions improved by grafting of cucumber to fig leaf gourd (*Cucurbita ficifolia* L.) (Pogonyi *et al*., 2005).

Cucumber 'Adrian' was grafted onto three rootstocks of *Lagenaria siceraria*, *Cucurbita maxima* × *C. moschata*, and zucchini. Results showed that grafting improved total yield,leaves number, total soluble solids and titratable acidity.

The effect of grafting is highly dependent on the choice of rootstock (Goreta *et al.*, 2014 ; Dadashpour *et al*., 2017) to improve growth and fruit production. The benefits of grafting on vegetative growth have been reported for cucurbit-type crops (Goreta *et al.*, 2014) but using the endemic cucurbits as a rootstock for this family has not been extensively investigated.

Ammonium, nitrate and urea are the three forms of inorganic nitrogen that increase plant growth (Niu *et al*., 2011). Most plants prefer nitrate to ammonium or a combination of them. The optimal NO_3^-/NH_4^+ ratio depends on many factors such as plant species, plant growth and maturity stages, and environment condition (Marschner, 2012). Yet, little is known about how the NO_3^-/NH_4^+ ratio may affect grafted plant and their nitrogen metabolism.

Higher NO_3^-/NH_4^+ ratio produce higher biomass than other ratios in mesquite (*Prosopis velutina*) (Hahne and Schuch, 2006). Conversely, higher NO_3^- improves growth and flowering in *Phalaenopsis* orchid (Phalaenopsis) (Wang *et al.*, 2008). In some plants, N form has no significant effects on growth. For instance, the dry weight of shoots and roots, and root/shoot ratio were not affected by NO_3^-/NH_4^+ ratio in *Sophora secundiflora* (Niu *et al*., 2011). However, using high NO_3^- in plant nutrition is a risk for the environment and human health. Replacing NO_3^- in plant nutrition with an appropriate amount of NH4+ may alleviate these concerns. Plants usually grow on NO_3^- and NH_4^+ as nitrogen sources, which eventually influence the synthesizing of amino acids and proteins (Xing *et al*., 2015).

The optimum level of N and the best ratio of NO_3^-/NH_4^+ are required by each species according to their respective growth and productivity (Fernandez-Nava *et al*., 2010). On the other hand, the process of up taking NO_3^- or NH_4^+ has a substantial impact on the uptake of other cations and anions and rhizosphere-pH. When roots take up NO_3^- with a negative charge and NH_4^+ with a positive charge, they release a specific charged molecule to keep a balanced pH inside the plant cells. NH_4^+ reduces rhizosphere pH while NO_3^- increases pH (Marschner, 2012). High levels of NH_4^+ can also inhibit the uptake of cations such as calcium and magnesium (Siddiqi *et al*., 2002).

Ammonium application reduces the rate of iron deficiency but increases phosphate and sulfate uptake due to changing substrate pH. In contrast, nitrate reduces the absorption of those essential anions. Thus, most of the time, supplying the proper NO_3^-/NH_4^+ ratio results in the highest growth rates and plant yield by balancing nutrient absorption (Marschner, 2012).

Cucumber has a shallow root system and could not uptake nutrient well (Causin *et al*., 2004). Information on the influence of endemic accession as a rootstock on cucumber nutrient absorption and growth is limited. To better understand the effect of NO_3^-/NH_4^+ ratio for grafted cucumber, the present study was designed. The present study determined plant growth response of grafted cucumber using two endemic rootstocks from *Cucurbita pepo* L. fertilized with different ratios of NO_3^-/NH_4^+ under greenhouse conditions.

2. Materials and Methods

Production of rafte seedlings and experimenta design

The greenhouse experiment was carried out at the Isfahan University of Technology, Isfahan, Iran. *Cucurbita pepo* L. accession as the rootstocks were collected from the Babol (Babol), Isfahan region (Isfahan), and C. *sativus* 'Dominos' is a common cultivar used as scion. The ungrafted Dominos used as control. Total nitrogen in the nutrient solution was equal and comprised NO_3^-/NH_4^+ ratio 100:0, 25:75, 50:50, 75:25, or 0:100. Rootstock and scion seeds were sown in cocopeat and perlite (50/50 v/v). Scion seeds had been cultured 10 days before the rootstock seeds in cocopeat: perlite ratio of 1:1. Scion plants and rootstocks were cut beneath and above the first true leaves, respectively. The hole insertion grafting method as described by Lee (1994) was adopted. Firstly, true leaves and meristem tissue were removed at the growing tip of the rootstock

before making a slit across the growing point from the bottom of one cotyledon to the other side of the hypocotyl. The newly grafted cucumber plant was kept in a grafting chamber with approximately 65% relative humidity and exposed at 16 h fluorescent light at 25-30°C for two weeks. The grafted plants were moved to the greenhouse and maintained at 30-35°C and 60-65% relative humidity for one week to gradually adapt to the greenhouse conditions (Kashi *et al*., 2008). Fertilization with a half-modified Johnson nutrient solution was applied to the grafted plants including (mM): $MgSO_4$ (2), KH_2PO_4 (1), H_3BO_3 (50), $MnCl_2$ (10), $CaCl_2$ (1), $MnSO_4$ (10), $CuSO_4$ (1.5), $ZnSO_4$ (0.8), Na_2MoO_4 (0.4), $Co(NO_3)_2$ (0.1), KNO_3 (10), $FeCl_3$ (0.1), EDTA (0.3) and H_3BO_3 (50.5) mM (Jones, 2005). Ten (10) days after adaptation of the grafted plants, they were treated with the NO_3^-/NH_4^+ ratios on a daily basis. The control treatment was irrigated with a complete Johnson nutrient solution. The plants were kept in the nutrient solution at varying NO_3^-/NH_4^+ ratios with 5 min airing in every 15 min in 2liter container. The EC and pH of the nutrient solution were kept at 2.0±0.2 dS m^{-1} and 6.0±0.3, respectively, by adding HNO_3 and H_3PO_4 into the nutrient solution. Plants were maintained for six more weeks before final harvest.

Data collection

Greenness. Chlorophyll index was measured with portable SPAD (SPAD-502 plus, Minolta, Japan).

Growth trait assay. All the leaf and nod number were counted. The shoots of seedlings were separated from the roots and after recording the fresh weight, they were oven dried at 70°C to constant weight. Root volume was measured by change in water volume in a graduated container (Haghighi *et al*., 2012). Shoot and root length measured by ruler and the stem thickness was measured using a pair of caliper.

Photosynthesis traits assay. Gas exchange parameters including photosynthesis rate, transpiration, stomata conductivity, and intercellular CO_2 of stomata were measured by a portable photosynthesis meter (Li-Cor Li-3000, USA) on a sunny day. Photosynthetically active radiation (PAR) intensity was 1000 µmol $m^{-2}\cdot s^{-1}$ and CO_2 concentration was 350 µmol·mol. The same leaves of each plant was used for chlorophyll measurement using chlorophyll meter (SPAD-502, Minolta Corp., NJ, USA). Mesophyll conductance (mmol CO_2 m^{-2} s^{-1}) was measured according by using the formula: Photosynthetic rate/sub-stomatal CO_2 concentration (Ahmadi and Siosemardeh, 2005).

Phenolic content. The Folin-Ciocalteu method was used for measuring the total phenolic content of the root exudate. The absorbance was measured at 725 nm with a spectrophotometer (UV 160A- Shimadzu Corp., Kyoto, Japan) (Motamedi *et al*., 2019).

Prolin. Plant leaves were homogenized in 3% sulfosalicylic acid at 4°C. Then the solution incubated and centrifuged at 5000 rpm for 20 min. The supernatant was mixed with 2.5% ninhydrin, 60% phosphoric acid (v/v) and 1 ml of glacial acetic acid (100%). The absorbance was measured at 518 nm by spectrophotometer (UV 160A- Shimadzu Corp., Kyoto, Japan) (Bates *et al*., 1973).

Total amino acid. Total amino acids measured by high-performance liquid chromatography (HPLC). Samples were hydrolyzed with 6 M HCl and 10 mg phenol (for protection of tyrosine) at 110 °C for 24 h. HPLC system was equipped with MD-1510 diode-array detector and set to 263 nm (λmax). The samples were injected with a 20 µL loop using a 7125 valve (Rheodyne, Cotati, CA) onto a Purospher RP-18 column and operated at 25°C with a flow rate of 1.0 mL/min using 50 mM acetate buffer (pH 4.2) as eluent A and acetonitrile as eluent B (El-Abagy *et al*., 2014). The level of amino acids present in 100 g of leaves.

Protein. Na-phosphate buffer (pH 7.2) was used to homogenized 1 g fresh leaf samples then centrifuged at 4°C. The absorbance of the supernatants and dye measured using a spectrophotometer (UV 160A-Shimadzu Corp., Kyoto, Japan) at 595 nm. Bovine serum albumin (BSA) used as protein standard (Bradford, 1976).

Nitrate reductase enzyme. The activity of nitrate reductase enzyme was measured according to the method proposed by (Cazetta and Villela, 2004). The amount of 400 mg of leaf samples was placed in a phosphate solution (100 mg, pH=7.5) containing 4% propanol and potassium nitrate, and stored in darkness for an hour at a temperature of 30°C. Then, 1 mL of the solution containing sulfanilic acid was dissolved in 2 ml of chloride acid and 1 mL of naphthylethylene diamide solution (0.02%) and after 20 min, the absorbance was measured at 540 nm wavelength.

Sodium nitrite ($NaNO_3$) was used to prepare the standard solution and the enzyme activity was calculated based on µmol nitrite/gr FW h.

Nitrate concentration. The leaf nitrate content was determined following the procedures described by Atanasova (2008).

Nutrient concentrations. The amount of calcium was measured by an atomic absorption device (model: Perkin Elmer, AA200) (Sharifi *et al*., 2016). zK concentration was determined by atomic absorption after digestion with HCl (Murillo-Amador *et al*., 2007). Nitrogen concentration of leaves measured by Kjeldahl method. Phosphorus was estimated by the vanadomolybdo phosphoric acid colorimetric method at 460 nm (Estan *et al*., 2005).

Ca concentrations. Twelve fruits for each treatment when reached market ripe harvested at the end of the experiment for measurements. Four leaf samples, consisting of young, fully expanded leaves were collected, washed thoroughly with tap water, gave a final rinsing with deionized water, dried at 65°C to constant weight. The extraction of Ca from the plant tissue material was performed using 1 N HCl after dry ashing at 550°C for five h. The amount of calcium was measured by an atomic absorption device (model: Perkin Elmer, AA200) (Sharifi *et al*., 2014).

K, Mg, and P concentrations. The concentrations of K, Mg, and P were measured (Shield Torch System, Agilent 7500a). Meanwhile, phosphorus was estimated by the vanadomolybdo phosphoric acid colorimetric method at 460 nm (Sharifi *et al*., 2014). P was colorimetrically determined using a spectrophotometer (UV 160A- Shimadzu Corp., Kyoto, Japan).

Statistical analysis

The factorial experiment was arranged in a completely randomized design with 10 replications. Data were analyzed with Statestix 8 (Tallahassee FL, USA) and treatment means were separated using the least significant difference (LSD) test at the 5% level of significance when the analysis of variance indicated significant difference between treatments at P≤0.05.

3. Results

The ANOVA in Table 1 showed that all growth and development characteristics were affected by NO_3^- /NH_4^+ ratio and rootstock.

The interaction of NO_3^-/NH_4^+ ratio and rootstock significantly affected all measured variables except rootstock stem thickness and root volume. Except for leaf greenness, all physiological parameters were affected by NO_3^-/NH_4^+ ratio and rootstock and their interaction (Table 2).

Table 1 - Analysis of variance effect of NO_3:NH_4 ratio a rootstock on growth characteristics of cucumber

Source	df	Shoot length	Rootstock stem thickness	Number scion leaves	Number scion nodes	Shoot fresh weight	Root fresh weight	Root volume	Root length	Shoot dry weight
NO_3:NH_4 ratio (N)	4	59.92 **	3.03 **	7.23 **	12.88 **	24.69 **	1.08 **	2.44 **	100.49 **	0.06 **
Rootstock (R)	2	290.70 **	6.51 **	36.87 **	24.01 **	14.03 *	1.16 **	2.94 **	92.88 **	0.26 **
NxR	8	37.40 **	1.67 ns	6.19 **	4.03 *	24.73 **	0.64 **	2.04 ns	44.01 **	0.09 **
Error	30	26.78	0.40	3.44	1.60	3.40	0.05	0.11	5.07	0.01
CV		20.31	14.22	28.98	20.89	31.31	38.97	39.76	18.63	24.10

NS, *, ** not significant, significant at 5% or 1%, respectively.

Table 2 - Analysis of variance effect of NO_3:NH_4 ratio on physiological characteristics of cucumber

Source	df	Greeness	Photo-synthesis	Tran-spiration	Inter-cellular CO_2 concentra-tion of stomata	Stomata conductivity	Meso-philic conductivity	Nitrate reductase activity	Proline	Root phsenol	Protein	Nitrate	Amino acid
NO_3:NH_4 ratio (N)	4	14.34 NS	9.59 NS	3.01 NS	19146.7 NS	0.0041 NS	0.0051 NS	0.006 NS	2.33 NS	1.61 NS	3307 NS	0.0000013 NS	35460 NS
Rootstock (R)	2	3.19 NS	11.17 NS	1.97 NS	34138.4 NS	0.0043 NS	0.0033 NS	0.013 NS	1.77 NS	1.56 NS	29073 NS	0.000075 NS	251790 NS
NxR	8	23.42 **	19.16 **	4.11 **	13750.9 **	0.005 **	0.005 **	0.003 **	2.39 **	2.05 **	59005 **	0.000015 **	236095 **
Error	22	0.92	9.17	9.17	9.17	9.17	9.19	9.17	9.18	9.17	30518	0.000043	72919
CV	38	12.52	4.69	7.41	3.23	0.16	0.15	0.02	0.65	0.08	17.18	11.08	24.17

NS, *, ** not significant or significant at 5% or 1%.

The main effect of nutrient absorbtion was not affected by NO_3^-/NH_4^+ ratio and rootstock, but the interaction showed significant changes (Table 3).

The main result of the N source indicated that the thickest rootstock stem was at 75/25 NO_3^-/NH_4^+ ratio and the most significant root volume was influenced by treatment 50/50 NO_3^-/NH_4^+ ratio. The ungrafted plants had the least root volume and Babol has the most root volume (Table 4).

Shoot length increased when the ratio of NO_3^-/NH_4^+ increased, which led to higher NO_3^- in shoot length compared to the root length (Fig. 1A and B). The shoot length was highest in the 0/100 and then in the 75/25 of NO_3^-/NH_4^+ ratio in all rootstock. It seemed the root had the best growth in the 50/50 NO_3^-/NH_4^+ ratio, and when the NO_3^-/NH_4^+ ratio was increased by more than 75% of total-N, the root length decreased.

Shoot and root fresh and dry weights were not

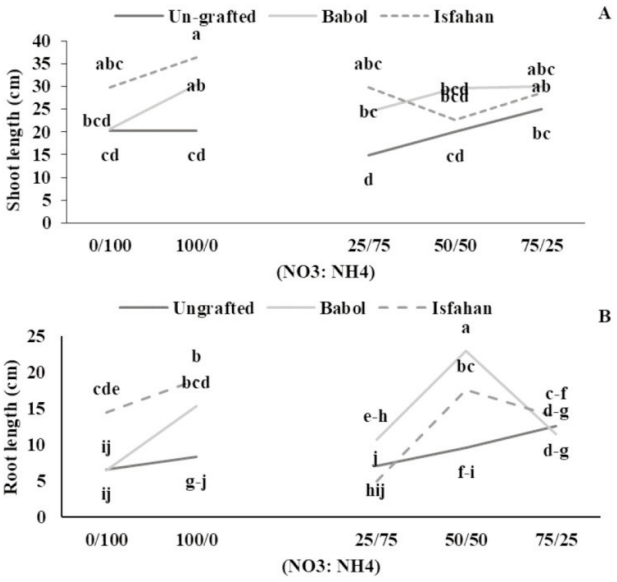

Fig. 1 - The interaction effect of different rootstocks and NO_3:NH_4 ratio on A) shoot length and B) root length.

Table 3 - Analysis of variance effect of NO_3:NH_4 ratio on nutrient concentration of cucumber

Source	df	N concentration	P concentration	K concentration	Ca concentration	Mg concentration
NO_3:NH_4 ratio (N)	4	230. 9 NS	244.56 NS	43.12 NS	78.12 NS	6.12 NS
Rootstock (R)	2	23.45 NS	150.01 NS	35.93 NS	602.45 NS	13.08 NS
N×R	8	26.54 **	111.77 **	4.65 **	81.12 **	4.20 *
Error	22	5.27	67.38	3.23	76.32	1.35
CV	38	25.62	13.04	15.50	23.97	44.38

NS, *, ** not significant, significant at 5% or 1%, respectively.

Table 4 - Effects of NO_3:NH_4 ratio and rootstocks on root volume and cucumber rootstock stem

Treatments	Rootstock stem thickness (cm)	Root volume (mm^3)
NO_3:NH_4 ratio		
100/0	4.51 b	0.35 c
25/75	3.62 c	0.47 bc
50/50	4.50 b	1.85 a
75/25	5.46 a	0.80 b
0/100	4.40 b	0.78 b
Rootstock		
Un-grafted	3.71 b	0.49 b
Babol	4.66 a	1.40 a
Isfahan	5.13 a	0.67 b

NS, *, ** not significant or significant at 5% or 1%.

improved with only NO_3^- or NH_4^+ (Fig. 2A-D). The Babol rootstock caused the best shoot and root growth in the 50/50 NO_3^-/NH_4^+ ratio. Ungrafted plant showed the lower growth in all the treatment N ratios. It seemed that the different NO_3^-/NH_4^+ ratio did not affect the growth in ungrafted plants. Isfahan rootstock increased both shoot and root growth in the 75/25 NO_3^-/NH_4^+ ratio. The highest root and shoot growth were seen in the Babol and the 50/50 NO_3^-/NH_4^+ ratio compared to all the other treatments.

The numbers nodes and leaves increased by increasing the NO_3^- portion of the nutrient solution for the grafted plants, but there were no significant changes in the ungrafted plants in the different NO_3^-/NH_4^+ ratios (Fig. 3A and B). The lowest number of

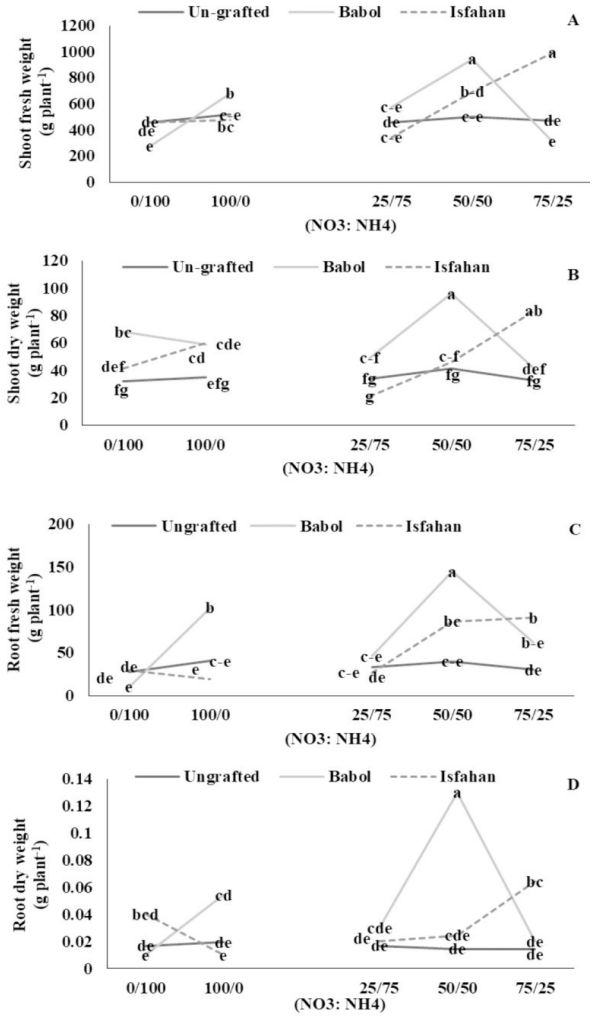

Fig. 2 - The interaction effect of different rootstocks and NO_3:NH_4 ratio on A) shoot fresh weight, B) shoot dry weight, C) root fresh weight and D) root dry weight.

leaves and nodes was seen in the ungrafted plants. These findings were in line with the increase in shoot length, which was higher in treatments with increased NO_3^-/NH_4^+ ratio for grafted plants.

Photosynthesis was more significant in both grafted cucumbers in all the NO_3^-/NH_4^+ ratio compared with the ungrafted plant, and it seemed that it is not related to the changes of the greenness index (Fig. 4A). The greenness index did not change between treatments significantly, except for the high increase in Babol from the50/50 NO_3^-/NH_4^+ ratio. Furthermore, the photosynthesis rate increased in the 50/50 NO_3^-/NH_4^+ ratio in the ungrafted cucumber (Fig. 4B).

Transpiration in all the rootstock was increased by the 25/75 NO_3^-/NH_4^+ and decreased with increasing NO_3^-/NH_4^+ ratio in the nutrient solution. It seemed

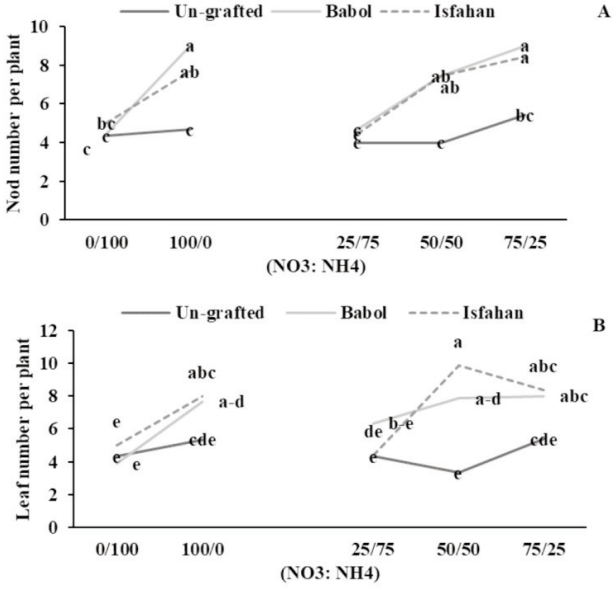

Fig. 3 - The interaction effect of different rootstocks and NO_3:NH_4 ratio on A) number of leaves and B) number of nodes.

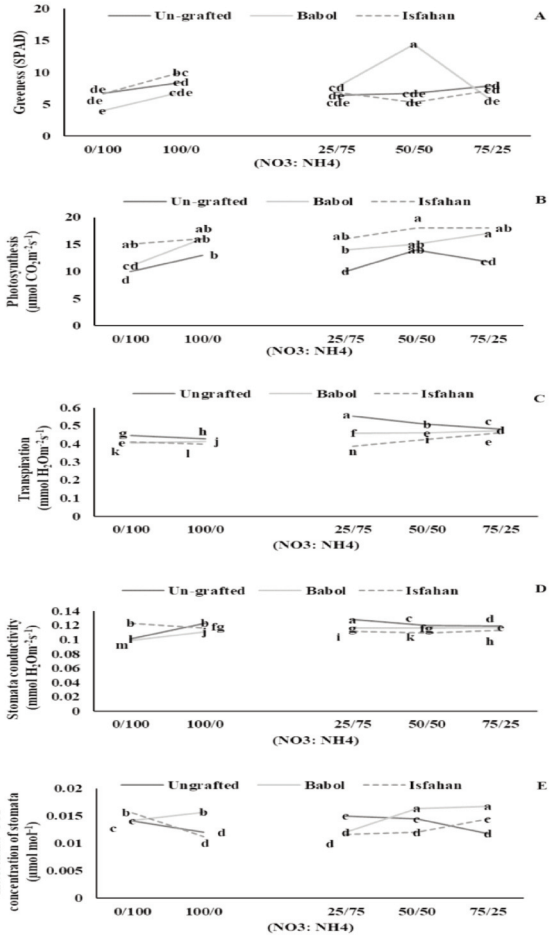

Fig. 4 - The interaction effect of different rootstocks and NO_3:NH_4 ratio on A) Greenness (SPAD), B) photosynthesis, C) transpiration D) stomata conductivity, and E) intercellular CO_2 concentration of stomata.

that by increasing transpiration in all plants stomata conductivity was increased by the25/75 NO_3^-/NH_4^+ treatment (Fig. 4C).

Stomata conductivity was highly raised in the Isfahan rootstock and the ungrafted plants (Fig. 4D). The stomata conductivity was enhanced in the ungrafted cucumber, especially at 25/75 NO_3^-/NH_4^+. Conversely, the CO_2 concentration in the stomata reduced in the 25/75 NO_3^-/NH_4^+ and increased with NO_3^- increment in the nutrient solution (Fig. 4E).

Phenol exudate of the root was highest in 25/75 NO_3^-/NH_4^+ and decreased by increasing the NO_3^- portion (Fig. 5A). It was highest in the ungrafted compared to the grafted cucumber plants. The highest proline content was seen in the 100/0 and the 25/75 NO_3^-/NH_4^+ and decreased by increasing the NO_3^- concentration (Fig. 5B).

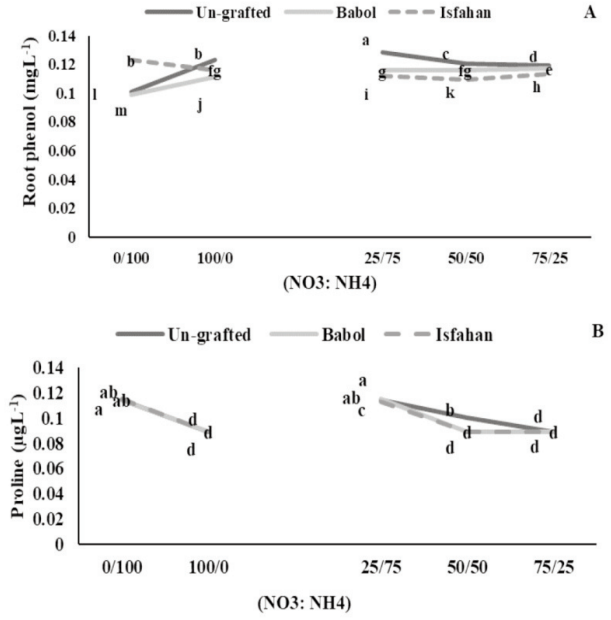

Fig. 5 - The interaction effect of different rootstocks and NO_3:NH_4 ratio on A) proline and B) root phenol.

Nitrate reductase activity was highest in the 25/75 NO_3^-/NH_4^+ and was reduced by increasing the NO_3^- portion. The lowest NO_3^- concentration was recorded by the 0/100 and the 25/75 NO_3^-/NH_4^+. Nitrate concentration was higher in the 0/100, and 100/0 NO_3/NH_4 and was the same in between grafting plants in 25/75 and 50/50 NO_3/NH_4 (Fig. 6B). The amino acid was higher in grafted cucumber, especially in 5/75 and 50/50 NO_3/NH_4. Protein content was higher in 50/50 NO_3/NH_4 in all rootstock and ungrafted (Fig. 6A).

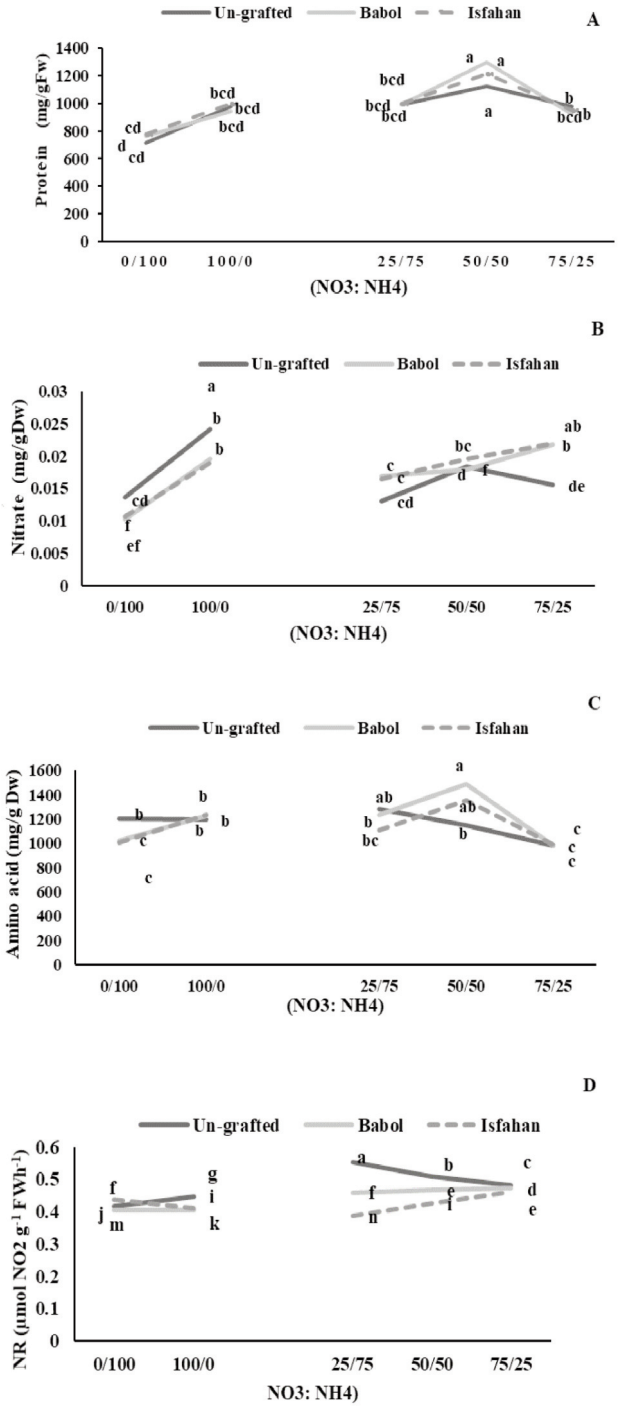

Fig. 6 - The interaction effects of different rootstocks and NO_3:NH_4 ratio on A) protein, B) nitrate and C) amino acid D) nitrate reductase (NR) activity.

It seemed that the most nutrient absorption was recorded by the Babol and the Isfahan rootstock, especially in the 100/0, 75/25 followed by the 50/50 NO_3^-/NH_4+ for N and K (Fig. 7). Conversely, the highest Ca absorption was recorded by the 50/50 NO_3^-/NH_4^+ and to lesser extent by the 75/25 NO_3^-/NH_4^+.

The Mg and P absorption were less absorbed, but highest in the Babol rootstock.

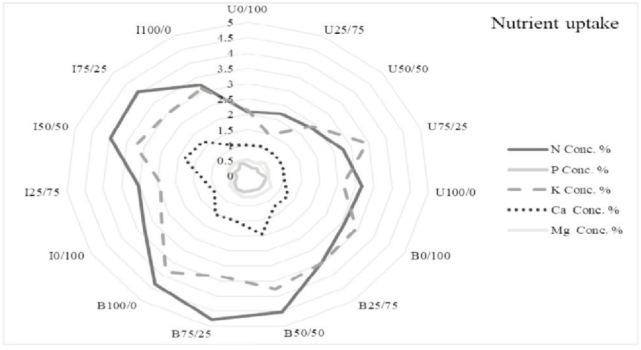

Fig. 7 - The effects of different rootstocks on nutrients (N, P, K, Ca and Mg) absorption in different NO_3:NH_4 ratio.

4. Discussions and Conclusions

The effect of NO_3^-/NH_4^+ *and grafting on the cucumber growth*

A wide range of morphological and physiological characteristics are influenced by scions, rootstocks and their interactions (He *et al*., 2009). In this study, it was observed that growth in terms of shoot and root fresh and dry weights, root and shoot lengths, numbers of node and leaves were increased by the 75/25 treatment. However, some parameters were not significantly affected. All the growth parameters were improved by the 75/25 treatment in Isfahan rootstock. On the other hand, the best result in plant growth improvement was seen in the 50/50 treatment for Babol. These results revealed that different rootstock act differently in different NO_3^-/NH_4^+ ratio to promote growth. In all the growth parameters, the ungrafted cucumber was lower than the grafted ones and Babol showed a better or similar increase than Isfahan rootstock. When using NO_3^- or NH_4^+ alone, plant growth was not significantly different except for the numbers of node and leaves and root length in the grafted cucumber following application of NH_4^+ alone. It noted that the use of both sources of N individually was not economical. Less plant growth was seen in the ungrafted cucumber by using NO_3^- or NH_4^+ alone compared to the grafted cucumber.

Different rootstocks have different root size and different absorbance abilities which can affect vegetative growth rates. Rootstocks improve photosynthetic ability and increase yield in grafted plants (Massai *et al*., 2004). Rootstocks improve growth in plant by improving nutrient uptake, hormonal status and root growth (Lee *et al*., 2010). Grafting, especially Babol rootstocks, contributed to better vegetative growth of 'Dominos' due to higher root distribution, perhaps resulting in more nutrient uptake. It should be considered that increasing number of nodes is a sign for more yield because the flower initiate in nodes so the more node means more flowers and fruits as obtained with grafted cucumber.

The effect of NO_3^-/NH_4^+ *and grafting on the metabolism of cucumber*

Results revealed that grafting on the Isfahan and Babol showed the same effect on N metabolism, i.e., NR activity decreased and NO_3^- concentration increased in plants treated with the 75/25 and the 100/0 NO_3^-/NH_4^+.Protein and amino acids contents of leaves was increased at the 50/50 treatment in grafted cucumber and protein showed the same trend like amino acid. It can be concluded that the healthiest cucumber plant was obtained from the 50/50 treatment in the grafted cucumber although the most nitrate metabolism was achieved by treatment 75/25 NO_3^-/NH_4^+. Despite the effect of rootstock on growth, it was seen that the rootstock has no difference in the metabolism N in the cucumber plants. The other reason for the promotion of plant growth by changing the NO_3^-/NH_4^+ ratio could be that after NR reduced NO_3^- to nitrite, it was changed to ammonium, and amino acids were produced, which can later combine to produce proteins (Haghighi *et al*., 2012). On the other hand, nitrate through producing active forms of cytokinins, as an osmolyte in vacuoles, stimulates leaf function and growth, causing cell extension and improved growth (Wang *et al*., 2008). Increasing root length and cytokinin production helped the plant to absorb more water and nutrients to improve vegetative growth (Haghighi *et al*., 2016 a).

The effect of NO_3^-/NH_4^+ *and grafting on the stress photosynthesis traits of cucumber*

Photosynthesis was not affected by N ratio but increased by grafting. Stomata conductance, internal CO_2 of stomata and transpiration increased with increasing NO_3^-/NH_4^+ ratio and was reduced by grafted cucumber. It seemed changes in photosynthesis traits were more related to stomata status, which can be associated with the rootstock.

Photosynthesis was improved with grafting due to an efficient root system of the rootstock with regards to nutrient uptake compared to the ungrafted plants

(Lee *et al*., 2010). Furthermore, more vigorous rootstocks could absorb more water and nutrients. Consequently, photosynthesis improved when these rootstocks were used compared to non-grafted plants as reported previously by Haghighi *et al*. (2016 b). In all NO_3^-/NH_4^+ ratios, grafting reduced stomata conductivity, which resulted in lower transpiration and improved water use efficiency so plants could deal with challenged conditions more efficiently (Duan *et al*., 2001).

In conclusion, the performance of the different cucumber plant accessions used as rootstocks i.e. nutrient absorption and growth parameters varied. The two accessions used in this study i.e. Isfahan and Babol responded similarly to N metabolism and photosynthesis traits. Therefore, it seems that different rootstock has the same physiology but different growth pattern due to their pre-existing genetic differences. More noticeably, we found that, shoot and root fresh and dry weights, root and shoot lengths, nodes, and number of leaves were increased by the 75/25 ratio of NO_3^-/NH_4^+. Grafting on the Isfahan and Babol increased nitrate reductase activity and NO_3^- concentration, protein content, amino acids content of leaves and photosynthesis parameters. Our findings suggested that the grafting 'Dominos' on Babol endemic rootstock using 50/50 NO_3^-/NH_4^+ ratio achieved better vegetative growth, which may result in better yield.

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Effect of harvest time on seed germination and seedlings growth of Sour orange and Mexican lime under *in vitro* conditions

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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: The aim of this research was to determine the best time to harvest the fruits for seed production which would ultimately lead to the production of citrus rootstocks of optimum quality. The sour orange and Mexican lime fruits were harvested on 7 and 5 occasions, respectively. The very first fruits were harvested 80 days after flowering and subsequent harvests were gathered every 30 days. An *in vitro* experiment was carried out in a completely randomized design, with four replications and 20 seeds in each replication. Based on fruit growth curve the time of fruit harvest affected seed germination (percentage and rate) and seedling growth (stem and root length, fresh and dry weight of stems, roots and leaves). The results showed that the best time to harvest the fruits of sour orange and Mexican lime was 230 and 170 days after flowering, respectively, which led to maximum seed germination (Mexican lime 100% and sour orange 85%) and seedling growth. The highest root, stem and leaf fresh and dry weight was also obtained at 230 and 170 days after flowering in sour orange and Mexican lime respectively.

1. Introduction

Citrus is an important genus of subtropical fruit trees, with substantial roles in the economy of many countries (Iglesias *et al*., 2007; Tercan and Dereli, 2020). Among different citrus species, the fruits reach maturity at different times of the season and as a result, the harvest time of fruits usually lasts several months (Orbović *et al*., 2011; Deterre *et al.,* 2021). It has been reported that a variety of physiological factors in citrus fruits, including fruit color change, sugar concentration and acid content affect the quality and marketability of fruits. However, there is insufficient information about the effects of seasonal changes on the fruit seeds, their germination potential and seedling vigor (Moulehi *et al.,* 2012; Orbović *et al.,* 2013). Many citrus cultivars that are selected for the production of high quality fruits do not have suitable root systems and, thus, it is highly rec-

ommended that these cultivars be grafted onto desirable rootstocks (Zhu *et al.,* 2020). Previous reports suggest that more than 20 traits of a grafted plant are affected by the rootstock, including drought tolerance, nutrient uptake, growth vigor, tree size, root penetration depth, tolerance to disease, amount of yield, fruit size and quality (Zhu *et al.,* 2013; Khoshbakht *et al.,* 2015). In southern regions of Iran, among the various citrus rootstocks, Mexican lime (*Citrus aurantifolia* L.) and sour orange *(Citrus aurantium* L.) are the most widely used due to their special characteristics. Mexican lime has high growth vigor and yield (Haji Vand and Lee Abdullah, 2012) and Sour orange is resistance to root rot, tolerance to calcareous and salinity soils and has deep root system (Louzada *et al.,* 2008; Etehadpour *et al.,* 2020). Citrus rootstocks are mostly propagated by seed.

Seed germination is a vital stage in the plant life cycle (Bakhshandeh *et al.,* 2017). Citrus growers often face many problems such as poor seed germination and great mortality rate of seedlings during the nursery stage (Dilip *et al.,* 2017; Chaudhary *et al.,* 2019). Citrus seeds usually do not have dormancy, they can germinate quickly and are considered as short-lived seeds (Khopkar *et al.,* 2017), their germination rate decreases as the seeds lose moisture (Hassanein and Azooz, 2003). Therefore, the seeds have low capacity of storability and should be sown quickly after extraction from the fruit (Khopkar *et al.,* 2017). Nursery operations to establishment of plant are very dependent on the high germination rate and growth of seedlings (Alouani and Bani-Aameur, 2004) so that they will reach the proper size for a short time and be ready for grafting, which can ultimately reduce the cost of growing grafted citrus plants (Girardi *et al.,* 2005).

An important factor that determines seed quality is the physiological maturity of seeds. Maximum germination (%) is reached when seeds are at their physiological maturity stage, which is associated with an optimum presence of nutrients in seeds to support the growth of seedlings with good vigor (Murrinie *et al.,* 2019). It has been also reported that the growth and maturity of fruits can affect seed germination percentage (Abbasi and Heidari, 2011; Mombeini *et al.,* 2011; Bareke, 2018). According to our knowledge, there is a lack of information about the best stage and time to harvest the fruits of sour orange and Mexican lime for producing seedling rootstocks. The aim of this research was to assess the effects of fruit harvest time, taking into account the fruit growth curve of sour orange and Mexican lime, on seed germination and seedling growth vigor under *in vitro* condition.

2. Materials and Methods

Plant materials and research site

This research was carried out in the laboratory of plant tissue culture and biotechnology, Faculty of Agriculture, Shiraz University. The seeds of two *citrus* species Mexican lime and sour orange were collected from an orchard belonging to the Citrus Research Institute, Larestan (27.66° N, 54.38° E, altitude 900 meters above sea level). The maximum and minimum temperatures and rainfall on an average of ten years in the region are 43.5°C, 4°C and 203 mm, respectively. The climate is characterized by mild winters and warm summers.

Seed samples were taken from fruits harvested at different times during the growing season. Sampling began from 80 days after flowering (June 1) when the seeds formed in fruits. Subsequent samples were taken on a monthly interval. The total time span considered for the harvest of sour orange and Mexican lime were 260 and 200 days after flowering, respectively. All sour orange fruits were harvested from a 10-year-old tree and all Mexican lime fruits were from an 8-year-old tree. The fruits were harvested from the same tree throughout the experiment. After harvesting the fruits, they were transferred to the laboratory in order to measure their diameter, length, and weight. Data were reported as the mean value of 10 fruits.

Effect of harvest time on in vitro seed germination

The treatments included 7 and 5 harvest times for sour orange (80, 110, 140, 170, 200, 230 and 260 days after flowering) and Mexican lime (80, 110, 140, 170 and 200 days after flowering) species, respectively. After separating from the fruits, the seeds were soaked in water for 12 hours and then washed with water and a few drops of dishwashing liquid for a few minutes to remove the gelatin-like material around the seeds. Then, the protective layer of the seeds was removed. The seeds were placed in vials containing water and a few drops of dishwashing liquid for 15 minutes to remove surface contaminants. After that, they were disinfected under sterile conditions by immersing in 70% alcohol for 30 seconds and then in 15% common bleach (containing 5.25% sodium

hypochlorite) for 15 minutes. Then, the seeds were washed three times in sterile distilled water. The seeds were then cultured on liquid MS medium (Murashige and Skoog, 1962) without any plant growth regulator and a filter paper was used to prevent them from being submerged. The mentioned medium was fortified with 30 g/l of sucrose, and the pH of the medium, before autoclaving (at 121 ° C and 15 psi) was regulated on 5.8.

The jars containing the cultured seeds were taken to the growth chamber in dark conditions (25±2°C). Within an initial period of 30 days, the percentage and rate of germination were measured by the Maguire (1962) method.

$$\text{Germination (\%)} = \frac{\Sigma\text{number of germinated seeds}}{\text{Total number of seeds)}} \times 100$$

$$\text{Germination rate} = \frac{\text{number of seeds until n-1 day}}{\text{number of days}}$$

In vitro plantlets growth

After 30 days in darkness, all cultures were maintained in a growth room at 25±1°C under a 16/8 h (light/dark) photoperiod of 45-50 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes and with 55-60% relative humidity. After 4 weeks, we measured growth indices such as stem and root length, number of leaves, fresh and dry weight of stems, roots and leaves

Experimental design and data analysis

The experiment was performed in a completely randomized design with 4 replications. In each replication, 20 seeds were checked for the percentage and rate of germination. Then, within the germinated seeds, 5 seedlings per replication were used for measuring growth characteristics. Statistical analyzes of the data were carried out using SAS 9.4 software and mean comparison was performed using LSD ($P \leq 0.05$). Microsoft Excel 2013 was used to draw the figures.

3. Results

The analysis of variance (Tables 1 and 2) showed that the fruit harvest stage significantly affected seed germination indices (germination rate and percentage) and seedling growth (root and stem length, number of leaves, fresh and dry weight of leaves,

Table 1 - Analysis of variance of the effect of harvest time on sour orange and Mexican lime on seed germination and seedling growth *in vitro* condition

	Source of variance	df	Germination percentage	Germination rate	Root length	Stem length	Number of leaves
Sour orange	Harvest time	6	5167 **	0.01 **	84.17 **	40.62 **	30.12 **
	Error	21	72.32	0.00006	3.82	1.49	0.87
	CV (%)	-	10.18	17.34	15.98	17.15	6.76
Mexican lime	Harvest time	4	3870 **	0.004 **	30.62 **	41.82 **	19.17 **
	Error	15	120	0.00008	3.55	1.17	1.88
	CV	-	10.12	10.86	9.32	14.59	11.28

**, significant at the level of 1 % probability using LSD.

Table 2 - Analysis of variance of the effect of harvest time on growth characteristics of sour orange and Mexican lime seedlings *in vitro* condition

	Source of variance	df	Root Fresh Weight	Stem Fresh weight	Leaf Fresh weight	Root dry weight	Stem dry weight	Leaf dry weight
Sour orange	Harvest time	6	1.04**	0.214**	0.926**	0.0796**	0.102**	0.100**
	Error	21	0.0003	0.0002	0.0004	0.00009	0.00005	0.0002
	CV (%)	-	10.21	2.4	5.85	12.95	3.38	4.25
Mexican lime	Harvest time	4	1.736**	0.268**	1.176**	0.141**	0.031**	0.055**
	Error	15	0.0004	0.0004	0.0005	0.0002	0.0002	0.0002
	CV	-	8.19	7.82	7.37	4.35	5.48	7.01

**, significant at the level of 1 % probability using LSD.

roots and stems) in both studied species (P≤0.01).

Fruit growth characteristics

Both citrus fruits species exhibited a simple sigmoid growth curve based on fruit dimensions and weight (Fig 1 A and B respectively). The growth curve in the first stage, i.e. 140 and 110 days after flowering showed a slow growth in sour orange and Mexican lime, respectively. Fruit growth at this stage is mostly a manifestation of cell division. The fruits in the second stage, from 140 to 200 days after flowering in sour orange and from 110 to 170 days after flowering in Mexican lime showed rapid growth and cell enlargement and water accumulation in fruit tissues. In the third stage, from 200 to 260 after flowering in sour orange and from 170 to 200 after flowering in Mexican lime, fruit growth had reduced growth rate and, accordingly, the process of non-climacteric ripening began in fruits.

In vitro germination characteristics

The highest percentage of seed germination was observed when sour orange harvested at 230 and Mexican lime at 170 days after flowering (Fig. 2 A and D), both of which are significantly higher than final stage of harvest. The lowest germination percentage of sour orange and Mexican lime was observed in the seeds of fruits harvested at 170 and 80 days after flowering, respectively (Fig. 3 A and C). It is also noteworthy that the seeds obtained from sour orange in the first three stages, namely 80, 110 and 140 days after flowering, were not able to germinate.

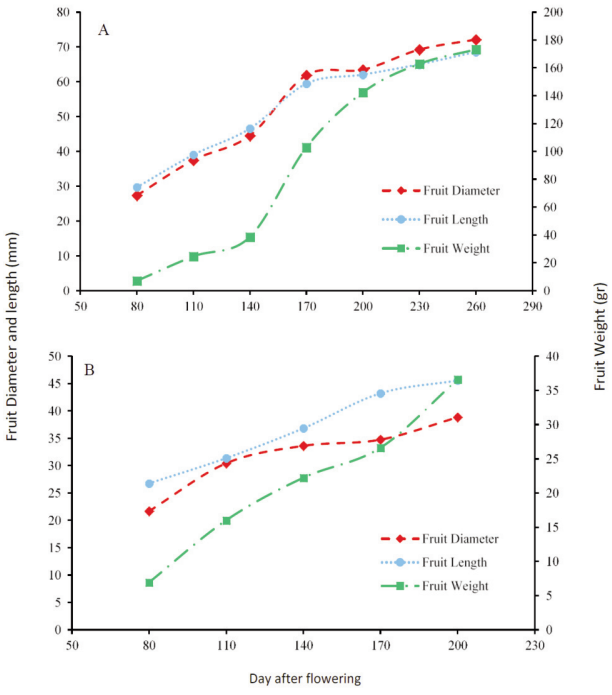

Fig. 1 - Fruit growth curve based on fruit weight, diameter and length of sour orange (A) and Mexican lime (B).

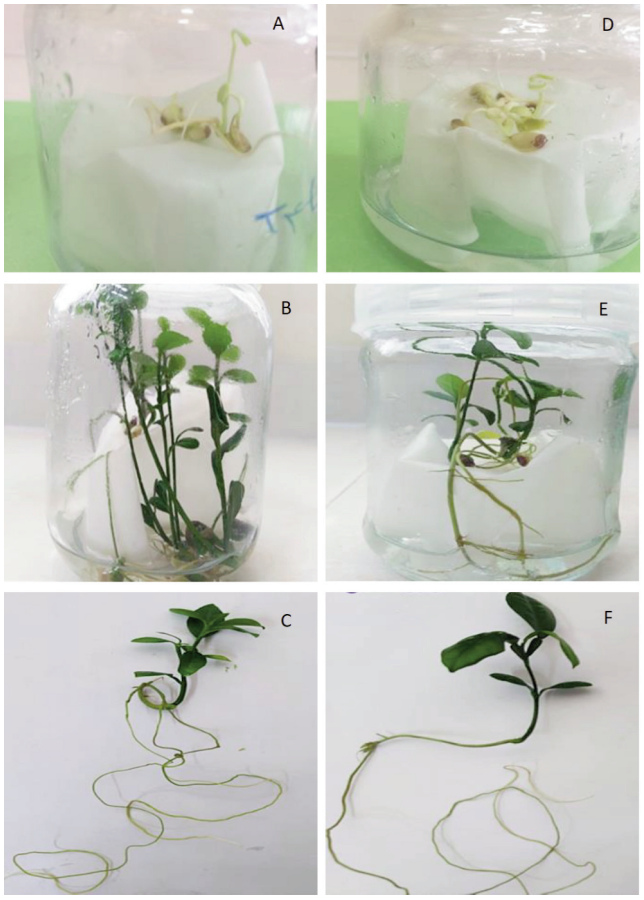

Fig. 2 - *In vitro* seed germination and plantlet growth. Germinated seeds of Mexican lime (A) and Sour orange (D) in darkness, 170 and 230 days after flowering respectively. Plantlets growth of Mexican lime (B, C) and Sour orange (E, F) after 4 weeks in light, 140 and 230 days after flowering respectively.

By increasing the harvest time, the germination rate increased in both species. In sour orange, the highest germination rate occurred at 230 days which was significantly 3.13 times higher than 170 days after flowering. In Mexican limes, the maximum seed germination rate occurred at 170 days which was significantly increased 1.57 times compared to 80 days after flowering. The lowest germination rate was observed in seeds of fruits harvested 170 days after flowering in sour orange and 80 days after flowering in Mexican limes (Fig. 3 B and D)

In vitro seedling growth indices

Fruit harvest time in both citrus species had a significant effect on seed growth indices (stem length,

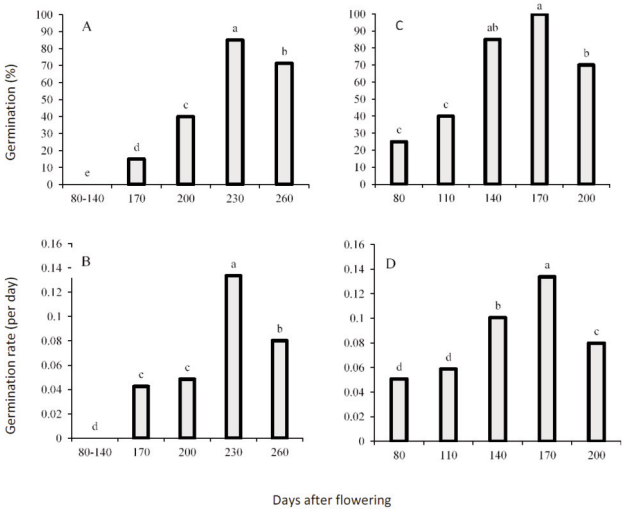

Fig. 3 - Effect of harvest time on germination percentage and germination rate of sour orange (A, B and Mexican lime (C D), under *in vitro* conditions. Means with the same letter are not significantly different at 1% probability using LSD test.

root length and number of leaves) (Fig. 4). In sour orang, the uppermost number of leaves were observed in the produced seedlings from the seeds of fruits that had been harvested 260 days after flowering which was significantly different from the 170 and 200 days (Fig. 4 C). However, there was no significant difference between the two treatments of 260 and 230 days after flowering.

In Mexican lime, the produced seedlings from the seeds of the fruits harvested 170 days after flowering indicated the higher number of leaves (9 leaves/seedling) which was significantly different compared to the produced seedlings from 80 (3.75 leaves /seedling) and 200 (6.75 leaves/seedling) days after flowering (Fig 4 F).

In sour orange, the *in vitro* seedlings of the 230 day after flowering showed the highest root length (10.75 cm) which did not show significant different compared to 260 days after flowering. Regarding Mexican lime, the highest root length occurred in the seedlings of 170 days after flowering (12.5 cm) which was significantly higher than those from 80 days after flowering. (Fig. 4 A and D). In both sour orange and Mexican lime seedlings, stem length increased with increasing the harvest time. In sour orange, stems of the seedlings related to 260 days after flowering showed the highest length of 7.25 cm which was not significantly different compared to 230 days after flowering (Fig 2 B and C). In Mexican lime, the seedlings of the 170-days harvest treatment indicated the maximum stem length of 11.75 cm which was significantly higher than the other harvest times (Fig 2 E and F) (Fig. 4 B and E).

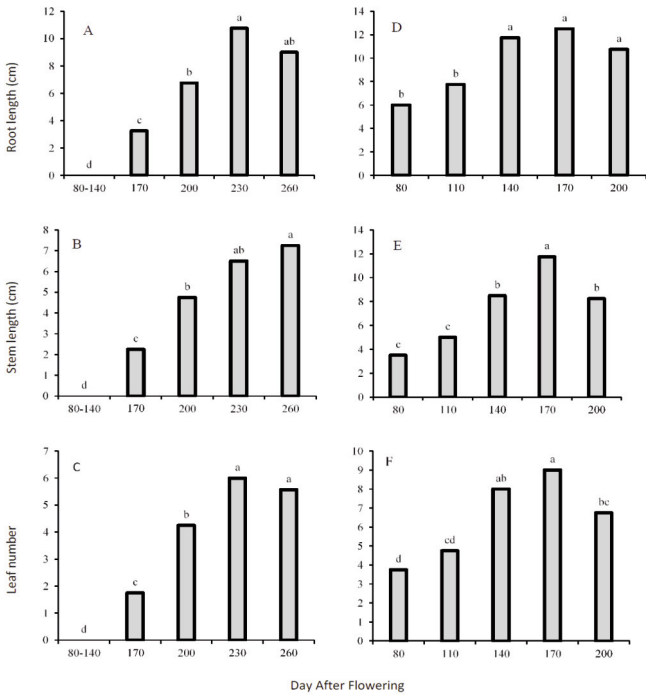

Fig. 4 - Effect of harvest time on seedlings root length, stem length and leaf number of sour orange (A-C) and Mexican lime D-F) under *in vitro* conditions. Means with the same letter are not significantly different at 1% probability using LSD test.

The results showed that a significant increase occurred in the fresh and dry weight of seedlings by increasing the harvest time. In sour orange, the produced seedlings in 230-day harvest treatment time indicated the highest fresh and dry weights of roots, stems and leaves which were significantly greater than other treatments (except in root fresh weight and leaf dry weight that was not significant different between 230 and 260 days after flowering) (Fig. 5 A-F). Regarding Mexican lime seedlings, with increasing the harvest time the fresh and dry weight of all organs increased. The highest fresh and dry weights of roots, stems and leaves were occurred in the seedlings of the 170-day harvest period, however with increasing harvest time to 200 days all measured traits decreased (Fig. 6 A-F).

4. Discussion and Conclusions

In this research, different stages of fruit harvest affected seed and rate of germination and seedling

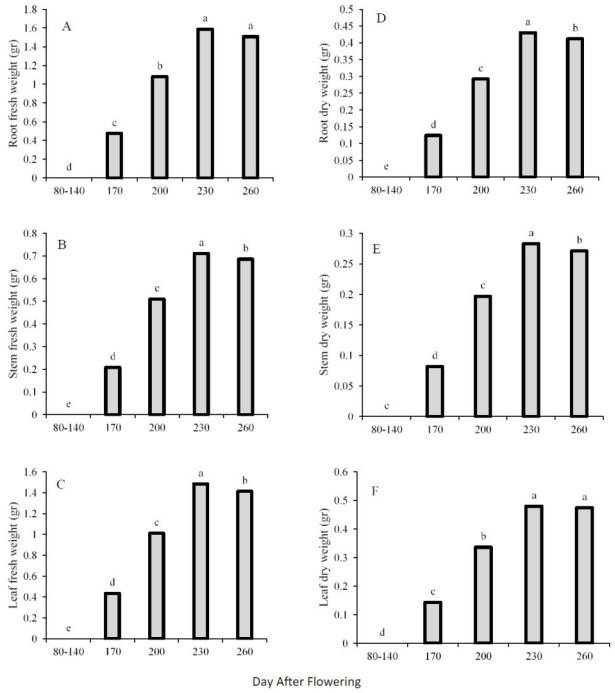

Fig. 5 - Effect of harvest time on seedlings root, stem and leaf fresh weight (A-C) and dry weight (D-F) of sour orange under *in vitro* conditions. Means with the same letter are not significantly different at 1% probability using LSD.

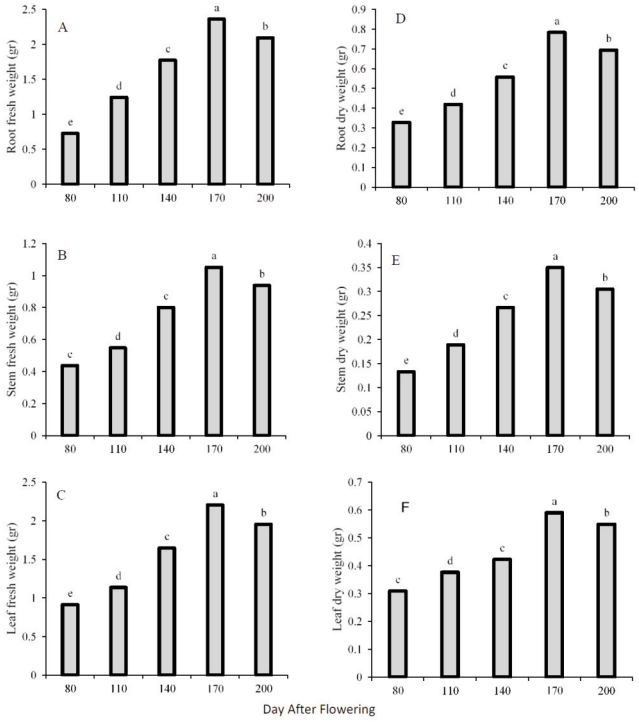

Fig. 6 - Effect of harvest time on seedlings root, stem, and leaf fresh weight A-C and dry weight (D-F) of Mexican lime. Means with the same letter are not significantly different at 1% probability using LSD test.

growth in both species (sour orange and Mexican lime). It has been demonstrated that by increasing the number of days after flowering to 230 days in sour orange and 170 days in Mexican lime, germination and seedling growth factors improved (Murti and Upreti, 2003; Kondo *et al*., 2004).

The results showed that the fruit growth of sour orange and Mexican lime based on fruit weight and dimensions have a single sigmoid growth, which is divided into three stages (Tadeo *et al.,* 2008). In the first phase of fruit growth, which is approximately between the flowering onset and June-drop, the rate of fruit growth is slow but cell division is high. The second is a period of rapid growth in which the size of the fruit increases through cell enlargement and accumulation of water. As these two growth stages end, the growing fruits change from the consumption phase to the storage stage (Mehouachi *et al.,* 1995). In the third stage, growth stops and the fruits undergo a non-climacteric ripening process (Mehouachi *et al.,* 1995; Tadeo *et al.,* 2008). Regarding fruit growth curve, our results showed that in order to achieve quality seeds and produce strong seedlings, the best time to harvest sour orange and Mexican lime fruits was in the beginning of third stage of fruit growth, about 230 and 170 days after flowering, respectively. Early harvest may reduce seed quality due to the partial development of basic seed structures, while late harvest may lead to reduced seed quality because of aging. Mombeini *et al.* (2011) reported that the highest seed germination occurred in sour orange at 250 days after flowering before full fruit ripening and then reduced until ripening, which is in line with our results.

It has been documented that one of the possible reasons for the differences in the physiological potential of seeds is related to changes in the embryo and endosperm of seeds at different stages of growth and development (Tekrony, 2003). The seed reaches its maximum potential for germination at the stage of physiological maturity, when more nutrients are available to support seedling growth and vigor (Murniati *et al.,* 2008). However, Murniati *et al.* (2008) also reported that papaya seeds extracted from fruits before full ripening (30-40% yellow color of the fruit skin) had maximum germination and growth.

Another study indicated that physiological maturity is a genotypic trait that is influenced by environmental factors. Environmental conditions during seed growth and maturity, including temperature, envi-

ronmental stresses, and nutrient deficiencies, affect seed quality (Mahesha *et al.,* 2001). In the process of seed development, various mechanisms occur from fertilization to physiological maturity and into the phases of cell division, development and then the phase of nutritional storage in seeds. There is usually an increase in the dry weight of seeds and finally a decrease in seed moisture due to changes in cell membrane structure and enhanced levels of enzyme synthesis, necessary for successful seed germination (Bareke, 2018). Theoretically, it can be said that during physiological maturity, the germination percentage of seeds increases and reaches a maximum when the seeds reach their maximum dry weight (Orbović *et al.,* 2013). In another study, the relationship between the germination percentage of grapefruit seeds and sour orange were evaluated from fruits harvested at the beginning of the season. Studies have shown that when a seed reaches physiological maturity, the seed vigor becomes consistently high throughout the harvest season (Fucik, 1978).

The produced seedlings from the seeds extracted from fruits at 230 and 170 days after flowering in sour orange and Mexican lime, respectively, had better growth than the seedlings produced from the harvested seeds at the final stage of fruit maturity. Accordingly, seedling growth indices such as fresh and dry weight stems, root and leaves were significantly higher at a stage before the last stage of harvest. It has also been reported that structural and chemical changes in fruits and seeds are associated with germination vigor and seedling growth indices (Abbasi and Heidari, 2010; Mombeini *et al.,* 2011). Orbović *et al.* (2013) stated that the ability of grapefruit seeds to germinate at the end of the season is a physiological manifestation of a change in hormonal balance in the fruits, which is largely associated with a slight decrease in abscisic acid levels in the seed. It has been also reported that Valencia orange seeds showed a great peak in ABA (Abscisic Acid) concentration at 150 days after flowering (in stage II) and this increment obviously decreased at 188 days after flowering while, amount of IAA (Indole-3-acetic acid) increased (Kojima 1995). It is possible the better seedlings growth in the beginning of third stage of growth be due to the increase of IAA content of the seeds.

In conclusion, we found that the fruit harvest stage can have a significant effect on seed quality for rootstock production in citrus. Fruit growth (length, diameter and weight) was affected by harvest time in sour oranges and Mexican lime. The best seed germination characteristics were obtained at the onset of the maturation stage in both citrus species (230 and 170 days after flowering, in sour oranges and Mexican lime, respectively), followed by the highest seedling growth. But, seed germination and seedling growth parameters were significantly reduced in 30 days after maturation.

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Stem and leaf anatomical and physiological characteristics of 'Colín V-33' avocado seedlings

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Abstract: The anatomical and physiological structure of the 'Colín V-33' avocado stem and leaf is described from samples from plants obtained from seed in order to identify genotypes and early selection parameters in a rootstock improvement program for avocado. Eighty-nine plants of 12 months of age were used, where a total of 25 anatomical variables of the stem, leaf, and physiological of leaf were evaluated. A cluster analysis was conducted that generated a hierarchical dendrogram that suggested six groups of plants. Furthermore, from the 25 variables, eight were selected as discriminant when performing a canonical discriminant analysis, the variables that most discriminated for the first canonical component were: stem diameter and density of xylem vessels, for the second: thickness of the stem epidermis, temperature of the stem leaf and stomata length, while for the third: thickness of the cambium, transpiration rate, and stomatal conductance. The genotypes showed a great variation between the groups, the characteristics of these indicated that the genotypes of Group 4 showed some that could be related to small or dwarf plants (smaller stem diameter, high density of xylem vessels, a higher rate of transpiration and stomatal conductance). In contrast to the genotypes of Group 3 which presented opposite characteristics in the previous variables, being able to associate with vigorous plants. The anatomical traits of the stem showed to be highly related to the behavior of the avocado plants. Associating genotypes with physiological and anatomical variables in leaf and stem can have great value for the selection of rootstocks at an early stage of development.

1. Introduction

Fruit trees used for the establishment of plantations are formed by a variety/rootstock combination, where the variety provides the productive part and the rootstock provide the root system of the tree (Dolgun *et al*., 2009). The rootstocks provide a simplified management of the orchard,

increase the productivity, ensure the survival of the trees, control the vigor conferring dwarfism and allowing the use of high planting densities and the water balance of the plant (Solari *et al*., 2006), influence the qualitative and nutritional attributes of the fruits (Remorini *et al*., 2008), also influence the scion development, as well as its adaptation to different types of soil, water stress conditions and other climatic conditions (Giorgi *et al*., 2005), as well as salt stress tolerance (Massai *et al*., 2004).

Selection of the rootstock and the cultivar or varieties to be exploited is an economically important decision, since the degree of productivity and quality of production will depend on the variety/rootstock combinations (Pinochet, 2010), these combinations are in function of the degree of affinity in terms of vascular connections between the graft and the rootstock, because vascular regeneration is a complex process that includes the differentiation of xylem and phloem (Aloni *et al*., 2010).

Avocado production in Mexico is based on the use of seed-derived rootstocks which generate large trees that, over time, complicates the agronomic management. For this reason, one of the priorities in avocado research is to find dwarf cultivars that are highly productive, with good fruit quality and resistant to pests and diseases in order to establish high-density plantations and reduce production costs (Sánchez-Colín *et al*., 1992). The use of rootstocks in intensive avocado cultivation is of great importance and rootstocks for this crop have been reported to influence the size of the tree and its productivity (Bergh and Whitsell, 1962). Potentially dwarfing rootstocks such as 'Wilg' and 'Colin V-33' have been evaluated against 'Duke 7', for their ability to limit the growth of 'Hass' (Roe *et al*., 1995).

'Colín V-33' avocado is a Mexican selection that has low vigor and an expanded growth habit (Roe *et al.*, 1995), considered as a possible dwarf rootstock since it has been used as an interstock, with reported reduction of 43% of the tree height on 'Fuerte' (Barrientos Priego *et al.*, 1987) and 'Hass' (Barrientos-Villaseñor *et al.*, 1999). On the other hand, given the genetic variability for the height of the seedlings of this cultivar (Rubí Arriaga, 1988), it makes it a valuable material for the improvement of dwarf avocado rootstocks. In this regard, Barrientos-Priego *et al*. (1992) have found that the cultivar seedlings produce some dwarfing individuals when used as rootstocks.

Efforts have been made mainly with 'Colín V33' for the selection of dwarf rootstocks that meet the needs of the new production systems, where stomata density has been proposed as a possible pre-selection index (Barrientos-Pérez and Sánchez-Colin, 1982; Barrientos-Priego and Sánchez-Colín, 1987).

Other important features to be investigated are the ones that contribute to the hydric status of the plant and its productivity, in which a series of physiological, anatomical and morphological characteristics of the stem can be measured. At the physiological level, gas exchange can be studied (Vilagrosa *et al.*, 2010) and to determine the affinity between variety/rootstock combinations, the anatomical structure of xylem can be studied (Sory *et al*., 2010; Leal-Fernández *et al*., 2013).

The anatomical characteristics of the dimensions of vessel elements and the proportions of xylem and phloem, in stems, are important to be able to define the amount of water that can be transported through them, since as the tissues become larger and the presence of smaller diameter of the vessels (higher pressure for water movement), the amount of water transported will be greater, therefore, there will be a better adaptation of the plants to low humidity conditions (Vasconcellos and Castle, 1994; Reyes-Santamaría *et al.*, 2002).

Reyes-Santamaría *et al*. (2002) found vessel elements with smaller diameters, for the avocado genotypes that have less vulnerability to drought. The stomatal density (ED) and the thickness of the epidermis are characteristics that may be related to drought tolerance (Baas, 1982), as they are the most exposed anatomical characteristics of the plant, which represent the last link in the transpiration torrent towards the atmosphere (Faust, 1989).

The use of avocado seedling rootstocks of a local type called "Criollo" is very common but rarely studied from their anatomical and physiological characteristics on stem and leaf, variables that can be useful as a preliminary study to understand their possible role when grafted. The objective of this research was to describe the anatomical and physiological structure of the stem and leaf of plants derived from avocado seed of 'Colín V-33', to select individuals at the seedling level with distinctive characteristics.

2. Materials and Methods

The research was carried out in a greenhouse of the Experimental Field of the Chapingo Autonomous

University, in Chapingo, State of Mexico, located at 19° 29'25.7'' and 98° 52'24.5'' with an altitude of 2240 meters above sea level.

Plant material

As plant material, 89 avocado seedling plants of 'Colín V-33' were used, that were donated by the Germplasm Bank of the Salvador Sánchez Colín Foundation-CICTAMEX, S.C. located in Coatepec Harinas, State of Mexico. The seeds were established in a black bag of caliber 600 of 26 cm x 35 cm with perforations in the first lower third; using soil, perlite and compost as a substrate (3:1:1; v:v:v), watered twice a week, and located in a glass greenhouse with oscillating temperatures between 35 ± 4°C.

Leaf gas exchange variables

To each of the plants the eleventh leaf, fully expanded and healthy, counted from the base towards the apex of the plant was selected as recommended by Barrientos-Priego *et al*. (2003). Once marked, the variables CO_2 assimilation rate (A), transpiration rate (E), leaf temperature, internal CO_2 concentration and, stomatal conductance was evaluated. A punctual measurement was made between 11:00 and 13:30 hours a day with an infrared gas analyzer (model CI-340, CID Bio-Science). This was done during three days of the month of September of the year 2017 and the measurement was taken in the same plant order every day to avoid more variation in the data taken for each plant. The average of the three values obtained was used in the analysis.

The water use efficiency index (WUE) was calculated based on the variables CO_2 assimilation (A) and transpiration rate (T), using the formula WUE = A/T.

Leaf anatomical variables

After the measurements of gas exchange variables, on the same leaf previously marked, an impression of the underside of the middle part of the lamina using silicone for dental impressions (Exactoden) was taken. By applying transparent nail varnish on the (negative) impression, the positive impression was obtained, which was placed on a slide and fixed with a coverslip.

In the positive impressions of each sample, the image area was calculated with an object micrometer, stomatal density (SD) per mm^2 and epidermal cell density (ECD) per mm^2 were determined. These variables were evaluated in five fields [400x, Ayala-Arreola *et al*. (2010)] in a Motic B3 Professional Series microscope, with the adaptation of a Moticam 480 camera with a 16 mm adapter. In addition, 10 stomata were measured in each sample. Stomata counts, epidermal cells and stomata length measurement were performed with the help of ImageJ 1.52a image analyzer.

With all this information the stomatal index (EI) was calculated, which is equal to:

$$EI = [SDE/ (SD + ECD)] \times 100.$$

Stem anatomical variables

Transverse stem samples were obtained from plants of approximately 1 cm each, which were fixed in 96 % ethanol:100 % glacial acetic acid (2:1; v:v) and processed in an automatic tissue exchanger (Tissuematon Fisher) with 2-ethoxyethanol (cellosolve) and xylene, then transfer to paraffin (55°C) staying 72 hours inside a stove. The paraffin pyramid was made according to Sass (1968) and in a rotary microtome (American Optical, model 820), transverse cuts were made with a thickness of 10 µm. The cut sections were stained for 30 min at room temperature in a mixture of equal volumes of 0.1% aqueous solutions of safranin and fast green, then washed in distilled water for 5 minutes and washed in 2 changes of absolute alcohol for 2-3 min (Bryan, 1955). The stained sections then mounted on slides with coverslips by means of Haupt adhesive and 10 % formalin (Sass, 1968).

Fifteen fields were observed in each preparation per replication (three repetitions). For which the 4x, 10x and 40x lenses were used as appropriate, in a Motic B3 Professional Series microscope, with the adaptation of a Moticam 480 camera with a 16 mm adapter and digital images were obtained. The area of the images was calculated with a slide micrometer and then the cell layers of the tissues and the dimensions of xylem vessel elements were measured with the help of the ImageJ 1.52a image analyzer.

The tissues evaluated were epidermis, parenchyma, phloem fibers, phloem, cambium, xylem, and pith. The thickness of each layer and the total diameter of the stem was measured. For the xylem dimensions number of vessel elements per area (vessel density), vessel element area, major axis of vessel element, minor axis of vessel element, cell wall thickness of two contiguous vessel elements, roundness index (RI) of vessel and Feret diameter were obtained.

Statistical analysis

A cluster analysis was performed using Ward's minimum variance agglomeration method to generate a hierarchical dendrogram (Núñez-Colín and

Escobedo-López, 2011). The resulted dendrogram was divided according to Hotelling's pseudo-statistical t^2 (Johnson, 1998). A canonical discriminant analysis (CDA) with the Mahalanobis distance was performed to determine the most discriminating variables with the greatest importance that describe the groups (Núñez-Colín and Escobedo-López, 2014). All statistical analysis were performed with the SAS V.9.2 statistical package (SAS Institute, 2009).

3. Results and Discussion

In order to explore the homogeneity within each variable evaluated and based on the coefficients of variation (CV), it was determined that the physiological variables of leaf: CO_2 assimilation rate, stomatal conductance and water use efficiency index (WUE), were the characteristics with the greatest variation (Table 1), presenting CV of 60.84%, 57.65%, and 62.5%, respectively. These characteristics being less stable within the plants studied (more heterogeneous). On the contrary, all the anatomical variables of stem and leaf showed lower coefficients of variation that were below 28%, so they could be considered more homogeneous but still show contrasting features (Fig. 1 and 2).

Cluster analysis

Based on Hotelling's t^2 pseudo-statistic (Fig. 3), the grouping by Ward's method showed the identification of six groups of plants (Fig. 4). Group 1 composed of 21 plants, Group 2 by 9, Group 3 with 13, Group 4 with 13, Group 5 with 16 and Group 6 with 17 plants (Table 2).

In the obtained dendrogram a partition of six groups was used approximately at a cut-off distance R^2 semi-partial close to 0.050 (Fig. 3). The greater distance corresponds to Group 4 confirming that are different from the rest, that was also reinforced with

Table 1 - Mean values of some leaf and stem anatomic variables and leaf gas exchange variables of 'Colín V-33' avocado seedling plants. The highest values are presented in bold for the coefficient of variability

Variables	Maximum	Mean	Minimum	CV (%)
Epidermis (µm)	1.72	1.12	0.74	18.43
Parenchyma (µm)	36.00	19.79	12.48	21.66
Phloem fibers (µm)	22.96	15.99	9.19	20.36
Phloem (µm)	66.46	39.72	21.46	22.56
Cambium (µm)	10.69	7.14	4.24	21.01
Xylem (µm)	115.22	76.05	28.40	25.42
Pith (µm)	98.49	60.02	19.41	27.20
Stem Ø (µm)	197.78	152.08	107.85	14.36
Vessel density by area (vessels/µm^2)	6.13	3.11	1.73	25.20
Vessel area (µm^2)	2688.49	1868.91	1096.34	16.99
Mayor axis of vessel (µm)	40.59	29.40	20.27	14.68
Minor axil of vessel (µm)	22.80	17.63	12.05	15.04
Wall thickness of two cells (µm)	3.57	2.80	2.19	11.06
Roundness index	0.59	0.35	0.18	24.03
Feret Ø	87.80	49.53	30.63	18.48
Leaf temperature (°C)	28.53	26.52	23.23	4.47
CO_2 assimilation rate (umol·m^{-2}·s^{-1})	3.97	1.28	-0.06	60.84
Transpiration rate (mmol·m^{-2}·s^{-1})	2.00	0.89	0.32	45.69
Stomatal conductance (mmol·m^{-2}·s^{-1})	105.18	33.04	9.33	57.65
Internal CO_2 concentration (ppm)	352.87	232.58	67.33	20.53
WUE	4.69	1.56	-0.19	62.50
Stomatal density (stomata/mm^2)	317.65	190.66	105.88	19.80
Epidermis cell density (cells/mm^2)	1267.65	818.88	582.35	17.56
Stomatal index (%)	25.16	19.03	11.78	13.89
Stoma length (µm)	19.64	15.99	13.11	8.60

WUE= Water use efficiency. CV= Coefficient of variability.

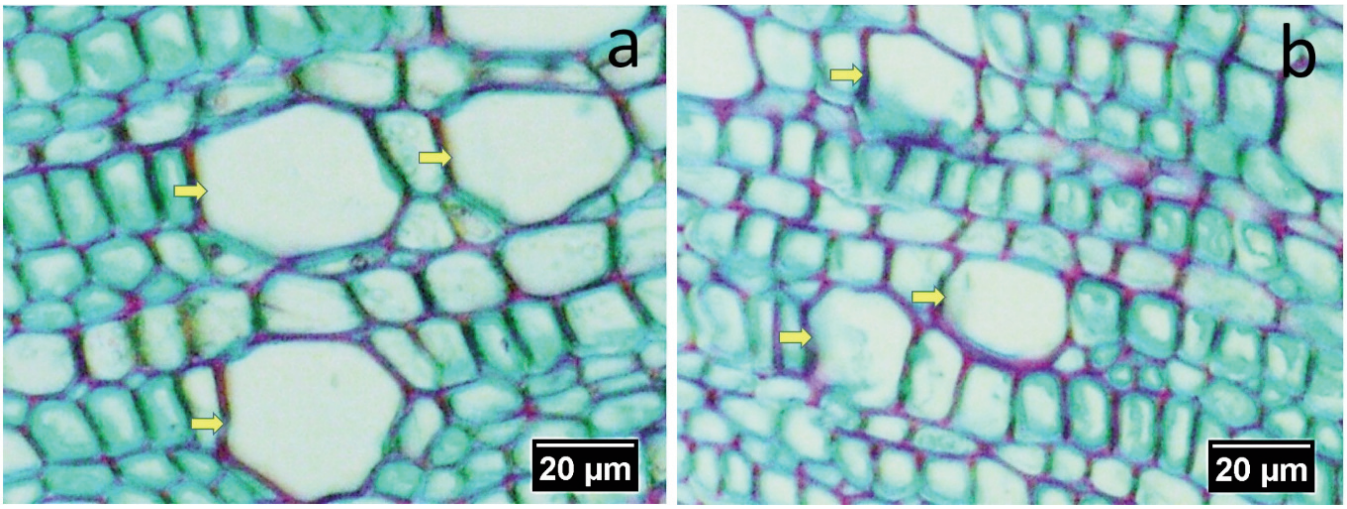

Fig. 1 - Transverse section of xylem tissue of main stem from plant numbered as 110 (a) and 88 (b) of 'Colin V-33' avocado seedlings. Arrows show vessel elements.

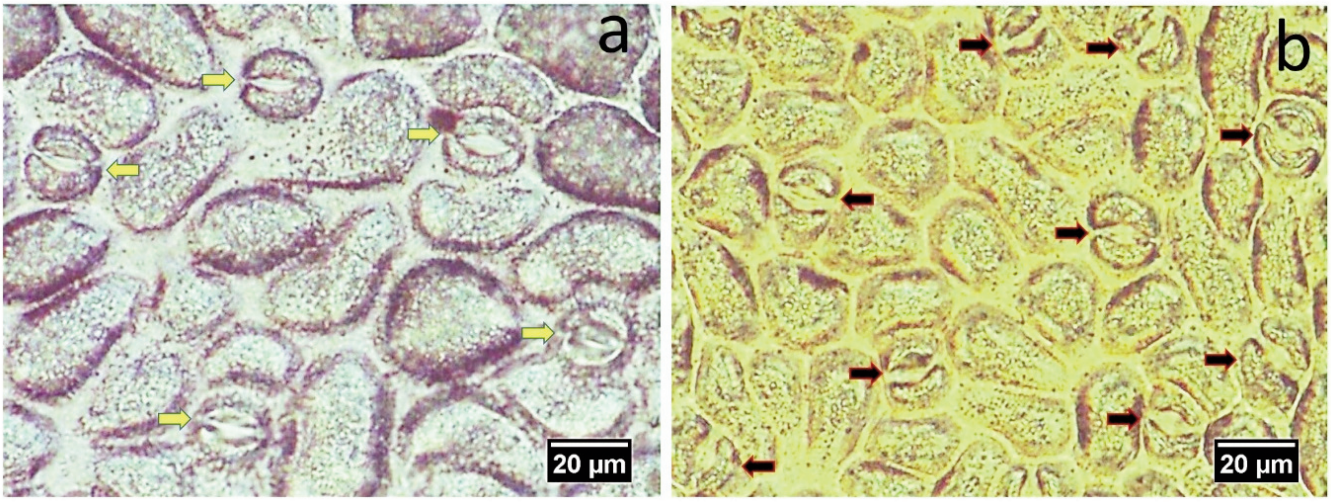

Fig. 2 - Epidermis replicates of leaf lamina from plant numbered as 162 (a) and 96 (b) of 'Colin V-33' avocado seedlings. Arrows show stomata.

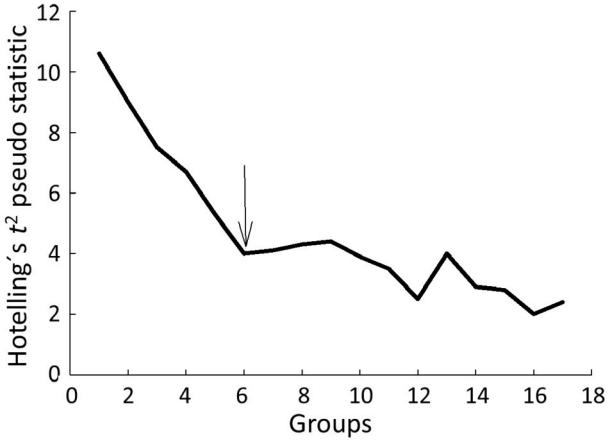

Fig. 3 - Hotelling's pseudo-statistical t^2 and the recommended number of groups as a proof of dendrogram division using physiological and anatomical of leaf and stem variables of 89 'Colin V-33' seedling plants.

plants were developed. Generally, plants with a greater number of relatively small vessel elements are associated with drought-resistant genotypes (Vasconcellos and Castle, 1994; Reyes-Santamaría *et al*., 2002; Núñez-Colín *et al*., 2006).

The CC 2 represented 28.90% of the total variation and was associated with: epidermis thickness, leaf temperature, and stoma length (Table 3 and 4). The anatomical stem variable (epidermis thickness) present in CC 2 is associated with drought resistance (Baas, 1982) and it is possible to relate it as acquired adaptation, to tolerate prevailing environmental conditions of the greenhouse (38°C). On the other hand, the size of stomata is one of the anatomical variables of the leaf that could be sensitive to the change in environmental conditions (Hetherington and

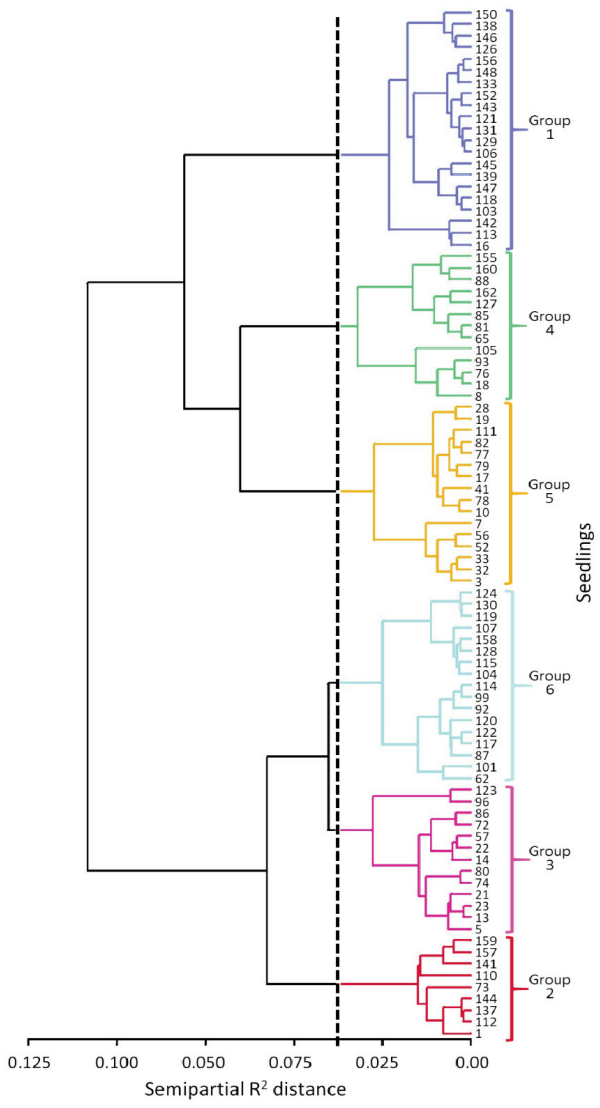

Fig. 4 - Cluster by the Ward method into six groups formed by the anatomical and physiological characteristics of the stem and leaf of 'Colín V-33' avocado seedlings. The black dotted line represents the dendrogram cut according to Hotelling's pseudo-statistic t^2.

Woodward, 2003). Consequently, these characteristics can be considered as representative of the adaptation to the environment of the plant, as a constant feature of taxonomic value, that the relationship between these two anatomical variables (epidermis thickness and stoma length) have with the variable air temperature.

The CC 3 was associated with the variables thickness of the cambium, transpiration rate, and stomatal conductance. Stomatal conductance refers to the control exerted by stomata on the rate of transpiration, representing the ability of a water molecule to diffuse through the leaf per unit of time. A minor stomatal conductance may be the expanation for lower CO_2 assimilation and at the same time a lower transpiration rate (Sholefield *et al*., 1980; Barrientos-Villaseñor *et al*., 1999).

Stomata size is considered a key physiological variable on stomatal conductance (Holland and Richardson, 2009), which is why the stomatic conductance use can be considered as a rapid test in genotype selection, as an indirect measure of stomatic density, since this can also be a way to perform a genotype separation towards dwarf types as proposed by Barrientos-Pérez and Sánchez-Colín (1987).

The *F* significance test of the Mahalanobis distance (Table 5) indicated that there are highly significant differences between the groups of plants at a level of $P<0.0001$.

To have a better and clearer visualization (distribution) of the groups of plants, the graphic representation was made in the first factorial plane with the first two canonical components (Fig. 5).

The graphic representation of the groups in the first factorial plane showed that the Group 4 is the only one that is isolated from the rest and was very different from the other five groups, with most of its

Table 2 - Groups formed according to the cluster analysis with 89 plants derived from 'Colín V-33' avocado seedlings, derived from anatomical and physiological characteristics of stem and leaf

Group	Seedings clustered
Group 1	16, 103, 106, 113, 118, 121, 126, 129, 131, 133, 138, 139, 142, 143, 145, 146, 147, 148, 150, 152, 156
Group 2	1, 73, 110, 112, 137, 141, 144, 157, 159
Group 3	5, 13, 14, 21, 22, 23, 57, 72, 74, 80, 86, 96, 123
Group 4	8, 18, 65, 76, 81, 85, 88, 93, 105, 127, 155, 160, 162
Group 5	3, 7, 10, 17, 19, 28, 32, 33, 41, 52, 56, 77, 78, 79, 82, 111
Group 6	62, 87, 92, 99, 101, 104, 107, 114, 115, 117, 119, 120, 122, 124, 128, 130, 158

Table 3 - Values of the canonical discriminant analysis of stem and leaf anatomical variables and physiological leaf variables of 89 'Colín V-33' avocado seedlings

Canonical component	Proper value	Variance (%)	Accumulated variance (%)	Canonical correlation	Approximated calculated *F*	*P>F*
1	47.504	39.91	39.91	0.909	4.93	<0.0001
2	34.405	28.90	68.81	0.880	4.02	<0.0001
3	18.683	15.69	84.50	0.807	3.09	<0.0001
4	13.703	11.51	96.01	0.760	2.45	<0.0001
5	0.4746	3.99	100	0.567	1.42	0.1419

Table 4 - Total canonical coefficients of the canonical discriminant analysis (CDA) of anatomical characteristics of the stem and leaf, as well of physiological characteristics of the leaf of 'Colín V-33' avocado seedlings. The highest values are presented in bold letters in three canonical components

Studied variable	Can 1	Can 2	Can 3	Can 4	Can 5
Epidermis (µm)	0.166274	0.677758	0.107810	-0.136872	0.076201
Parenchyma (µm)	0.250557	0.442521	-0.254777	-0.069388	0.232325
Phloem fibers (µm)	0.431579	0.577674	0.439686	-0.030758	0.020814
Phloem (µm)	0.432130	-0.365082	0.103285	0.028883	0.067597
Cambium (µm)	0.136514	-0.145255	0.545261	0.168825	-0.111704
Xylem (µm)	0.408444	-0.551603	-0.038947	0.244313	-0.159389
Pith (µm)	0.061974	0.521382	0.194067	0.087974	-0.233069
Stem Ø (µm)	0.601144	-0.122358	0.233650	0.369948	-0.235378
Vessel density by area (vessels/µm^2)	-0.642113	0.285704	-0.040889	-0.295962	0.234301
Vessel area (µm^2)	0.198248	0.296278	0.035828	0.557918	0.230066
Mayor axis of vessel (µm)	0.540765	-0.053647	-0.094319	0.682230	0.145237
Minor axil of vessel (µm)	0.464509	0.339487	0.238300	0.596176	0.043128
Wall thickness of two cells (µm)	0.262160	0.553793	0.219939	0.168747	-0.051240
Roundness index	0.521700	-0.516643	-0.056714	0.381362	-0.115487
Feret Ø	-0.415188	0.247956	-0.183468	-0.143826	0.263973
Leaf temperature (°C)	-0.055325	0.671497	0.197631	-0.080977	0.002190
CO_2 assimilation rate (µmol·m^{-2}·s^{-1})	-0.346322	0.209754	-0.090718	0.653934	-0.383860
Transpiration rate (mmol·m^{-2}·s^{-1})	-0.538189	0.110232	0.546988	0.242327	0.023917
Stomatal conductance (mmol·m^{-2}·s^{-1})	-0.530059	-0.016900	0.502987	0.237708	0.022604
Internal CO_2 concentration (ppm)	-0.035307	-0.155754	0.408420	-0.518668	0.491616
WUE	0.010946	0.092531	-0.434341	0.556483	-0.488497
Stomatal density (stomata/mm^2)	0.059033	-0.043825	0.165589	0.198190	0.023354
Epidermis cell density (cells/mm^2)	-0.032255	0.110568	0.025203	-0.166117	0.273310
Stomatal index (%)	0.098199	-0.132837	0.100880	0.400103	-0.233939
Stoma length (µm)	0.087852	0.636119	-0.014346	-0.140672	-0.173689

WUE= Water use efficiency index.

plants in the lower left quadrant (Fig. 5).

Groups 1, 2 and 6 are probably clustered because they are plants with anatomical and/or physiological similarities according to the variables evaluated in this study (Fig. 5). These groups are those that have dispersion in both planes, so they are considered the most heterogeneous groups within the plants studied.

Groups 3 and 5 were slightly more separated from the rest of the groups, but between them, there is some closeness (less dispersion), which could be inferred as similar groups according to the behavior of the variables evaluated (Fig. 5).

Table 5 - Mahalanobis distance and its significance, from anatomical characteristics of stem and leaf, as well of physiological characteristics of leaf from 89 'Colín V-33' avocado seedlings

Group	1	2	3	4	5	6
1	-					
2	18.20	-				
3	24.83 ***	36.28 ***	-			
4	37.84 ***	39.65 ***	55.11 ***	-		
5	22.39 ***	34.87 ***	16.52 ***	29.83 ***	-	
6	12.92 ***	22.02 ***	16.19 ***	36.78 ***	23.52 ***	-

*** Significant at P<0.0001 of probability.

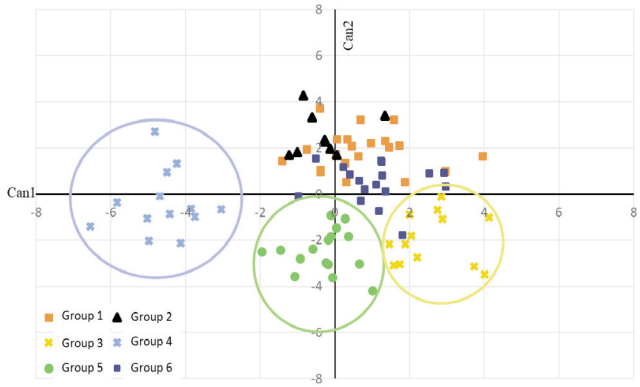

Fig. 5 - Dispersion of the six groups of 'Colín V-33' avocado seedlings in the first factorial plane of the first two canonical components of the Canonical Discriminant Analysis, derived from anatomical and physiological characteristics of stem and leaf.

In the pattern of dispersion of the plants in a three-dimensional graph formed by the three canonical components (CC), it was observed that group 4 is completely separated from the rest and is located towards the most negative values of CC 1 and CC 3 (Fig. 6), it was also observed that groups 3, 5 and 6 were not so separated from the rest of groups (less dispersed), such as 4, and if they could be easily differentiated in the graph located towards positive values of CC 1 and CC 2; on the other hand, the most dispersed groups were 1 and 2, mixing with each other, indicating that they probably have similar characteristics.

Group 1 is formed by plants with the greatest epidermis thickness and the greatest stoma length but the lowest stomatal conductance of all other groups (Fig. 6). The thickness of the epidermis is a characteristic that may be related to drought tolerance according to Baas (1982), as it is the most exposed anatomical characteristic of the plant along with the stomatic density, representing the last link of the transpiration stream into the atmosphere (Faust, 1989).

The plants of group 2 were characterized by having the cambium with less thickness, high average values of stem diameter and stomata length.

Group 3 was characterized by having plants with greater stem diameter, a lower density of vessel elements and shorter stomata length, low average values in transpiration and stomatal conductance. This group of plants is likely to be more vulnerable to physiological problems such as drought as reported by Reyes-Santamaría *et al*. (2002).

The plants in group 4 had the lowest values for stem diameter, but the highest density of xylem vessel elements, also the highest rate of transpiration and the highest stomatal conductance, this being the group that separated completely from the rest (Fig. 4). The results could indicate that the plants belong-

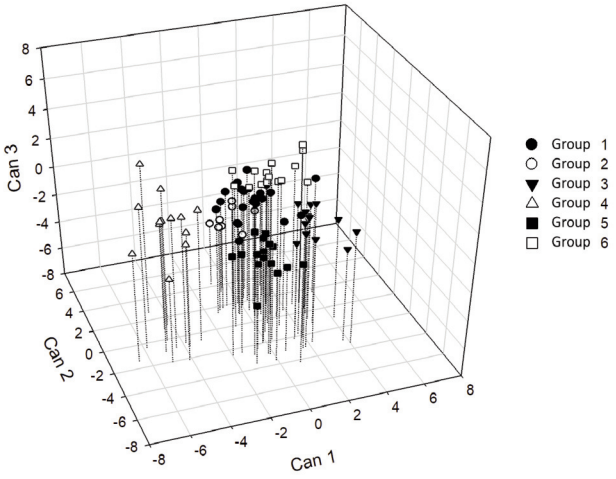

Fig. 6 - Dispersion of 89 'Colín V-33' avocado seedlings in the first three canonical functions based on physiological and anatomical variables of leaf and stem.

ing to this group probably use more water and can be more adapted to condition of more availability, because by decreasing the stomatal opening and closing it contributes to reducing the loss of water due to evapotranspiration (Núñez-Colín *et al*., 2006). Previous results (Sholefield *et al*., 1980; Barrientos-Villaseñor *et al*., 1999) indicated that there is a positive correlation between the variables CO_2 assimilation, stomatal conductance and transpiration rate, thus an increase in stomatal conductance promotes the increase in the transpiration rate and the photosynthetic rate (Damián-Nava *et al*., 2009).

Group 5 was represented by plants with the lowest thickness of the epidermis, the lowest leaf temperature, low stomatal conductance values, and transpiration. Group 6 (Fig. 5) was characterized by having the greater cambium thickness, high average values in the variables stem diameter, epidermis thickness, leaf temperature, stomata length, but low densities values of vessel elements, transpiration, and stomatal conductance.

Groups 1 and 6 were closest to each other, according to the Mahalanobis distances (Table 4), while the most distant groups were 3 and 4 (Fig. 5).

For any type of plant, the conducting tissue of the stem, the size of the xylem vessel elements, the percentage of the xylem and phloem, and the relationship between xylem and phloem, are anatomical features that define the transport capacity of water in plants. It has been observed in some tree species, that as the percentage of vascular tissues increases and the diameter of the vessels are smaller, the amount of water transported is higher, and this may be an indicator of greater adaptation from plants to low humidity conditions in the soil (Vasconcellos and Castle, 1994; Reyes-Santamaría *et al*., 2002). For the case of avocado rootstocks, it has been found that stems of 'Duke 7' (1248.7 μm^2) had narrowed xylem element cells diameters than 'Toro Caynon' (1536.1 μm^2), were 'Duke 7' showed higher daily sap flow (2.8 kg day^{-1}) compared to 'Toro Caynon' (2.0 kg day-1) on ungrafted plants and in the case of grafted plants it also increased the sap flow when 'Duke 7' was used (Fassio *et al*., 2009).

Several authors compared normal (vigorous) trees with dwarf trees of citrus, olive, mango, and *Copaifera langsdorfi* found that dwarf trees are characterized by narrow (small) vessel elements and a higher density of xylem vessel elements (Saeed *et al*., 2010; El Said *et al*., 2013; Rashedy *et al*., 2014; Longui *et al*., 2014, respectively) which has also been found in avocado (Reyes-Santamaría *et al*., 2002). The results found showed that the plants of Group 4 are the ones that had the highest densities of vessels and with this, probably less vessel element diameter, increasing transport and conferring a low probability of suffering cavitation and embolism (Núñez-Colín *et al*., 2006). On the other hand, Goncalves *et al.* (2007) and Tombesi *et al.* (2010) indicated that in cherry and peach dwarf rootstocks, respectively, the diameter of xylem vessel elements is smaller. It has been found in peach that large diameters of xylem vessel elements are found in the more invigorating rootstocks (Bruckner and DeJong, 2014). For all the above, it is inferred that genotypes belonging to Group 4 are small size plants or with dwarfing characteristics, this statement is reinforced by studies that showed that intermediate and small size plants frequently have a smaller stem diameter, such as the case of the Group 4 plants that had the lowest average stem diameter values (Fig. 7). These results show a similar trend to the study of López Jiménez and Barrientos Priego (1987) in trees of 'Colín V-33' where the trunks in dwarf trees had a circumference and average diameter smaller than the tall trees.

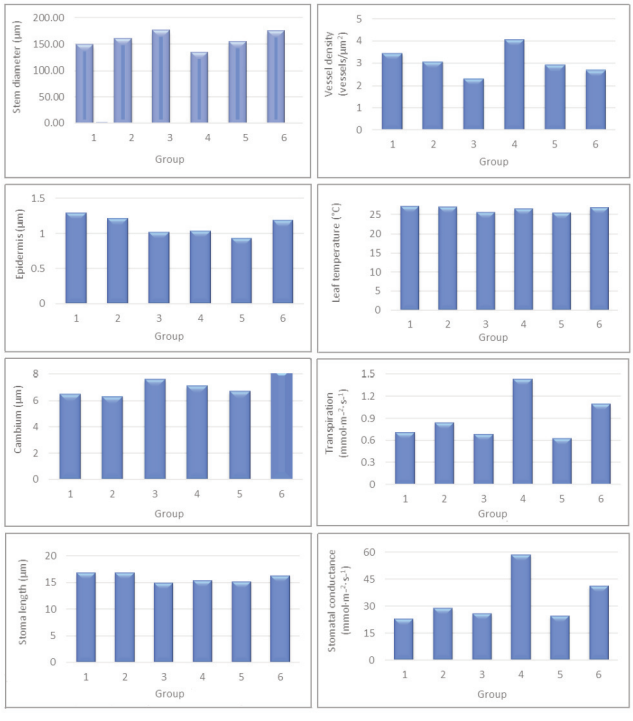

Fig. 7 - Discriminant variables of plants derived from 'Colín V-33' avocado seedlings in the six groups generated according to canonical discriminant analysis (ADC) and Ward cluster, derived from anatomical and physiological characteristics of stem and leaf.

The classification results show that 97.8 % of originally grouped individuals were correctly categorized (Table 6), which indicated that the six groups based on this are consistent, showing the stability of belonging to each group. Where only two individuals were atypical of their group. These results give certainty to the analysis performed and the congruence of the groups obtained.

4. Conclusions

The variables stem diameter, the density of vessel elements, thickness of the epidermis, leaf temperature, stomata length, thickness of the cambium layer, transpiration rate and stomatal conductance, discriminated the groups of 'Colín V-33' avocado seedling plants correctly.

The main correlations obtained were a positive correlation between the density of xylem vessel elements with the rate of transpiration and with stomatal conductance. These correlations indicated a linear relationship of these variables and this last can be used as an index of preselection to discriminate the vessel element density in seedlings.

A higher density of elements of xylem vessel elements and smaller stem diameter appear to be indicators of dwarf plants in avocado, so these characteristics could allow the selection of genotypes with behavior of this type of growth.

Group 4 genotypes presented anatomical and physiological characteristics probably associated with dwarf types unlike Group 3, which were plants with typical anatomical and physiological characteristics of vigorous plants.

To demonstrate the use of the different contrasting groups it is required further studies with grafted plants to determine their potential according to their rootstock characteristics.

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Table 6 - Summary of a priori resubstituting test using the linear discriminant function of 'Colín V-33' avocado seedlings and formed by anatomical and physiological characteristics of the stem and leaf

	Number of observations and percentage of classified by group						
Group	1	2	3	4	5	6	Total
1	20*	1	0	0	0	0	21
%	95.24	4.76	0.00	0.00	0.00	0.00	100
2	0	9*	0	0	0	0	9
%	0.00	100	0.00	0.00	0.00	0.00	100
3	0	0	12*	0	0	1	13
%	0.00	0.00	92.31	0.00	0.00	7.69	100
4	0	0	0	13*	0	0	13
%	0.00	0.00	0.00	100	0.00	0.00	100
5	0	0	0	0	16	0	16
%	0.00	0.00	0.00	0.00	100	0.00	100
6	0	0	0	0	0	17*	17
%	0.00	0.00	0.00	0.00	0.00	100	100
Total	20	10	12	13	16	18	89
%	22.47	11.24	13.48	14.61	17.98	20.22	100

*97.8 % of individual grouped and correctly classified.

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Zucchini squash production in conventional and organic cultivation systems

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Key words: *Cucurbita pepo* L., fruit quality, protected cultivation, sustainability, water use efficiency, yield.

Abstract: Organic production must be carried out following EU regulations and protocols. On the contrary, conventional cultivation instead can be carried out using the best agronomic approaches available and using the latest innovative resources. Organic cultivation is more widespread in permanent crops (olive and grape crops) than vegetable ones, and even less in protected cultivation systems, due to the high intensity production processes which render the application of organic growing protocols more complex. The comparison between the two systems of cultivation, organic and conventional, is difficult because the two cultivation methods are often carried out in different farms and hence in different environmental conditions. Cultivation using the two methods was conducted in a greenhouse from November to March 2017/2018. Results demonstrated that the total fruit yield zucchini squash in organic cultivation was not significantly different to the conventional one (43.2 Mg ha^{-1} and 46.4 Mg ha^{-1}, respectively). The agronomic inputs (fertilizers, fungicides, and insecticides) were higher in the organic cultivation system than conventional one. The water use efficiency was higher in the conventional cultivation system (150.6 kg m^{-3} ha^{-1}) compared to the organic one (147.6 kg m^{-3} ha^{-1}). No statistically significant differences were found for the fruit number per plant and for the marketable fruit at the end of the growing period. Significant differences for the harvest period were only detected for fresh weight, shape index, firmness, and titratable acidity. In conclusion, this work demonstrated that the organic system required higher inputs compared to the conventional cultivation. The extensive experience of the grower allowed for comparable yields between the two systems.

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1. Introduction

Both the demand for organic vegetables and cultivation areas have been increasing following market demand. Environmental benefits claimed by organic producers clearly contributed to building a positive consumer attitude towards organic. The primary request for organic produce is the absence of pesticide residuals or the presence of any agrochemical that is not allowed in the EU-defined organic protocols in regulation n. 834/2007 and 889/2008, and more recently, n. 848/2018. Organic

farming has become the fastest-growing agricultural sector, accompanied by a constantly increasing consumer demand for organic produce. The organic agriculture accounts for approximately 37.3 billion € in the European Union (EU) and it is the second-largest single organic market in the world. In 2018, the countries with the largest organic agricultural areas were Spain (2.2 million hectares), France and Italy (2.0 million hectares each) (Willer *et al*., 2020).

In Europe, the area dedicated to organic farming has increased in recent years, reaching 15.6 million hectares (Willer *et al*., 2020). The distribution of organic farmland by location is 15% in Spain, 12% in Italy, 11% in France, and 8% in Germany (Brzezina *et al*., 2017).

The total area of organic vegetables represents only 0.6% of the total area devoted to vegetables worldwide. Europe, with 184,373 ha, represents 47.6% of the total area and ranks first worldwide, followed at a distance by North America (73,238 ha). Italy with 60,732 hectares is at the second place worldwide after the United States (Willer *et al*., 2020).

Quality and safety of organic produce must be guaranteed at harvest and during postharvest. The restricted use of pesticides and fungicides, and the predominant use of organic matter for fertilization during cultivation may increase the microorganism contamination of the product. The organic rules are stringently applied to vegetable production because the growing cycles are short and the rapid turnover of crops requires frequent soil tillage, with a negative effect on the soil structure and organic matter content. Soil fertility can be maintained by frequent organic material supply and adequate crop rotation (Watson *et al*., 2002).

Organic agriculture is considered one of the best alternatives for sustainable and good quality food production (Aninowski *et al*., 2020). The comparison between organic and conventional cultivation systems is very difficult to perform because the agronomic management and strategies cannot be the same. Organic vegetable production must follow EU regulations, which outline specific protocols to follow and the agronomic choices are limited. Therefore, organic production is sometimes difficult, especially in environments with high levels of biotic stresses such as pests and diseases (Raigon *et al*., 2010). Agronomists can use only organic certified products and exploit the positive interactions among crops for controlling pests and diseases and for plant nutrition. The conventional vegetable cultivation system has a wide range of choices and the experience of the agronomist can play an important role in increasing the yield and quality of the products. Conventional cropping systems follow innovative technologies and year-by-year, new hybrids or cultivars can be adopted as well as new fertilizers, plant growth regulators, pesticides, etc. (Odegard and Van der Voet, 2014).

In organic cropping systems, the nutrients must be provided by certified organic fertilizers or through appropriate crop rotations (Thorup-Kristensen *et al*., 2012). In long term organic vegetable cultivation, the nutrients are provided by manure or by catch crops and intercrops. However, organic farming is strictly regulated by rules and laws thus allowing a better comparison of its performances with conventional farming methods, with and without the use of agrochemical inputs and/or the adoption of specific growing practices (Gomiero *et al*., 2011).

Organic farms which specialize in vegetable production have more difficulty compared to farms involved in livestock and mixed production. These difficulties are represented by the lack of manure produced in the farms and the supply of organic matter must be provided by green manure that represents a loss of a cultivation cycle.

In many studies (Raviv, 2010; Campanelli and Canali, 2012; Rahmann *et al*., 2017) the great majority of organic systems are refer to open field conditions; only recently such alternative organic systems of production have been tested in protected conditions (Tittarelli *et al*., 2017). In fact, organic greenhouse production is still a small sector of the organic industry and constitutes only a small proportion of total greenhouse production (Gamliel and Van Bruggen, 2016).

The different studies that have compared organic and conventional production systems have provided inconsistent results with regard to the sensorial quality and nutritive value of fruits (Bourn and Prescott, 2002; Lester, 2006; Zhao *et al*., 2006) but organically grown foods have lower pesticide residues (Trewavas, 2004). This is not surprising because comparing the effect of organic and conventional farming systems on fruit quality is inherently difficult due to the wide range of factors that can potentially affect crop composition such as climate, soil conditions, cultivar, soil type, planting date, harvesting time, and growing seasons (Goldman *et al*., 1999; Adam, 2001; Magkos *et al*., 2003). In other studies, however, where organic vegetables were compared to conven-

tional ones, a higher concentration of health promoting components has been found (Brandt and Mølgaard, 2001; Rembialkowska, 2003, 2007). Rembiałkowska (2000) found a higher content of total sugars in organically produced vegetables (carrots, sugar beet, red beetroot, potatoes, spinach, savoy cabbage). Hallmann (2012) showed that organic tomatoes presented a higher ratio of reducing sugars/organic acids, and contained significantly more total sugars, vitamin C and total flavonoids, 3-quercetin rutinoside, and myricetin in comparison with the conventionally-grown fruits.

The main difference between conventional and organic cultivation systems is that conventional agricultural systems are continuously evolving due to the introduction of innovative techniques, while organic cultivation must follow fixed protocols that are revised at an interval of several years. In general, organic farming is represented by an articulated series of variables related to the biotic and abiotic factors affecting growth and the final product (Lester and Saftner, 2011). The physical, chemical and biological/nutritional attributes of soils, the irrigation management and water quality, the crops/genotypes and the growing cycles, the harvesting, handling and storage methodologies are the main variables which affect organic and conventional produce quality.

Zucchini squash and pumpkins within the three major species of *Cucurbita* are important crops worldwide. In the Mediterranean region, and in particular in Italy, zucchini squash (*Cucurbita pepo* L.) is an important commercial crop, both in the open field and in the greenhouse. Zucchini squash is generally cultivated in soil under greenhouse conditions for off-season production, but in the last years soil-less cultivation has been strongly developed because it improves the product quality and increases plant defenses against diseases (Van Os *et al*., 2002). For these reasons, greenhouse zucchini crops are usually cultivated during two growing seasons (Spring-Summer and Summer-Autumn seasons) to respond to the high demand for this fresh vegetable in national and international markets (Rouphael and Colla, 2005).

The aim of this work was to compare the productivity and inputs (fertilizers, insecticides, and fungicides) of conventional and organic zucchini squash cultivation systems carried out in a greenhouse. Both farms were in the same geographical area allowing for a comparison under reduced environmental interferences so that differences could be attributed to the crop management systems.

2. Materials and Methods

Greenhouse conditions

Zucchini squash (*Cucurbita pepo* L.) 'Sibilla' was grown under conventional and organic procedures, commonly adopted in the Sicily Region for zucchini squash production. The experiment was conducted in 2017/2018 in two 240 m^2 unheated polyethylene tunnels located in Syracuse (36°59.1' N, 15°12.6' E, 30 m above the sea level), Sicily, Italy: one devoted to organic (20 years under organic regime) and other to conventional horticulture systems. Plants were grown under natural light conditions. The mean temperature was 16.5 °C and the mean relative humidity levels were 75.5%. The total radiation levels ranged from 4.5 to 14.6 MJ m^{-2}. Zucchini squash seedlings were transplanted at the two-leaf stage on 2nd November 2017 for both methods of cultivation, in rows 1.1 m apart, with an along-row spacing of 0.8 m, giving a planting density of 0.88 plant m^{-2}. Preliminarily, bottom fertilization was performed with cattle manure at a dose of 1500 kg ha^{-1} in organic system and a total amount of phosphorus (P_2O_5 as triple super phosphate), and potassium (K_2O as potassium sulfate) and one-third of the nitrogen (as ammonium sulfate) were applied for the conventional cultivation system. Specifically, 120 kg ha^{-1} of N, 45 kg ha^{-1} of P_2O_5, and 265 kg ha^{-1} of K_2O were added.

The following products were used during the preparation of the soil and cultivation of the plants:

Organic cultivation system system

Pre-trasplant: Siveg GR (Biolchim S.P.A.); 6: 6: 12 Orga Kem (Biolchim S.P.A).

After transplanting: NOV@ (Biolchim S.P.A.); Folicist® (Biolchim S.P.A.); zsdqdaEDTA Zinc (Biolchim S.P.A.); Keliron® (Biolchim); Bio Energy® VEG (Biolchim S.P.A.); Glibor Ca (Biolchim S.P.A.); Mn sulfate (Biolchim S.P.A.); Protamin Cu 62 (Fertilgest); Mg sulphate (Biolchim S.P.A.); Fylloton (Biolchim S.P.A.); Cremalga (Biolchim S.P.A.); Microfol mix (Biolchim S.P.A.); Mg sulphate (Biokimia International S.r.l.).

Fertigation: NOV@ (Biolchim S.P.A.); Bio Energy® VEG (Biolchim S.P.A.); Glibor Ca (Biolchim S.P.A.); Mg sulphate (Biokimia International S.r.l.).

Foliar application: NOV@ (Biolchim S.P.A.); Bio Energy® VEG (Biolchim S.P.A.); Fylloton (Biolchim S.P.A.); Folicist® (Biolchim S.P.A.); Cremalga (Biolchim S.P.A.); Glibor Ca (Biolchim S.P.A.); Mg sulphate

(Biokimia International S.r.l.).
Pesticide: Sulphur 95% (Mannino S.P.A.).

Conventional cultivation system

Pre-trasplant: Siveg GR (Biolchim S.P.A.); 6: 6: 12 Orga Kem (Biolchim S.P.A).

After transplanting: Phostart Zn (Biolchim S.P.A.); Urea sulfate 70 (Fertilgest); Fulvumin (Biolchim S.P.A.); Keliron® (Biolchim S.P.A.); Kemical® (Biolchim S.P.A.); 20.20.20 fertilizer (Valagro S.P.A); Protamin Cu (Fertilgest).

Foliar fertilizations: Microfol® Mix (Biolchim S.P.A.); Urea sulphate low biuret (Fertilgest); Nitrocam® (Biolchim S.P.A.); Loker® (Biolchim S.P.A.); Green-Go 12.8.24+10 (Fertilgest); Magnitron (Biolchim S.P.A.); Fulvumin (Biolchim S.P.A.).
Foliar application: Nitrocam® (Biolchim S.P.A.); Kriss (Biolchim S.P.A.); Rizzamina® 42 (Fertilgest).

Pesticide: Karma® 85 (Certis Europe, Italia); Tiovit® JET (Syngenta Italia).

Similar types of machinery were used in both cultivation systems. The final stage of cultivation involved the harvesting of zucchini squash fruits, which was performed manually and so did not affect the relative environmental performance of conventional and organic systems (Table 1).

Organic cultivation system

The zucchini squash cultivation was performed following the procedures described in EU n. 834/2007 and 889/2008.

In this system Siveg GR and 6: 6: 12 Orga Kem were applied at 35 cm depth in pre-transplant, respectively at doses of 400 and 1500 kg ha^{-1}; after the spreading, the products were appropriately topped up.

During the preparatory phase of the organic growing media, in addition to the background fertilization, biostimulants and nutrients were added according to the following scheme:

- seventh day: NOV@ (15 L ha^{-1}), Folicist® (2 L ha^{-1}), EDTA Zinc (2.5 L ha^{-1}) and Keliron® (2 kg ha^{-1});
- twentieth day: NOV@ (10 L ha^{-1}), Folicist® (1.3 L ha^{-1}), Bio Energy® VEG (20 L ha^{-1}), Glibor Ca (3.5 L ha^{-1}) and Mn sulfate (5 kg ha^{-1});
- thirtieth day: Protamin Cu 62 (3.5 L ha^{-1}), Mg sulphate (7 kg ha^{-1}) and Keliron® (3.5 kg ha^{-1}).

The following top dressings were applied 10 and 20 days after transplanting:
tenth day: Fylloton (1.5 L ha^{-1}), Folicist® (1 L ha^{-1}), Cremalga (1 L ha^{-1}) and Microfol mix (1 kg ha^{-1}).
twentieth day: Cremalga (1 L ha^{-1}), Mg sulphate (1.5 kg ha^{-1}) and Folicist (1 L ha^{-1}).

During cultivation, fertigation and foliar application were performed starting from the fourteenth day and at two week-intervals.

The fertigation was carried out with the addition of NOV@ (15 L ha^{-1}), Bio Energy® VEG (20 L ha^{-1}), with Glibor Ca (7 L ha^{-1}), and Mg sulphate (20 kg ha^{-1}); Foliar application was performed with a solution containing NOV@ (15 L ha^{-1}), Bio Energy® VEG (20 L ha^{-1}), Fylloton (1.5 L ha^{-1}), Folicist® (1 L ha^{-1}), Cremalga

Table 1 - Cultivation procedures (inputs) of the organic and conventional systems per hectare of squash cultivation

Cultivation procedures (inputs)	Cultivation systems	
	Organic	Conventional
Land use (m^2)	10.000	10.000
Mean yield (Mg)	43.2	46.4
Irrigation		
PE irrigation hoses (m)	6667	6667
Submersible electric pump SHANKTY, QF 106/9 + pump motor HP 50 8''(h)	45	45
Water (m^3)	2100	2100
Fruit harvesting (h)	19	19
Machinery		
Harrowing (h)	8	8
Tillers (h)	8	8
Fertilizer spreader (h)	5	5
Spreading plastic mulching (h)	16	16
Soil tillage (h)	15	15
Spreading plastic tunnel coverage (h)	98	98
Plastic maintenance	28	28

(1 L ha^{-1}), Glibor Ca (4.5 L ha^{-1}), and Mg sulphate (2.5 kg ha^{-1}).

Manual weed control was carried out twice, to eliminate the weeds that grew during the cultivation period.

The defence against Oidium was carried out weekly with the use Sulphur 95% (35 kg ha^{-1}), after 3-4 days from the foliar fertilization (Fig. 1).

Conventional cultivation system

In this system Siveg GR and 6: 6: 12 Orga Kem, as for the organic system, were applied in pre-transplant, respectively at doses of 400 and 1500 kg ha^{-1}; after the spreading, the products have been appropriately topped up. After transplanting, the following products were applied on the indicated day:

seventh day after transplantation: Phostart Zn (20 L ha^{-1}), Urea sulfate 70 (10 L ha^{-1}) and Fulvumin (15 L ha^{-1});

fifteenth day: Keliron® (3.5 kg ha^{-1}), Kemical® (15 L ha^{-1}) and Fulvumin (15 L ha^{-1});

twenty-eighth day: the 20.20.20 fertilizer) (20 kg ha^{-1}), Fulvumin (10 L ha^{-1}) and Protamin Cu (3.5 kg ha^{-1}).

In the same period two foliar fertilizations were applied;

tenth day: Microfol® Mix (1 kg ha^{-1}) and Urea sulphate low biuret (5 kg ha^{-1});

twentieth day: Nitrocam® (2 L ha^{-1}) and Loker® (3 L ha^{-1}).

Weekly fertigation and foliar application were applied during plant cultivation. Fertigation was performed with Green-Go 12.8.24+10 (35 kg ha^{-1}), Magnitron (15 kg ha^{-1}) and Fulvumin (10 L ha^{-1}), until the end of crop production. Foliar application consisted of of Nitrocam® (3 L ha^{-1}), Kriss (0.85 L h^{-1}), and Rizzamina® 42 (1.8 kg ha^{-1}).

Defence against Oidium was carried out weekly with the use of Karma® 85 (3 kg ha^{-1}), and Tiovit® JET (0.3 kg ha^{-1}) after 3-4 days from the foliar fertilization. Manual weed control was carried out as described for organic cultivation system (Fig. 1).

Sampling procedure and measurements

The fruits were harvested every two days and those obtained at the beginning, in the middle and at the end of the harvest were transported to the laboratory of the Department of Agriculture, Food and Environment Science (Di3A) of Catania University (Italy), and immediately analyzed.

Agronomic data and fruit physical parameter from the greenhouse experiment such as plant productivity, fruit weight, shape index, color, thickness epicarp etc. were measured. Water Use Efficiency (WUE) was calculated as yield/water consumed (kg m^{-3}) (Yaghi *et al.*, 2013).

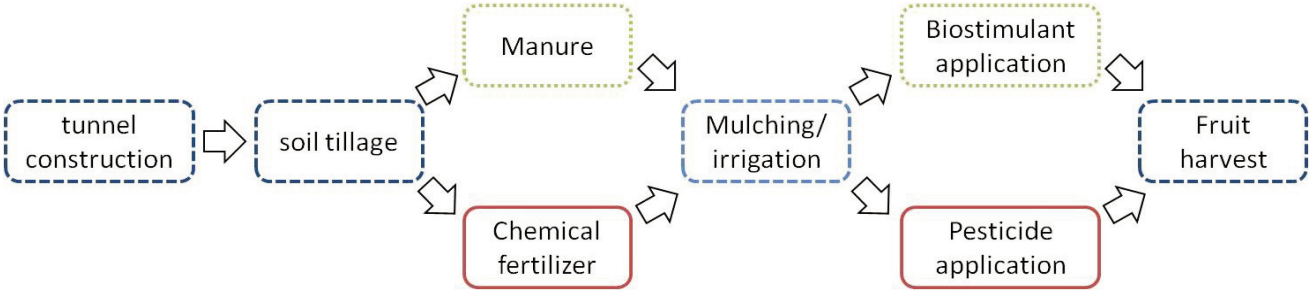

Fig. 1 - Processes included in the conventional and organic cultivation systems.

The epicarp and mesocarp color was measured using a Chroma Meter CR-200 (Konica Minolta, Japan) based on light reflectance. The color was expressed using the Commission Internationale de l'Eclairage (CIE) system where the L*, a* and b* values represent the lightness, green-red and blue-yellow, respectively. The dry matter (DM) content was obtained by drying samples in a thermo-ventilated oven at 70°C to constant weight.

The firmness of the zucchini squash was measured using a compression test based on the resistance of the fruit to deformation in the middle portion using a texture analyzer (TA.XT2i, Stable Micro Systems Ltd., Godalming, UK) incorporating a 2 mm diameter probe. Eighteen recordings were performed for each treatment. The values were expressed as the maximum shear force (N).

Titratable acidity (TA) was measured by titration with a solution of sodium hydroxide 0.1 mol L^{-1}, up to the point of phenolphthalein turning, and expressed as meq L^{-1} of citric acid. Total soluble solids (TSS, °Brix) were read in a digital refractometer with automatic compensation for temperature (model Brix PR-

1, Atago CO., Ltd, Tokyo, Japan).

Statistical analysis

The experiment was conducted as a randomized complete-block design with three replications to compare two cultivation methods: conventional and organic. Each experimental unit consisted of six plants (18 plants for cultivation methods). The statistical analyses were performed using CoStat version 6.311 (CoHortSoftware, Monterey, CA, USA); pairwise comparisons for productivity parameters were done using t-test for means of samples with unequal variances. Two-way ANOVA for quality and color parameters was used. The differences between the means were determined using Tukey's test ($P<0.05$). Interaction effects were calculated using the Tukey's test at a 5% level of significance.

3. Results

Crop productivity

The harvest period lasted nine weeks, with the first and last harvest dates on the 16th December 2016 and 7th March 2017, respectively; in total, 57 harvests were done during this period for both cultivation methods. The average harvesting interval was 1.4 days during the cultivation period.

The total fruit yield of conventional zucchini squash was similar to the organic cultivation method (46.4 Mg ha^{-1} and 43.2 Mg ha^{-1} respectively) (Fig. 2). No statistically significant differences were found for the fruit number per plant at the end of the growing period (21.5 fruits $plant^{-1}$ in conventional cultivation and 22.9 fruits $plant^{-1}$ in organic cultivation). Similarly, no significant differences between the conventional and organic cultivation were recorded for the marketable fruit yield plant: 4.1 and 3.8 kg $plant^{-1}$, respectively (Table 2). The percentage of unmarketable fruit weight was 3.5% and 2.6% in organic and conventional, respectively, without significant differences.

Water use efficiency and external inputs

The water use efficiency (WUE) was higher in the conventional than in organic cultivation system with 150.6 and 147.6 kg m^{-3} ha^{-1}, respectively. In the conventional production system, the amount of fertilizers used were 4.8 kg Mg^{-1} ha^{-1} and 0.3 L Mg^{-1} ha^{-1}, solid and liquid, respectively (Table 2). The organic vegetable production showed higher fertilizers input compared with conventional cultivation system, with 11.4 kg t^{-1} ha^{-1} and 0.5 L t^{-1} ha^{-1}, solid and liquid, respectively. For plant protection purposes, solid fungicides were used in both growing systems.

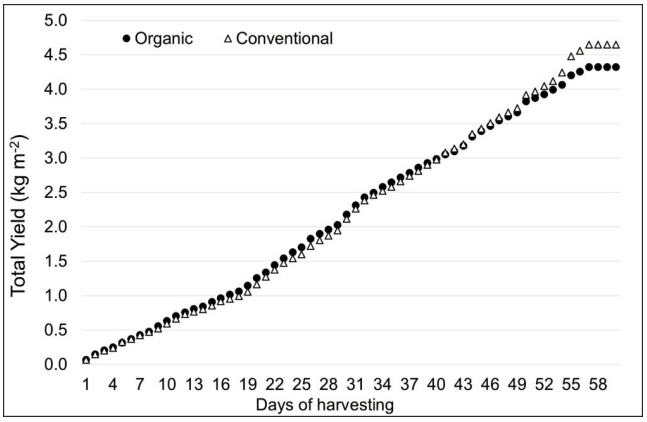

Fig. 2 - Cumulative trends of zucchini squash yield (kg m^{-2}) during the harvesting period (60 days) under conventional (•) and organic (Δ) cultivation methods.

The amount of fungicides used was almost indistinguishable, with 0.11 kg Mg^{-1} ha^{-1} used in the conventional cultivation system as compared to 0.12 kg Mg^{-1} ha^{-1} applied in the organic cultivation regime. Solid insecticides used were 103-fold higher in organic cultivation system compared to the conventional one, while the liquid insecticides were 4.8-fold higher in the organic compared to the conventional cultivation system.

Fruit quality parameters

During the cultivation period, three samples of

Table 2 - Productivity parameters of zucchini squash grown under different cultivation methods

Method of cultivation	Fruit yield (Mg ha^{-1})		WUE (Kg m^{-3} ha^{-1})	Fruit number (n. $plant^{-1}$)	Fruit yield plant (kg)
	Early	Total			
Conventional	8.0±0.6	46.4±3.1	150.6 a	21.5±0.8	4.1±0.4
Organic	7.9±0.7	43.2±2.9	147.6 b	22.9±0.9	3.8±0.4
Significance	NS	NS	*	NS	NS

Means within columns separated using t-test ($P<0.05$).

fruits were taken for quality evaluation (one at the beginning, one approximately in the middle, and one at the end of production).

Significant differences for fresh weight, shape index, firmness, and titratable acidity were only detected for the harvest period (Table 3). No significant difference for dry biomass percentage was found (Table 3).

With regard to the total soluble solids content, the conventional cultivation method showed an effect of interaction (Cultivation methods x Harvesting time): the fruits of the plants cultivated using the organic method have maintained, for the entire cultivation period, higher values, while those harvested in conventional cultivation have shown a reduction at the end of the growing period (by 7%) (Table 3 and Fig. 3).

Measurements of surface color demonstrated significant differences between the cultivation methods only with regard to L* mesocarp, which was reduced: the fruits obtained in the organic method have recorded a uniformity of the values, while those obtained in conventional cultivation method have shown lower values corresponding to the second harvest (Table 4 and Fig. 4).

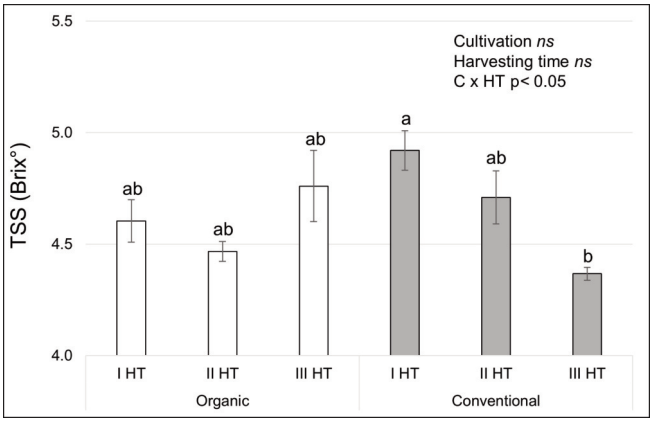

Fig. 3 - Interactions between the two cultivation methods, Organic (I, II, III HT) and Conventional (I, II, III HT), and harvesting time on TSS (Brix°). The vertical bars indicate ± S.E. of means (n=18). Columns denoted with the same

4. Discussion and Conclusions

In greenhouses, where intensive cultivation systems are widely used, the differences between conventional and organic approaches are especially evident. Organic production in greenhouse operations is a challenging task (Gamliel and Van Bruggen, 2016).

The production of vegetables in organic conditions presents more technical challenges than conventional cultivation, because many practices, such as the use of non-natural agrochemicals, are not permitted in organic production under the regulations of many countries (Raigon *et al*., 2010). Consequently, organic production is sometimes difficult, especially in environments with high levels of pests and disease pressure.

Organic cultivation systems that are specifically dedicated to the vegetable production do not have their own manure supply and must buy a majority of their most of agronomic inputs, as such as fertilizers, biocontrol agents, natural compounds, and biostimulants for controlling pests and diseases and for plant nutrition from the market. Results demonstrate that

Table 3 - Quality parameters of zucchini squash grown under different cultivation methods

Factors		Fresh weight (g)	Shape index	Firmness (N)	% DM	TSS	Titratable acidity
Method of cultivation (C)	Harvesting Period (H)						
Conventional		188.7±6.6	4.7±0.1	12.3±3.3	6.01±0.1	4.7±0.1	0.6±0.0
Organic		183.5±7.8	4.6±0.1	12.5±2.7	5.99±0.1	4.6±0.1	0.7±0.0
	I	158.7±3.0 b	4.6±0.1 b	11.6±1.2 b	6.00±0.1	4.8±0.1	0.7±0.0
	II	200.6±3.8 a	4.9±0.0 a	12.2±2.2 b	6.01±0.1	4.6±0.1	0.7±0.0
	III	199.0±2.5 a	4.4±0.1 c	13.4±1.4 a	6.00±0.0	4.6±0.1	0.6±0.0
Significance							
Cultivation (C)		NS	NS	NS	NS	NS	NS
Harvesting period (H)		***	***	***	NS	NS	NS
C x H		NS	NS	NS	NS	*	NS

Values are means of main effects of method of cultivation and harvesting period.
The statistical analysis was two-way ANOVA;
NS not significant; * significant at P<0.05; *** significant at P<0.001. The values in the same column followed by the same letter are not significantly different at P<0.05 (Tukey's test).

Table 4 - Color parameters (L*, a* and b*) in epicarp and mesocarp of zucchini squash grown under different cultivation methods

Factors		Epicarp			Mesocarp		
Method of cultivation (C)	Harvesting Period (H)	L*	a*	b*	L*	a*	b*
Conventional		56.8±0.1	-27.3±0.1	10.8±0.1	63.4±0.2 b	-29.6±0.1 a	14.3±0.3 b
Organic		56.8±0.1	-27.1±0.1	10.6±0.1	64.0±0.1 a	-29.8±0.1 b	14.6±0.2 a
	I	56.9±0.1	-27.5±0.1 b	11.0±0.1 a	64.1±0.1 a	-30.0±0.0 b	15.1±0.1 a
	II	56.8±0.1	-27.0±0.0 a	10.5±0.0 b	63.3±0.4 b	-29.5±0.1 a	14.6±0.1 b
	III	56.7±0.1	-27.1±0.1 a	10.6±0.1 b	63.7±0.1 ab	-29.5±0.1 a	13.7±0.1 c
Significance							
C		NS	NS	NS	**	**	*
H		NS	***	***	**	***	***
C x H		NS	NS	NS	*	NS	NS

Values are means of main effects of method of cultivation and harvesting period.
The statistical analysis was two-way ANOVA;
NS not significant; * significant at P<0.05; *** significant at P<0.001. The values in the same column followed by the same letter are not significantly different at P<0.05 (Tukey's test).

the grower, after years of cultivation, found a wide range of agronomic inputs that allowed for the highest yield that was similar to the conventional farm.

In literature, it is well-known that mineral elements released by the organic fertilizers are not promptly available and the lag of time may reduce growth and yield. This problem can be compensated by higher inputs of specific organic fertilizers and biostimulants.

The differences observed for crop yield between organic and conventional growing systems range from 5 to 34%, while on the average, organic cultivation can reach about 80% of the yield of conventional cultivation but with substantial variations depending on the growing system and site characteristics (De Ponti *et al*., 2012; Meier *et al*., 2015; Ciaccia *et al*., 2019). Some studies, however, point out that the effect of cultivation method disappears when the results are converted to absolute dry matter, because often differences are due to water content (Pieper and Barrett, 2009). The yield in organic cultivation system was about 30 Mg ha^{-1} and is similar to those observed in other open field cultivation experiments with yields averaging 30.7 Mg ha^{-1} (Conti *et al*., 2015). Colla *et al*. (2002) found similar results to our in tomato, with no differences in yield between the organic and conventional cultivation methods, whereas a lower yield was found in the organic growing system compared to the conventional one for zucchini (Maggio *et al*., 2013). A strong yield reduction of about 25% was observed in summer zucchini squash grown using organic fertilizers (Dasgan and Bozkoylu, 2007). Conventional mineral nutrition inputs can provide nutrients when plants really need them, while organic fertilizers or matrixes release nutrients following degradation kinetics that usually cannot promptly satisfy plant requirements. This long-term effect of the organic nutrient tools can slow down plant growth and negatively affect the yield. However, there is little information about nutritional and sensorial quality (aroma and volatile organic compounds between the two growing systems), and food safety of organic versus conventional crops (Gennaro and Quaglia, 2003).

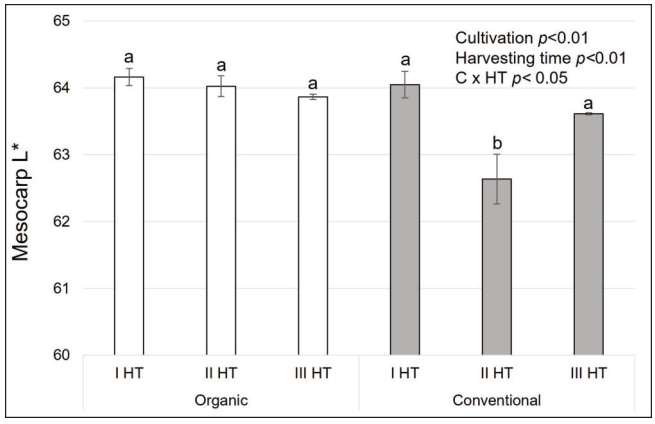

Fig. 4 - Interactions between the two cultivation methods, Organic (I, II, III HT) and Conventional (I, II, III HT), and harvesting time on mesocarp L* value. The vertical bars indicate ± S.E. of means (n=18). Columns denoted with the same letters are not significantly different, as determined by Tukey's test (P < 0.05).

Specific cultivars for organic cultivation are not available; in greenhouse tomato, the use of F_1 hybrid

cultivars was beneficial to the organic system, being superior to non-hybrids (Santa Rosa *et al*., 2019) as normally occurs in conventional cultivation systems.

Observation of consumer expectations on food quality presents the base for any successful food production system and marketing scheme. This is also true for fruits and vegetables which are increasingly valued as an important part of the diet (Péneau *et al*., 2006).

Appearance, colour, texture, and aroma are arguably the most important criteria used by consumers to evaluate the immediate quality of a product and thus, persuade them to buy it (Ragaert *et al*., 2004). In our experiment, quality parameters were not significantly affected by cultivation systems. Between the two cultivation systems, differences in fruit colour, firmness, and titratable acidity, were found in relation to the harvesting date. In analogous comparison experiments, fruit color between organic and conventional cultivation showed higher L*, a*, and b* values in the organic cultivation system. These results can be ascribed to agronomic management techniques but also to varieties and different geographical cultivation areas (Armesto *et al*., 2020).

Many studies on the quality of organic vegetables indicate a higher nutritional value and a higher content of biologically active compounds in agricultural crops from organic farming (Brandt and Mølgaard, 2001). In other related vegetable crops, such as tomato or pepper, it has also been found that production under organic conditions has a significant effect on fruit composition, which normally consists of an increase in the content of antioxidants and minerals (Chassy *et al*., 2006; del Amor *et al*., 2008). For this reason, organic agriculture is considered one of the best alternatives for sustainable and good quality food production (Aninowski *et al*., 2020).

Our results highlighted that the main significant changes were observed in growth parameters. The product quality was mainly influenced by environmental conditions that changed according to summer weather. Therefore, quality changes were visible at the different harvesting dates. It is known that vegetable crops have higher requirements compared to other crops and short cycles require appropriate agronomic management. The higher inputs do not always provide a better quality or higher yield in organic system. Meta-analysis performed on different crops highlighted a wide variability among crops in both organic and conventional systems. The majority showed higher inputs in conventional cultivations system (Seufert *et al*., 2012). However, the evaluation of both systems can provide useful information only if the cultivations are performed in the same environments and differences can be really attributed to the agronomic managements.

Our results showed that zucchini squash crop can be grown in organic or conventional cultivation systems with no significant changes in fruit quality. The organic system reduced the yield even if higher inputs of agronomic tools were required for the crop management. As reported by Rouphael *et al*. (2015), understanding the functional links between cultural factors and physiological responses is an important requisite to enhance the quality of organic products.

The organic cultivation was able to give comparable yield to conventional one under protected cultivation. Almost all the analysed qualitative parameters of fruits were not statistically different between the two systems, except for the TSS and L-mesocarp, to underline the possibility to adopt organic procedures also in greenhouse. The obtained results highlighted the difficulties of performing a comparison between these two cultivation systems because of the different variables that can change. Our study was carried out in a geographic area where the organic cultivation for vegetable crops has been long established and the contemporarily presence of organic and conventional cultivation systems allowed us to perform a scientific and reliable study. In conclusion, this work demonstrated that the organic system required higher inputs compared to the conventional cultivation system. The extensive experience of the grower allowed for comparable yields between the two systems. However, further evaluations should be performed for understanding the economic and environmental sustainability of zucchini squash production in the two cropping systems.

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Short note

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Impact of Moroccan *Crocus sativus* L. tepals, corms, and stigmas extract on growth and photosynthetic pigments in tomato seedling

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Key words: Biostimulant, chlorophyll, Saffron by-product, *Solanum lycopersicum* L.

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Abstract: An experiment was carried out in a greenhouse to study the effect of aqueous extracts of *Crocus sativus* L. by-products on tomato plants. Three concentrations of tepals and corms were used by fertigation: 1 g/L, 2 g/L, and 3 g/L. The aqueous extract of the stigmas was used as a foliar application at 0.6 g/L. The experiment was carried out in a completely randomized block with three repetitions for each concentration. The concentration of tepal extract at 3 g/L significantly (p≤0.05) increased the plants' height, the chlorophyll a, b content in the leaf. The same results were observed for the foliar treatment with stigmas; however, there was no effect of tepal extract on the carotenoid content. On the other hand, the concentration 2 g/L of the corms extract had a positive impact (p≤0.05) in the chlorophyll b content while the concentration of 3 g/L increased the plant's height, the chlorophyll a (p≤0.05). Current results indicate that *Crocus sativus* by-products could improve certain physiological aspects of the recipient plants and be new and natural biostimulants.

1. Introduction

Saffron (*Crocus Sativus* L.) is a stemless, bulbous perennial plant that belongs to the Iridaceae family (Taylor et *al*., 2008). Moroccan surfaces grown from saffron are about 1000 ha, mainly in the mountains of the *AntiAtlas*. This production area takes more and more importance in other regions (Lage and Cantrell, 2009). In the production of saffron, for each kilogram of spices produced, about 53 kg of tepals are made (Maggi et *al*., 2012), and 90% of the total fresh weight corresponds to the by-products composed of tepals, which are generally discarded as waste (Menghini et *al*., 2018). Hundreds of corms too small for flowering to be replanted are dumped to obtain only 1 kg of dry stigmas (Smolskaite et *al.*, 2011). However, this biomass is a potentially significant source of bioactive compounds. Many researchers have focused their attention on valuing saffron by-products to increase crop profitability, such as floral bio-residues and

corms (Lahmass et *al.*, 2018). The enhancement of these properties of saffron floral bio-residues was evaluated on the antioxidant activity (Sanchez-Vioque et *al.*, 2012), the anti-inflammatory activity (Amin and Hosseinzadeh, 2015), the antifungal and cytotoxic activity (Zheng et *al*., 2011), and antibacterial activity (Shadmehri et *al*., 2019). On the other hand, the tepals are used in many industries, as active ingredients in various food (Tuberoso et *al*. 2016), in cosmetic formulations (Natalia et *al*., 2019), and as a potential resource of natural color thanks to the high content of anthocyanins for food applications, and biomedical (Shadmehri et *al*., 2019). Bioactive components of corms include proteoglycans and saponins, which have shown antifungal and antioxidant activity (Rubio-Moraga et *al.*, 2011). In a preliminary study, saffron stigmas showed a biostimulant and antifungal effect on the tomato plant (Khoulati et *al*., 2019). The applied treatments significantly improved plant height and positively affected the tomato fruit quality after enhancing the secondary metabolites' content.

These bioactive molecules of tepals and corms are supposed to have an effect biostimulant on recipients' plants. Therefore, the current study was planned to confirm the use of tepals and corms extract as a biostimulant on the morphological photosynthetic pigment parameters of tomato seedlings. The study will promote *Crocus sativus* L. by-product and produce new and natural biostimulants of plant growth.

2. Materials and Methods

Plant material

Tomato cultivar was sown in plastic trays until germination, at a temperature of 27°C ±2, 70% relative humidity, photoperiod of 16 h/8 h light/dark. At four leaves emergence, the seedlings were transferred to plastic pots (33x18 cm) containing 60% sand, 35% peat (Floragard 50/50, v/v), and 5% gravel and maintained at the optimum temperature (28/ 20°C, day/ night) and natural daylight under greenhouse in the northeast of Morocco (34°50'33" N, 2°10'18" W). The containers are placed on 100 cm apart rows and 60 cm within rows. There was no fertilizer application, and all the plants received the same volume of irrigation water.

One week later, the first treatment is applied. Tepals and corms extracts at three different concentrations (1 g/L, 2 g/L, and 3 g/L) were used both as fertigation with a 200 mL volume by one plant and 0.6 g/L used foliar application by the saffron stigmas. Distilled water was taken as a control treatment. Each replicate set contained three seedlings, and the experiment had three replications in a completely randomized trial. After one week from the first application, the treatments were repeated, keeping the same concentration and method as the first application. One week after the second application, and when the biostimulant effect was visually observed, samples were collected to determine the morphological and photosynthetic pigment indices.

Aqueous extract preparation

Saffron stigmas originating from Taliouine (Morocco) were purchased, milled, and conserved at -20°C until each use. The dried tepals were harvested during the 2019 production in the Tinissane region in the northeast of Morocco (34°50'33" N, 2°10'18" W). The tepals were milled and stored at - 20°C until each use. The corms were obtained from Taliouine and kept at -20°C after drying. Before each application, the concentration of tepals and corms powder used for the study left 24 h in 1 L of distilled water for extraction in the dark for each concentration: 1 g/L, 2 g/L, and 3 g/L. For stigmas foliar application, 0.6 g extracted in 1 L of the distilled water 24 h in the dark.

Quantification of the main components of extracts

- The anthocyanin contents of tepals were measured according to the protocol explained by Ganjewala et *al*. (2008). The absorbance (Abs) of the samples was measured at 530 nm, and the results were expressed in milligram equivalent cyanidin 3-glucoside by 100 grams of dry matter (mg CGE/100g DM). Flavonol's contents were determined by measuring the absorbance at 360 nm, and the results were expressed in milligram equivalent quercetin 3-glucoside by 100 grams of dry matter (mg QGE/100g DM).
- The total polyphenol of tepals was determined using the Folin-Ciocalteu method (Velioglu *et al.*, 1998). The total polyphenols concentrations were expressed in milligrams of gallic acid equivalent to 100 grams of dry matter (mg GAE/100 g DM).
- The content of crocin, picrocrocin, and safranal of saffron stigmas was determined following ISO 3632 (ISO, 2003).

Morphological indices of tomato seedling

Plant height was recorded using a measuring tape in cm. Shoot fresh weight was measured immediately

after rooting up the plants using an electronic balance. To determine the dry weight, samples were oven-dried for 24 h at 80 °C, and the value of the samples recorded in grams.

Photosynthetic pigments

The chlorophyll content and carotenoid were determined by crushing 0.2 g fresh leaf sample in 20 mL of 80% acetone and placed at room temperature for 48 h in the dark. A spectrophotometer was used to observe the absorbance at 663, 645, and 652 nm of the samples (Lichtenthaler, 1987). Chlorophyll a, b, and total chlorophyll calculated using the following formula:

$$\text{Chlorophyll a (Chl a)} = (12.7 \times Abs663) - (2.69 \times Abs645)$$
$$\text{Chlorophyll b (Chl b)} = (22.99 \times Abs645) - (4.68 \times Abs663)$$
$$\text{Total chlorophyll} = \text{Chl a} + \text{Chl b}$$

$$\text{Total carotenoid content} = \frac{(1000\ Abs470 - 1.82\ Chla - 85.02\ Chlb)}{198}$$

Statistical analysis

All figures and statistical analyses were performed using SPSS Statistics 17.0 software. The data were expressed as the mean ± standard deviation (SD) of triplicate independent experiments and analyzed using a one-way analysis of variance (ANOVA). $p \leq 0.05$ was treated to be statistically significant. The Student-Newman-Keuls (SNK) test was used to classify averages using different letters for significant differences.

3. Results

The main components of extracts

The anthocyanins, flavonols, and polyphenols contents of tepals were 43.66 ± 0.79 mg CGE/100 g DM, 0.19 ± 0.21 mg QGE/100 g DM, and 672.73 ± 1.02 mg GAE/100 g DM, respectively. Total crocin, safranal and picrocrocin contents in the saffron sample are 184.56 ± 2.19 mg/g, 4.89 ± 0.18 mg/g, and 62.43 ± 0.37 mg/g, respectively.

Morphological indices

Variations in the morphological data revealed the significant effect of tepals, corms, and stigma extracts on tomato seedling growth in terms of concentrations used (Figs. 1, 2). For the application of tepal extracts by fertigation, the highest plant height was recorded for plants fertigated with 3 g/L followed by 2 g/L. In contrast, the lowest values were observed in

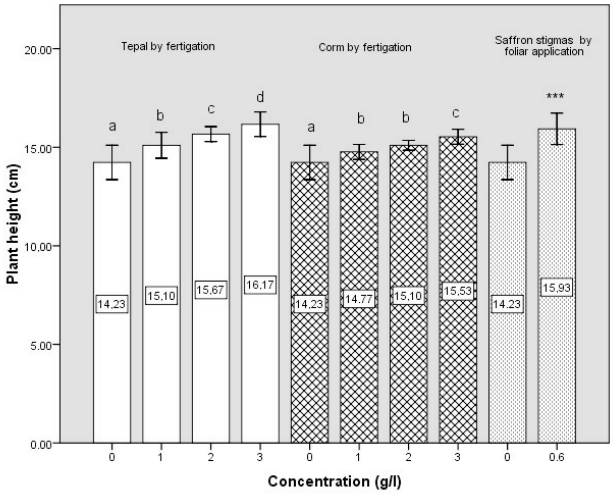

Fig. 1 - Saffron stigmas, tepals, and corms extracts affect the height of the tomato seedling. Measurements were made one week after the last treatment. Data were recorded for a total of three plants by treatment replicated three times. The concentration of saffron was represented as 0.6 g/L. The concentrations of tepals and corms are defined as 1 g/L, 2 g/L, and 3 g/L. 0: control. Bars represent averages and error lines one standard deviation. The difference in the letters indicates significant differences at $p \leq 0.05$ based on the SNK test. ***: the significative difference at $p \leq 0.001$.

Fig. 2 - Tepal, corm, stigma extracts application to influence the tomato plant's plant height. The pictures were taken one week after the last treatment. 0 represents control treatment. A) treatment with petal extract by fertigation (2 g/L, 3 g/L). B) treatment with corm extract by fertigation (1 g/L, 2 g/L, 3 g/L). C) foliar application by saffron stigma extract at 0.6 g/L.

control seedlings. Concerning the treatment of corms highest plant was recorded for plants fertigates with 3 g/L followed by 2 g/L and 1 g/L compared to the control. However, the treatment by stigmas foliar application showed a significant difference at p≤0.001 compared to the control (Figs. 1, 2). Table 1 represents fresh shoot weight, dry shoot weight, fresh root weight, and dry root weight. As compared to control, the fresh and dry weight of seedlings were increased with tepal application and reached a significant level at 2 g/L followed by seedlings applied with 3 g/L. However, the same concentrations of applied tepal increased the weight of root fresh and root dry at 2 g/L, followed by 3 g/L. However, no effect of the application of the different concentrations of corm extract observed.

Photosynthetic pigments

The effect of tepals and corms on the chlorophyll contents of tomato leaves is depicted in figure 3. As it can be observed, the various applications influenced significantly enhanced the chlorophyll a, b, and total chlorophyll content. Concerning the use by tepal fertigation, the high value was recorded for the concentration of 3 g/L with an increase of 45.96 % for Chla and 28.71 % for Chlb, compared to the control. The application by corm fertigation at 3 g/L has increased Chla and Chlb concentrations of 37.12 % and 15.22 %, respectively (Fig. 3), compared to the control. On the other hand, the three corm extract concentrations significantly increased the carotenoid content (Table 1). However, no effect was observed on the carotenoid content after fertigation by the three different tepals (Table 1).

For foliar application by saffron stigmas, the Chla, Chlb, and carotenoid content significantly increased 11.59 % for Chla, 56,84 % for Chlb, and 55,69% for

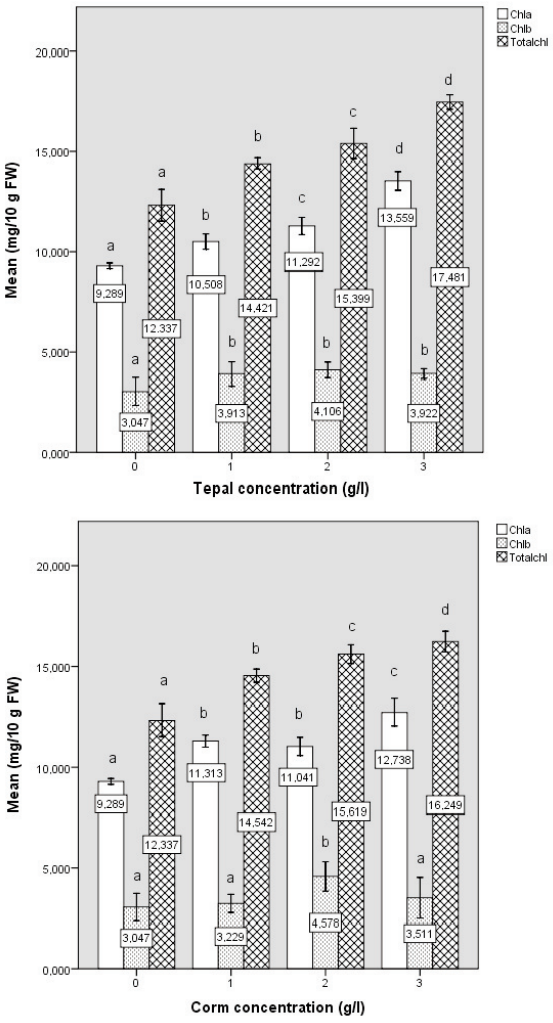

Fig. 3 - Effect of tepal and corm extract on pigment system of tomato leaves. Data are the mean ± standard deviation of three replicates. The column followed by different letters shows a significant difference at p ≤ 0.05 significance level between treatments according to the SNK test. FW= fresh weight. Chla= chlorophyll a; Chlb= chlorophyll b; Totalchl=: total chlorophyll. 0= control.letters are not significantly different, as determined by Tukey's test (P<0.05).

Table 1 - Influence of tepal and corm application on the root and shoot weight of tomato plants and carotenoid leaves

Application	Concentration (g/L)	Root fresh weight (g)	Root dry weight (g)	Shoot fresh weight (g)	Shoot dry weight (g)	Carotenoid (mg/g FW)
Control	0	0.185±0.289 a	0.0192±0.004	1.72±0.135 a	0.132±0.017	2.431±0.108 a
Tepal	1	0.195±0.009 a	0.021±0.001 *	1.68±0.355 a	0.118±0.036	2.507±0.209
	2	0.261±0.341 b	0.0323±0.005 *	2.641±0.564 b	0.218±0.046 *	2.703±0.335
	3	0.212±0.013 a	0.0283±0.008 *	2.332± 0.156 ab	0.218±0.046 *	2.985±0.337
Corm	1	0.138±0.427	0.017±0.003	1.436±0.613	0.119±0.060	2.98±0.652 b
	2	0.141±0.018	0.019±0.003	1.516±0.548	0.107±0.045	2.889±0.147 b
	3	0.142±0.519	0.016±0.004	1.495±0.591	0.01±0.044	2.708±0.15 b

The difference in the letters indicates significant differences at p≤0.05 based on the SNK test.
* = significative difference at p≤0.05.
FW= fresh weight.

carotenoid (Fig. 4, Table 1). However, no significant difference was reported from the different tepal extract concentrations on the carotenoid content (Table 1). The different concentrations of the corm extract participated in the increase of the Chla and Chlb content but not in the same way as the tepal extracts; the highest value in Chla in plants treated with 3 g/L of the extract corms is 12.73 mg/10 g FW and while the highest value in Chla in plants treated with 3 g/L of the tepal extract is 13.55 mg/ 10 g FW. However, a significant difference is observed in the carotenoid content of plants treated with extracts of corms, while this difference in carotenoid is absent in plants treated with tepal extracts. Chlorophyll content, especially Chlb of plants treated with the application of stigmas extract at 0.6 g/L, increased. The carotenoid content also increased after treatment with the stigma extract.

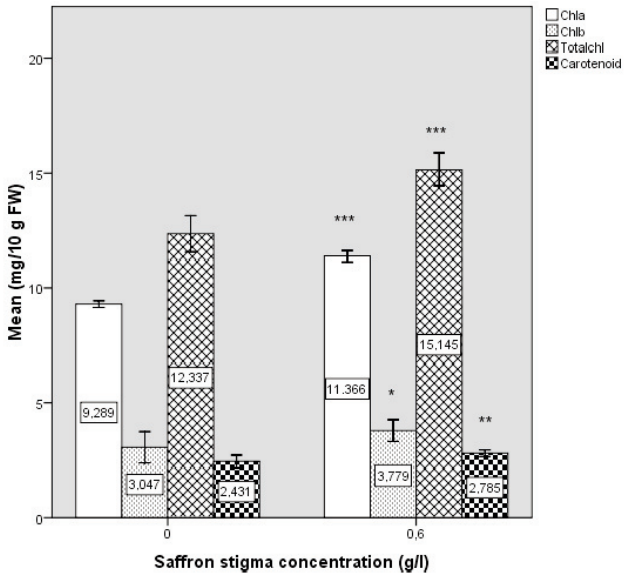

Fig. 4 - Effect of saffron stigmas on pigment system of tomato leaves by foliar application. Data are the mean ± standard errors of three replicates. ***= the significative difference at p≤0.001. *0 the significative difference at p≤0.05. FW= fresh weight. Chla= chlorophyll a; Chlb= chlorophyll b; Totalchl= total chlorophyll. 0= control.

4. Discussion and Conclusions

The present study's objective was to valorize the tepals, corms, and stigmas of *Crocus sativus* L. and take advantage of its bioactive components to use them as a biostimulant alternative to chemicals products. Therefore, three tepals and corm extract (1 g/L, 2 g/L, and 3 g/L) are chosen to assess their effect by fertigation in some morphological and biochemical parameters of tomato seedling under greenhouse conditions in a completely randomized block.

The application of tepal, corm, and stigmas extracts has influenced the growth of the plant. Tepal has been reported as a protein source, fiber, fats, and essential minerals (K, Ca, and P) necessary for plants' growth (Fahim et *al.*, 2012; Khazaei et *al.*, 2016). Tepals are also rich sources of phenolic and biologically active compounds such as flavonoids (kaempferol, rutin, quercetin, luteolin, hesperidin, and bioflavonoids), tannins, and anthocyanins (Kanakis et *al.*, 2006; Srivastava et *al.*, 2010). The increased growth of tomato plants suggests that tepal extract may act as a promoter of plant growth with a 3 g/L concentration, which contains 1.308 mg CGE/L of anthocyanin and 20.181 mg GAE/L of polyphenol. Corms also are a rich source of phenolic, flavonoid, and especially saponin (Rubio-Moraga *et al.*, 2013), likely to participate in increased growth characteristics of the treated plants in the results of current research. Saffron stigmas contain nitrogenous substances, anthocyanins, glycosides, monoterpenes, aldehydes, flavonoids, vitamins, volatile oils, proteins, carbohydrates (Amin and Hosseinzadeh, 2015). Also, some studies indicated the presence of micro-nutrients in saffron such as Zn, Mn, and certain amino acids (Priscila del Campo et *al.*, 2009; D'Archivio et *al.*, 2014), which favored the development of the plant height and that the current results strongly corroborated with our previous study (Khoulati et *al.*, 2019) with a concentration of 0.6 g/L, which contains 110.73 mg/L of crocin, 2.93 mg/L of safranal, and 37.45 mg/L of picrocrocin in the extract used in this study.

Photosynthesis is one of the primary processes of plant metabolism impacted by external conditions (Kalaji et *al.*, 2017). Chlorophyll is a critical component of photosynthesis which absorbs sunlight (Hörtensteiner and Kräutler, 2011). It occurs in chloroplasts as green pigments in all photosynthetic plant tissues (Mazumder and Paul, 2014). Chla, the primary photosynthetic pigment of photosystems I and II, converts the energy of light. Chlb is an accessory pigment that absorbs light's energy (Petit et *al.*, 2012).

On the other hand, carotenoids are also essential for plants because they protect the photosynthetic apparatus from light-mediated stress. Carotenoids participate in a wide range of physiological process-

es, including growth, development, and plant responses to environmental stimuli, and protect plants against photo-oxidative damage (Racchi, 2013).

The application of tepal and corm extract by fertigation, and saffron extract by foliar application, significantly improved the chlorophyll content. The tepal extract increased the Chla content in a linear manner (R^2 = 0.947); the increase in the Chla content correlated with the rise of the concentration of the tepal extract (r = 0.973) (Fig. 5). This increase in chlorophyll content may result from reduced chlorophyll degradation, which may be related mainly to polyphenol in the extract and many other molecules that can increase the pigment content. They can also activate enzymes responsible for the regulation and photosynthetic reduction of carbon and the chloroplast protection against oxidative damage. Also, they can include compounds that play a photoprotective role by scavenging reactive oxygen species. The exogenous application of biostimulant has been shown to activate specific genes involved in the transcription of proteins for photosynthetic processes (Trevisan et *al.*, 2011) . However, higher chlorophyll content may involve growth stimulation and primary metabolic responses of treated plants compared to control plants (Shalaby and El-Ramady, 2014).

In this study, we concluded that the aqueous extract of tepals and corms at 3 g/L had a bio-stimulatory effect on tomato plants. The use of these extracts in specialized horticulture practices such as organic farming may be a better solution to ensure quality performance and does not present risks for the user and the consumer. Besides, these results present an advantage for the farmer practicing the culture of *Crocus sativus* by recovering the waste which they throw before like tepals.

However, future research needed to explore the influence on the treated plants' primary and secondary metabolites provides a platform to elaborate and study the biological activity of the extracts inside the treated plants to identify and later confirm the molecular patterns involved in the bioactive mechanism of these extracts.

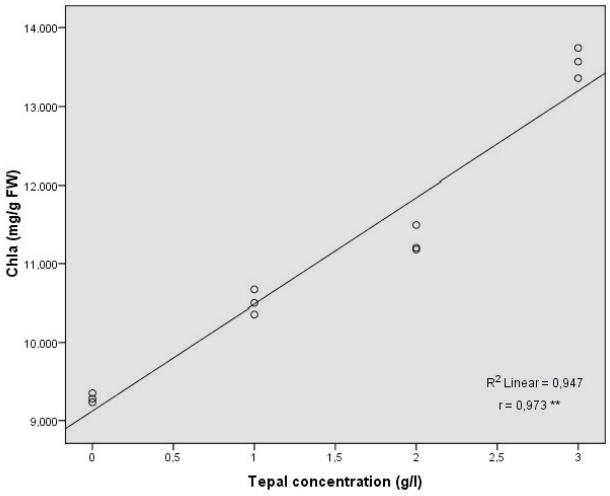

Fig. 5 - Linear regression curve between chlorophyll a, and the concentrations of tepal extracts. Chla= chlorophyll a. **= correlation is significant at the 0.01 level. R^2= R-squared.

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