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Optimizing of the quality of rose grown with varying ratios and periods of Red: Blue light-emitting diodes in commercial greenhouse

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ABSTRACT

Purpose: This research investigated the effect of different regimes of supplementary LED light on physiological and morphological traits of two cultivars of cut Roses. **Research method:** In this study, treatments included cultivars (Allstar and Dolcevita) and nine light regimes including (Control, LED night from 5 pm to 7 am, LED day from 7 am to 5 pm, LED night day (24 hours light) and LED dark (24 hours, without sunlight) which were all applied in two intensities of 2000 and 4000 lux. **Findings:** The results showed that the LED light regime had a positive effect on morphological traits such as the number of buds and mean harvest, length, diameter and fresh weight of flowers. LED day 4000 lux improved bud diameter and mean harvest by 18% and 112% respectively compared with control in Dolcevita cultivar. The light regime also caused a significant improvement in physiological characteristics so that in Allstar cultivar LED day 4000 lux, chlorophyll a, b, total, and carotenoid were increased by 66%, 60%, 63%, and 64% respectively compared with control. The vase life in Allstar cultivar by LED night day 4000 lux intensity and Dolcevita cultivar by LED day 4000 lux were 44.5% and 133.2% higher than the control treatment, respectively. **Research limitations:** There was no limitation. **Originality/Value:** The results showed that LED night day with 4000 lux intensity had the best results in vase life in Allstar cultivar and LED day supplementary light with 4000 lux intensity increased the quantity and quality characteristics of roses.

INTRODUCTION

Rosa hybdria is one of the most popular cut flowers. Several elements influence the quality and postharvest life of roses after they have been cut (Shi et al., 2021). These factors are classified into two categories: preharvest and postharvest. The most important preharvest stage parameters that have a significant influence on appearance quality, physiological features, and vase life of flowers are irrigation conditions, mineral nutrients, and the amount and quality of light. Among these elements, light is crucial for floral quality, biosynthesis, and pigment content (Rasouli et al., 2015).

Livadariu et al. (2023) state that LED lighting has become more prevalent in the past few decades for the commercial breeding of various economically important species in horticulture and agriculture. LED lights are long-lasting, have high radiative efficiency, and switch quickly. Furthermore, they enable the selection and customization of output spectrum characteristics, which meet the demands of the plant and allow for high-quality harvests including antioxidant capacity (Carotti et al., 2021). In recent studies by Song et al. (2022), it has been reported that different light wavelengths have the ability to regulate various plant processes, including photosynthesis, germination, flowering, biomass accumulation, and phytochemical synthesis. Research indicates that red light plays a crucial role in the development of the photosynthetic apparatus and influences morphogenesis through light-induced transformations of the phytochrome system. On the other hand, blue light can impact chlorophyll biosynthesis, stomata opening, and photomorphogenesis (Song et al., 2022; Sakurako et al., 2021). As red and blue light are the primary absorption peaks of photosynthetic pigments in plant leaves, they have a significant impact on plant photosynthesis. However, it is important to note that a single red or blue light is insufficient for normal plant growth. Studies suggest that a specific proportion of red and blue light is necessary for optimal plant growth (Song et al., 2022).

The effect of light on the proportion of plant hormones is one of the processes in the effect of light on plant growth and morphology. Kurepin et al. (2010) discovered that long-term culture under changing light conditions has an effect on internal hormone levels, particularly auxin, and induces homeostasis. Furthermore, it appears that high-intensity blue light damages or does not synthesize auxin. According to Cioć et al. (2022), the application of red and red:blue light in gerbera flowers led to a decrease in tissue auxin levels. In contrast, the use of blue LED light resulted in a lowered the shoot multiplication rate and their height, but it induced the highest content of gibberellins in the final stage of the culture.

According to Alsanius et al. (2017), red LED light 80% + blue 20% with 16 hours of illumination and intensity of 7000 lux improved sunflower plant height and stem diameter. Schroeter-Zakrzewska and Pradita (2021) discovered that Chrysanthemum plants grown under red + blue light had the highest leaf greenness index (SPAD) value and the shortest cuttings with the longest roots. Additionally, white + blue light significantly affected most of the growth parameters, except for plant height and the number of leaves.

Cut flower post-harvest senescence is a dynamic process involving physiological and biochemical changes that are governed by a cell death program. Carnation senescence's physiological and biochemical features have already been characterized, and conditions during mother plant growth, storage and handling, environment, and phytohormones all play roles in senescence regulation (Aalifar et al., 2020). Postharvest or vase life as a commercial attribute impacts the market's flexibility at any given time, particularly for cut flowers. The limited vase life of cut rose flowers is related to physio-chemical processes that influence aging. Water loss and wilting during transit have a significant impact on these characteristics.

Water scarcity and the resulting premature senescence result in poor cut flower quality and market loss, according to numerous reports (Alaey et al., 2011).

Optimizing the quality of roses in commercial greenhouses through different ratios and durations of LED lighting is an important research topic in agriculture today. As it has a significant effect on the quality and performance of roses in greenhouses. By determining the optimal ratio and duration of red:blue LED lighting, growers can ensure that their roses receive the right amount and type of light, which can lead to improved quality, marketability, and vase life. This, in turn, can lead to a reduction in energy consumption and an increase in profitability for producers. In this regard, the present research investigates the influence of supplemental LED light on the physiological and morphological features of two cut rose cultivars, “Allstar” and "Dolcevita," in a commercial greenhouse under different LED light regimes. The study also examines the effect of pretreatment of roses with LED on their vase life. The findings of this research can provide valuable insights into the optimal lighting conditions for rose growth and inform future research and innovation in the field.

MATERIALS AND METHODS

Culture conditions and light treatments

This experiment was conducted in the Sivan Energy commercial rose greenhouse in Dahagan, Isfahan province, as a factorial experiment in the form of a completely randomized design (CRD). This study included three replications and focused on two rose cultivars, Allstar and Dolcevita. Allstar and Dolcevita rose plants were imported from the Dutch company De Ruiter, Amstelveen, and the Netherlands, whose standard morphological characteristics are shown in Table 1 according to the manufacturer's information. Then, the hydroponic bed was prepared and the rose bushes, which were approximately 25 cm in size and had 2-3 leaves, were planted in the perlite bed with a distance of 19 cm between the bushes. To start establishing the plants, they were watered for two weeks, and then NPK fertilizer (20-20-20) was given to the rose plants for one month and the plants were fed daily with the nutrient solution, the information of which is included in Table 2 (Nikbakht & Ashrafi, 2019)

Light regime treatments (red wavelength 60%+blue wavelength 40%) included 9 levels: Control (without using LED light), LED night (5 pm to 7 am) with two intensities of 2000 and 4000 lux+sunlight, LED day (7 am to 5 pm) with two intensities of 2000 and 4000 lux+ sunlight, LED day and night (24 hours of light) with two intensities of 2000 and 4000 lux+sunlight and LED dark (24 hours without sunlight) with two intensities of 2000 and 4000 lux (Table 3).

Five months after the growth and full establishment of the plants, LED lamps were placed at a distance of approximately one meter from the substrate to provide the desired light intensity on the leaf surface. The light intensity of 2000 (35µmolm^{-2} s^{-1}) and 4000 (75µmolm^{-2} s^{-1}) lux was adjusted by adjusting the amount of use of these lamps in the panels, and the wavelength of the lamps used was 660 (red), and 460 nm (blue) (Fig. 1 and Fig. 2). The control treatment received only sunlight and no LED light was applied. In the LED night treatment, LED treatments applied during the day and night, the light intensity of the LED lamps was 2000 and 4000 lux along with the sunlight intensity. But in the LED dark treatment, the plant environment was darkened with black covers to minimize the effect of sunlight intensity, then the light regimes were applied on Allstar and Dolcevita rose cultivars with three repetitions, each repetition including six rose bushes. The light treatments were applied for about three months and during this period we had six to seven peak harvests, where the measured parameters were obtained from the last harvest.

Table 1. Specifications of Allstar and Dolcevita cultivars in standard conditions.

Cultivar	Shape	Bud length (cm)	Stem length (cm)	Vase life (days)	Production in square meters per year (flower branch)
Allstar		5.4-5.5	40-60	10-12	180-220
Dolcevita		5-6	50-80	7-10	150-200

Table 2. Hydroponic nutrition solution formula in this experiment.

(µM)	Micro elements	(mM)	Macro elements
30-50	Fe	11/25-11/5	NO_3
5-6	Mn	1-1/5	NH_4
4-5	Zn	1/25-1/3	H_2PO_4
30-40	B	4/5-5	K
0/60-0/75	Cu	3/5-4	Ca
0/5-0/6	Mo	1/25-1/3	Mg
1/6	EC	1/25-1/3	SO_4
5/5	pH	1/25-1/3	HCO_3

Table 3. Introduction of experimental treatments.

Cultivar	Light regimes
Allstar/Dolcevita	Control (Without LED light)
	LED night(5 pm to 7 am) with 2000 lux intensity + sunlight
	LED night(5 pm to 7 am) with 4000 lux intensity + sunlight
	LED day (7 am to 5 pm) with 2000 lux intensity + sunlight
	LED day (7 am to 5 pm) with 4000 lux intensity + sunlight
	LED night day (24 hours light) with 2000 lux intensity + sunlight
	LED night day (24 hours light) with 4000 lux intensity + sunlight
	LED Dark (24 hours, without sunlight) with 2000 lux intensity
	LED Dark (24 hours, without sunlight) with 4000 lux intensity

LED include red wavelength 60% + blue wavelength 40%

Fig. 1. LED lamps installed in the greenhouse.

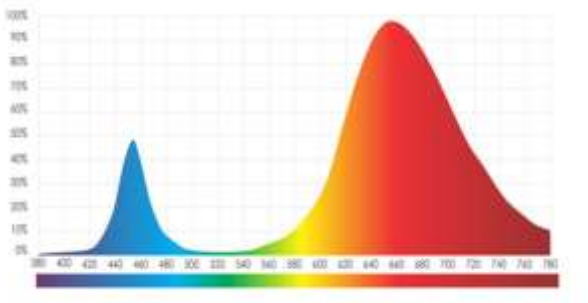

Fig. 2. Wavelengths used in LED boards.

Harvesting and measuring the growth parameter

Throughout the experimental stages from February 15 for 10 weeks in order to check the effect of the treatments, the number of flowers that were produced in each treatment from each plant was picked and recorded. The number of buds was counted one week after the treatment, regardless of whether the resulting buds changed into harvestable flowers, and the counts were averaged for each repetition individually and recorded weekly for ten weeks, with the results given for each replication after averaging. The length of the stem from under the sepal to the cut point (ten weeks) was also measured with a ruler and reported for each replication.

For each repeat of three flowers, the weight of cut flowers was measured in three phases at two-week intervals using a scale with an accuracy of 0.01 g for 30 cm long cut flowers, and its size was measured for each repetition and reported after averaging. After weighing three flowers for each replication, they were packaged in paper envelopes and placed in a 70-degree oven for 48 hours to determine the dry weight of cut flowers with a length of 30 cm. The number of leaves on each bloom was counted in each repeat and averaged for each repetition, and this experiment was repeated ten times with a one-week interval. The leaf area of the plants was measured using an Alborz Andisheh Technologies Co. leaf area measurement device, model Winarea-UT-11, in each replication of 8-10 leaves.

Physiological factors

A sample of 5 g of the tissue was homogenized with 80% acetone; the residue was filtered and adjusted to 10 mL; the absorbance reading was done at 476, 646, and 663 nm using the spectrophotometer (Model UV 160A- Shimadzu Corp., Kyoto, Japan) (Arnon, 1949). The content of carotenoids was calculated based on the formula (1) presented by Pérez -Grajales et al. (2019).

$$\text{Total chlorophyll} = \text{Chlorophyll a} + \text{Chlorophyll b}$$

$$\text{Carotenoids} = 100(A476) - 3.27(\text{mg/g Chl. a}) - 104\ (\text{mg/g Chl. b})/227 \qquad (1)$$

The spectrometric method by Rapisarda et al. (2000) was used to determine anthocyanin. Fresh leaves (1.0 g) were crushed with 20 mL alcohol (60 %; pH = 3.0) and heated the samples on hot water for 2 h; after cooling the samples certain volume of sample solution was used for reading at 535 nm anthocyanin content (mg/100 g FW).

The chlorophyll index of mature plant leaves was used by the chlorophyll meter (SPAD-502 plus, Japan). For this goal, three readings were carried out from each plant on three separate leaves (a total of 9 readings per replicate), and then the average was registered (Dezhabad & Haghighi, 2020). Maximum photochemical quenching Fv/Fm was measured by chlorophyll fluorescence (model OS-30, Minolta Corp). The portable fluorescence monitoring system (Hansatech Instruments LTD RS232) was used for chlorophyll fluorescence determination. The instrument clamp was placed on each leaf for 30 min to complete the dark adaptation. The initial (F0), maximum (Fm), and maximum quantum efficiency of the photosystem-II (Fv/Fm) were reported according to Maxwell and Johnson (2000).

Vase life experiment

The appropriate flowers were selected early in the morning using sterilized scissors and immediately placed in water. They were then taken to Isfahan University of Technology and placed in a cold room at 4°C for 8 hours to absorb water and cool the flowers. The next morning, all flowers were cut at a distance of 30 cm from the end of the flower bud and immediately placed in 500 ml containers containing distilled water that had been disinfected

with 70% alcohol at a temperature of 24±2°C and a humidity of 40±5%, and were kept in 13 hours of light and 11 hours of darkness.

To determine carbohydrates content, the method of Hedg and Hofreiter (1962) was used. In this way, 100 mg of the sample was placed in a boiling bath with 5 mL of 2.5 N hydrochloric acid for 3 hours. Then, a few drops of 2% sodium carbonate were added to each sample. Add 25 mL of distilled water to the resulting solution to reach a volume of 50 ml. 0.1 mL of the resulting solution was removed and made up to 10 ml with distilled water. In the next step, 2 mL of anthrone reagent was added to the solution and placed in a boiling water bath for 1 minute. Then the resulting solution was cooled at the laboratory temperature and its light absorption was read at 630 nm by a spectrophotometer model (Shimadzu UV106A) (Hedge & Hofreiter, 1962). The amount of carbohydrates in the studied sample was estimated in terms of mg/g of dry sample weight using a standard curve. 0, 0.2, 0.4, 0.6, 0.8, and 1 ml of standard were used to prepare the standard curve.

The vase life of the flowers was considered from the time of harvesting to the bending of the neck or falling of the petals and was expressed as a number of days (Shi et al., 2021).

The speed of flower opening was measured by calipers on days 0, 2, 5, and 8, and the maximum amount of flower opening was subtracted from the diameter of the flower on day 0 and divided by the number of days, which was calculated in millimeters per day.

Flower water content was obtained by dividing the difference between the heavier and dry weight of the flower by its heavier weight. This experiment was performed twice in two weeks and for each repetition, the calculation and its numbers were reported separately.

Statistical analysis

The study applied following a factorial experiment in a complete randomized design (CRD) with three replications. The data underwent analysis using SAS program version 9.1 (SAS Institute, Cary, NC). The data performed a two-way analysis of variance (ANOVA), and the means were examined for statistical significance using the least significant difference (LSD) test at a significance level of P<0.05. Principal component analysis (PCA) was carried out using Statgraphics Centurion, Version XVI.

RESULTS AND DISCUSSION

The results of variance analysis are presented in Table 4. The main effects are not shown due to the significance of the interaction effect and the rising length of the article.

Table 4. Analysis of variance of main and interaction effects of treatments.

Source	df	Bud number	Mean harvest weekly	Cut flower height	Bud length	Bud diameter	Number of leaves	Stem diameter	Flower quality
Cultivar	1	0.4877 n.s	0.09086 **	145.366 n.s	5.59185 **	1.70951 **	37.9178 **	0.03393 **	0.42934 n.s
Light	8	10.1285 **	0.20967 **	188.908 **	0.80121 **	0.34802 **	8.0516 **	0.3337 **	3.26838 **
Cultivar×Light	8	0.5771 **	0.05788 **	102.617 *	0.03617 *	0.0469 *	3.2455 **	0.00503 **	0.60103 *
Error	34	0.0889	0.01218	53.881	0.0362	0.02864	0.2841	0.0004	0.25421
CV		6.2	10.4	13.52	3.68	4.56	5.43	2.23	6.51

ns: not significant, * significant at P ≤0.05 and ** significant at P ≤0.01 probability level, df: degree of freedom.

Table 4. (*Continued*).

Source	df	Greenness	Chlorophyll fluorescence	Fresh weight of cut flowers	Dry weight of cut flowers	Leaf surface	Thrips pest	Chlorophyll a of leaves
Cultivar	1	433.217 **	0.00089 n.s	390.48 **	11.6111 * *	2.325E+10 n.s	0.0741 n.s	0.00258 n.s
Light	8	51.644 **	0.00263 **	299.717 **	10.1983 **	2.655E+10 *	20.713 **	0.01543 **
Cultivar×Light	8	15.011 **	0.00074 *	38.426 **	1.1937 **	2.700E+10 *	0.4907 *	0.01058 **
Error	34	3.769	0.0005	3.684	0.2004	2.64E+10	0.1991	0.00125
CV		3.87	2.9	4.8	6.42	4.56E+02	9.41	11.57

ns: not significant, * significant at P ≤0.05 and ** significant at P ≤0.01 probability level, df: degree of freedom.

Table 4. (*Continued*).

Source	df	Chlorophyll b of leaves	Total chlorophyll of leaves	Carotenoids of leaves	Anthocyanin of petal	Carbohydrate of leaves	Vase life	Speed of flower bud opening	Flower water content
Cultivar	1	0.00435 n.s	0.02664 n.s	0.00139 n.s	708460 **	0.21426 **	13.0046 **	0.1057 n.s	0.01051 n.s
Light	8	0.0068 **	0.10137 **	0.00728 **	3398 *	0.24455 **	9.3542 **	0.80612 **	1.19501 *
Cultivar×Light	8	0.00282 *	0.06262 **	0.0045 **	8603 **	0.02369 **	0.9421 *	0.30697 *	0.45356 *
Error	34	0.00149	0.00994	0.00056	1603	0.00705	0.509	0.16335	0.89472
CV		16.69	12.15	12.13	27.29	7.58	6.67	16.39	1.14

ns: not significant, * significant at P ≤0.05 and ** significant at P ≤0.01 probability level, df: degree of freedom.

The effect of LED on morphological factors of two rose cultivar

The number of buds, flower harvest (weekly), cut flower height, bud length, bud diameter

In the interaction effects of the light regime with the cultivar, the Allstar produced the most bud number in the LED day 2000 lux intensity treatment, which was 37% more than the control. Dolcevita produced the highest number of buds with LED night 4000 lux which was 42% more than the control (Table 5). A positive correlation was detected at the 1% level between bud number and bud length, diameter of bud, number of leaves, flower number, greenness, fresh weight, dry weight, carbohydrate, and vase life.

The results of study Park and Jeong (2020) indicated that a 4-h supplementation of blue light with a wavelength of 450 nm during the photoperiod increases flower bud formation in chrysanthemum. Light treatments were found to increase the amount of collected buds and flowers in this study. Heo et al. (2003) found that irradiating cyclamen (*Cyclamen persicum*) with red-blue light at a 1:1 ratio with a light intensity of 5500 lux for 12 hours can enhance the number of flower buds compared to the control and other monochromatic kinds, as well as make the blooms survive longer. On the facility for approximately 20 days longer than the control.

The interaction effect of light treatments with cultivar, the highest number of flowers harvested in Allstar was observed in LED dark 2000 lux treatment, which was 17% compared with the control. In the Dolcevita, the highest number of harvested flowers was observed in the treatment of LED day 4000 lux intensity, which increased by 105% compared to the control (Table 5). There is a positive correlation at the level of 1% with chlorophyll a, total chlorophyll, and relative water content at a 5% level, and a negative correlation was observed with thrips density index (was scored between lower 1 to higher 10 observation).

We can expect varied reactions in the formation of flowers in plants if we use different light spectrums and durations of light presence. The amount of internal auxin in plants can also be regulated by the quality of light. According to one study, the quality of light influences the activity of IAA oxidase and causes an increase in the length of roots and stems, which is most likely produced by a change in the amount of internal auxin in the plant (Iacona & Muleo, 2010). According to Hao et al. (2016), red-blue supplementary light at a ratio of 1:1 with a light intensity of 5000 lux on the cloud plant (Houstonianum cv. Blue Field Ageratum) increased the number of flowers. Gao et al. (2023) conducted an experiment which revealed a lower R:FR ratio of 54%:46% under natural light treatments compared to artificial light treatments. Interestingly, the high proportion of far-red light in natural light was found to promote the flowering number in *Crocus sativus* L. plants.

In the interaction effect of light treatment with cultivar, the highest cut flower height was observed in the Allstar cultivar in the treatment of LED dark 2000 Lux, which was 45% higher than the control. In the Dolcevita cultivar, the highest flower height harvested in LED night 2000 lux treatment was observed, which was a 40% increase in height compared with the control (Table 5). Thrips pest symptom and water content showed a positive and significant correlation at the 1% level, and with the weight, leaf area, and relative water content at the 5% level.

In the interaction effect of light treatment with cultivar, the maximum bud length was observed in the Allstar cultivar in LED night day with 4000 lux intensity, which was 20% more than the control. In the Dolcevita cultivar, the maximum bud length was observed in the LED night 4000 lux, which is a 13% increase in bud length compared with the control (Table 5). The bud length index with the indices of bud number, bud diameter, number of leaves, fresh weight, dry weight, and carbohydrate, a positive correlation was observed at the level of 1% and anthocyanin index at the level of 5%, a positive and significant correlation was observed between the traits.

In the interaction effect of light treatment with the cultivar, the largest bud diameter was observed in the Allstar cultivar in LED night 4000 lux treatment, which was 20% more than the control. In the Dolcevita cultivar, the maximum bud length was observed in LED night day 2000 lux, which is an 18% increase in bud diameter compared with the control (Table 5). The bud diameter with indicators of bud length, stem diameter, number of leaves, fresh weight, dry weight, and carbohydrates, a positive correlation was observed at the level of 1% and a positive and significant correlation was observed with the number of buds at the level of 5%.

Kurepin et al. (2010) discovered that long-term cultivation under different light conditions has an effect on internal hormone levels, particularly auxin, and causes homeostasis. Furthermore, it appears that high-intensity blue light destroys or does not produce auxin, altering the auxin-cytokinin ratio. Acetic acid is broken down, resulting in less longitudinal growth and less internode growth (Lee & Palsson, 1994). Alternating red and blue LED light that was on for 8 hours and off for 16 hours increased plant height in lettuce (*Lactuca sativa*) compared to illumination for 4, 2, and 1 hours (Fu et al., 2017).

Table 5. Interaction effect of LED light regimes and rose cultivar on some flower characteristics.

Cultivar	Light (LED day/night) Lux	Bud number	Mean harvest (weekly)	Cut flower height (cm)	Bud length (mm)	Bud diameter (mm)	Number of leaves	Stem diameter (mm)
	Control	4.25c	1.12ab	41.97e	44.90e	32.16e	7.08c	5.11f
	Night 2000	5.42ab	1.01bc	50.85d	48.10d	38.00bc	8.83b	6.13c
	Night 4000	5.41ab	0.92c	46.62de	50.80cd	38.63bc	9.19b	5.913d
	Day 2000	5.81a	0.94c	55.12cd	52.03c	34.70cd	10.38ab	6.75bc
	Day 4000	5.42ab	1.11ab	51.41d	48.45d	37.16bc	9.73ab	6.32c
Allstar	Night Day 2000	5.66ab	0.92c	58.42bcd	51.29c	35.16bcd	9.43b	6.68bc
	Night Day 4000	5.30bc	0.93c	55.19cd	53.83c	36.33bc	9.50b	6.27c
	Dark 2000	3.16d	1.29a	60.71ab	44.86e	34.03cd	8.63b	5.62e
	Dark 4000	3.08d	1.03bc	53.69cd	42.23e	32.06e	8.04bc	4.96g
	Control	4.47c	0.68d	47.21de	52.20c	34.91cd	9.75ab	5.68e
	Night 2000	5.21bc	1.04bc	66.28a	56.66b	39.63bc	12.40a	7.06ab
	Night 4000	6.36a	1.03bc	56.56cd	58.93a	40.00ab	11.90a	7.05ab
	Day 2000	5.70ab	1.13ab	54.00cd	57.23ab	40.00ab	10.71ab	6.91ab
	Day 4000	5.90a	1.44a	58.63bcd	55.70b	41.16a	10.83ab	6.88ab
Dolcevita	Night Day 2000	5.48ab	1.25ab	59.47abc	55.53b	41.30a	12.07a	6.90ab
	Night Day 4000	5.51ab	1.00bc	59.22abc	58.90a	40.73ab	12.03a	7.62a
	Dark 2000	2.33e	1.03bc	63.38ab	49.95d	38.84bc	7.83c	5.12f
	Dark 4000	2.03e	1.23ab	51.76d	43.80e	33.70c	8.39b	5.05f

In each column, the averages that have at least one letter in common do not have a significant difference at the 5% probability level based on the least significant difference (LSD) test.

Alsanius et al, (2017) reported that red LED light 80% + blue 20% with 16 hours of illumination and intensity of 7000 lux increased the plant height and stem diameter of common sunflower, which is consistent with the present study. The effect of red-blue LED light at a ratio of 1:1 with an intensity of 17,000 lux on tomato (*Solanum lycopersicum*) increased the diameter of the plant stem and the height and growth of the root (Li et al., 2017; Alsanius et al., 2017).

Number of leaves, stem diameter, flower quality, greenness index, and chlorophyll fluorescence

In the interaction effect of light treatment with cultivar, the highest number of leaves was observed in the Allstar cultivar in LED day 2000 lux treatment, which was 47% more than the control, and in the Dolcevita cultivar in LED night 2000 lux treatment, which was 27% more than the control (Table 5).

In the interaction effect of light treatment with the cultivar, the maximum stem diameter was observed in the Allstar cultivar in LED day 2000 lux treatment, which was 34% compared with control. In the Dolcevita cultivar, the maximum stem diameter was observed in LED night day 4000 lux, which is a 35% increase in the stem diameter compared to the control, (Table 5). The stem diameter index with the indicators of the number of buds, length of buds, length of buds, number of leaves, quality of flowers, fresh weight, dry weight, and carbohydrates, a positive correlation was observed at the level of 1%, and a positive correlation was observed with the index after harvest at the level of 5%.

In the interaction effect of light treatment with cultivar, the highest flower quality was observed in the Allstar cultivar in LED day 4000 lux, which was 24% compared with control. The highest flower quality was observed in the Dolcevita cultivar in night 400 lux intensity, which was a 22% increase in flower quality compared with the control (Table 6). The flower quality with the parameters of bud number, bud length, bud diameter, stem diameter, number of leaves, fresh weight, dry weight, carbohydrate, and total chlorophyll, it is significant correlation at 1% level and the vase life index is significant at 5% level.

In the interaction effect of light treatment with the cultivar, the highest greenness was observed in the Allstar cultivar in LED night day 2000 lux, which showed a 10% increase in greenness compared with the control (Table 6). The greenness index has a positive correlation with the number of buds at the level of 1%, and a significant positive correlation was observed with anthocyanin and after harvest at the level of 5%.

In the interaction effect of light treatment with cultivar, the highest chlorophyll fluorescence was observed in the Allstar cultivar in LED day 4000 lux intensity, which was 7% compared with control. The highest chlorophyll fluorescence index of 4000 lux was observed in the Dolcevita cultivar at daylight (Table 6). The chlorophyll fluorescence index with weekly harvest, chlorophyll a, and total chlorophyll had a positive and significant correlation, and a negative correlation with thrips was observed at the 1% level.

According to Schroeter-Zakrzewska and Pradita's (2021) experiment, leaf color plays a crucial role in the quality of ornamental plants. They showed that light color significantly affected the SPAD index value in Chrysanthemum plants. Specifically, exposure to white + blue light and white and blue lights resulted in an increase in the SPAD index value. However, red + blue light exposure resulted in the highest index of greening leaves (SPAD) value and the shortest cuttings with the longest roots.

Fresh weight, dry weight, leaf surface, thrips pest symptom

In the interaction effect of light treatment with cultivar, the highest fresh weight of cut flowers was observed in the Allstar cultivar in LED night 4000 lux treatment, which was 43% more than the control. In the Dolcevita cultivar, the heaviest fresh weight of cut flowers was observed in LED night day 4000 lux, which is a 33% increase compared with control (Table 6). A positive correlation was observed with the indices of the number of buds, bud length, stem diameter, dry weight, and carbohydrate at the level of 1% and with the indices of vase life, thrips pest at the level of 5%.

In the interaction effect of light treatment with cultivar, the highest dry weight of cut flowers was observed in the Allstar cultivar in LED night 4000 lux treatment, which was 39% compared with control. The highest dry weight of cut flowers was observed in the Dolcevita cultivar in LED night day 4000 lux intensity, which is a 33% increase compared with control (Table 6). A positive correlation of 1% was observed in dry weight with weekly harvest indices, bud length, bud diameter, stem diameter, number of leaves, fresh weight, and carbohydrate. This finding is consistent with the findings of Fan et al. (2013), who investigated the effect of different intensities of LED light (50 % blue and 50 % red) on tomato (*Solanum lycopersicum*). Schroeter-Zakrzewska and Pradita's (2021) study found that Chrysanthemum plants exposed to white + blue light had the highest fresh and dry weights, averaging about 65.2 and 38.1 g, respectively. In contrast, the plants exposed to red + blue light had the lowest fresh weight (46.9 g) and dry weight (26.3 g).

In the interaction effect of light treatment with the cultivar, the highest leaf surface was observed in the Allstar cultivar in LED night day 2000 lux, and in the Dolcevita cultivar, the highest leaf surface was observed in LED day 2000 lux intensity (Table 6). The leaf surface has no significant correlation with other indicators.

Table 6. Interaction effect of LED light regimes and rose cultivar on some flower characteristics.

Cultivar	Light	Flower quality	Greenness (SPAD)	Chlorophyll fluorescence (Fv/Fm)	F.W. of cut flowers (g)	D. W. of cut flowers (g)	Leaf surface (mm^2)	Thrips pest	Chlorophyll a of leaves (mg/g FW)
	Control	6.88bc	52.75ab	0.72c	5.63e	33.22bc	6723.60d	8.00a	0.27cde
	Night 2000	7.76ab	51.26ab	0.76ab	6.60c	37.75bc	10499.90bdc	5.66b	0.28cd
	Night 4000	7.77ab	52.30ab	0.75ab	8.03ab	46.21a	8779.30cde	5.33bc	0.31bc
	Day 2000	8.53a	54.23ab	0.77a	7.84ab	43.73abc	12148.80ab	4.66de	0.35b
	Day 4000	8.00a	53.70ab	0.77a	7.47bc	41.23abc	12146.60ab	5.00cd	0.45a
Allstar	Night Day 2000	7.99ab	58.10a	0.77a	7.56bc	43.42abc	13317.80a	5.66b	0.22e
	Night Day 4000	8.16a	53.33ab	0.77a	7.61bc	44.52abc	11865.50abc	5.00cd	0.36b
	Dark 2000	7.61ab	48.63bc	0.74b	3.80f	22.92c	9353.60cde	1.66e	0.23e
	Dark 4000	6.13b	51.22ab	0.74b	3.99f	23.04c	11416.30abc	2.00e	0.29cd
	Control	7.10c	50.33ab	0.75ab	6.47c	39.51bc	9925.00cde	8.00a	0.26de
	Night 2000	8.48a	50.50ab	0.77a	8.42b	48.52a	12345.70ab	5.00cd	0.23e
	Night 4000	8.70a	49.36bc	0.75ab	7.84ab	44.91abc	12124.20ab	5.66b	0.31bc
	Day 2000	8.23a	48.96bc	0.77a	8.10ab	45.12abc	13040.20a	4.00de	0.24e
Dolcevita	Day 4000	8.34a	50.13ab	0.77a	7.60bc	44.22abc	11613.50abc	5.33bc	0.32bc
	Night Day 2000	8.13a	48.53bc	0.77a	7.91ab	44.87bac	12225.40ab	5.51b	0.38ab
	Night Day 4000	8.35a	49.43bc	0.77a	8/60a	48.44a	12227.80ab	2.33e	0.38ab
	Dark 2000	6.39bc	47.26b	0.75ab	6.23c	37.35bc	10577.90bcd	2.00e	0.22e
	Dark 4000	6.63bc	39.90c	0.74b	5.70e	31.49bc	14024.00a	8.00a	0.30bc

In each column, the averages that have at least one letter in common do not have a significant difference at the 5% probability level based on the least significant difference (LSD) test. F.W.: Fresh weight; D.W.: Dry weight.

The number of leaves in the LED light regimes and light intensity both increased the number of leaves in the rose. This finding is consistent with Duong et al. (2000) that reported that using LED light 70% red + 30% blue with an intensity of 2600 lux in conjunction with sunlight on strawberry plants can increase the number of leaves, stem and root height, and dry weight compared to plants grown under fluorescent light. Naznin et al. (2019) found that in pepper, leaf number was significantly increased under 95% red light with 5% blue LED compared to 100% red LED.

The plant weight of mint (*Mentha sativa*) and basil (*Ocimum basilicum*) increased at LED light 70% red and 30% blue with 18 hours of lighting 4 times (Sabzalian et al. 2014). Tomatoes were grown under 1:1 blue and red LED light conditions, and the specific leaf surface area and total chlorophyll increased, resulting in better light absorption (Fan et al. 2013).

In the interaction effect of light treatment with cultivar, the highest thrips pest was observed in the control with an average of 8 out of 10 and the lowest thrips drop was observed in LED dark 2000 lux intensity with an average of 1.66. In the Delsovita cultivar, the highest thrips infestation was observed in control and the lowest thrips drop was observed in LED dark 2000 lux (Table 6). There was a positive correlation between the thrips pest index and the number of buds at the 1% level, and a negative correlation was observed with the number of flowers and chlorophyll fluorescence at the 1% level, and a significant corroletion observed with the greenness index, fresh weight, and dry weight.

Johansen et al., (2018) evaluated the phototactic response of *F. occidentalis* to yellow and blue sticky traps with blue LED in colored and white Alstroemeria varieties. They found that catches in blue traps with LED were 3.4 and 4.0 times higher compared to blue traps without LED. In contrast, catches in yellow traps with LED increased by 4.5 times compared to yellow traps without LED, but they were only slightly higher than those observed in blue traps without light and lower than catches in blue traps with LED. This difference is likely due to the reflection of blue light in the blue sticky traps, which produced a higher stimulus, while the blue light is mostly absorbed in the yellow traps.

Physiological factors chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, petal anthocyanin content

In the interaction effect of light treatment with cultivar, the highest chlorophyll a was observed in the Allstar cultivar in LED day 4000 lux, which was 66% compared with control. Delsovita cultivar in LED night day 2000 lux, which is a 46% increase compared with control (Table 6). The chlorophyll a with chlorophyll b, total chlorophyll, and carotenoid indices is significant at 1% level and with life after harvest at 5% level.

In the interaction effect of light treatment with cultivar, the highest chlorophyll b was observed in the Allstar cultivar in LED day 4000 lux, which was 60% compared with the control. The most chlorophyll b was observed in the Delsovita cultivar in LED night day 4000 lux, which is a 42% increase compared to the control (Table 7). The chlorophyll b with total chlorophyll, carotenoids, and after harvest, a positive correlation was observed at the level of 1%, and with the number of leaves and water content, a positive correlation was observed at the level of 5%.

In the interaction effect of light treatment with the cultivar, the highest total chlorophyll was observed in the Allstar cultivar in LED day 4000 lux, which was 63% compared with control. The highest total chlorophyll was observed in the Dolcevita cultivar in LED night day 4000 lux, which is a 44% increase compared to the control (Table 7). The total chlorophyll with carotenoid indicators and after harvesting, a positive correlation was observed at the

level of 1%, and with the content of flower water, carbohydrates, and total chlorophyll, a positive correlation was observed at the level of 5%.

In the interaction effect of light treatment with cultivar, the most carotenoid was observed in the Allstar cultivar in LED day 4000 lux, which was 64% compared with control. The most carotenoids were observed in the Dolcevita cultivar in LED night day 2000 lux, which is a 62% increase compared to the control (Table 7). A positive correlation was observed between carotenoids and total chlorophyll indices after harvest, at the level of 1%, and a positive correlation was observed with the content of flower water content and carbohydrates at the level of 5%.

In the interaction effect of light treatment with cultivar, the highest anthocyanin content was observed in the Allstar cultivar in LED day 2000 lux, which was 41% higher than the control. The highest anthocyanin content was observed in the Delsovita cultivar in LED night 2000 lux, which was a 113% increase compared with control (Table 7). The anthocyanin with greenness index at the level of 1% and with the vase life index, carbohydrate, and flower water content, a positive correlation was observed at the level of 5%.

Chlorophylls try to adapt to the environment in different environmental conditions so that the chlorophyll content changes to improve maximum photon absorption in different environmental conditions (Samuoliene et al., 2011). When a plant is exposed to blue light in addition to other wavelengths, its chlorophyll content increases (Hernandez, 2013). According to Wu et al. (2007), pigments, particularly chlorophyll and carotenoid, decreases in the dark. The duration of light exposure can also affect the chlorophyll content. However, it has been determined that red light causes carotenoid and anthocyanin accumulation while blue light causes chlorophyll accumulation. Anthocyanin biosynthesis is typically linked to blue light via cryptochromes. However, active phytochrome is required for the regulation of anthocyanin synthesis via cryptochromes, and this requirement can be related to the presence of phytochrome an or phytochrome b (Ahmad & Cashmore, 1997).

According to Zhang et al. (2020), increasing the exposure time to light results in an increase in chlorophyll and carotenoids in microgreens, which is consistent with the current study. Fan et al. (2013) found that exposing cabbage to a red-blue LED light with a 6:1 ratio of 8000 lux without sunlight for 12 hours on and 12 hours off increased carotenoids by about 70% when compared to mono spectral lights.

The effect of LED and cultivar on the vase life of two rose cultivars

Soluble carbohydrate, vase life parameters, and the speed of flower bud opening

In the interaction effect of light treatment with cultivar, the highest carbohydrate was observed in the Allstar cultivar in LED day 4000 lux, which was 31% compared with control. In the Dolcevita cultivar, the highest carbohydrates were observed in LED night day 4000 lux, which is a 142% increase compared with control (Table 7). The carbohydrate content has a positive correlation with the number of buds, bud length, bud diameter, stem diameter, number of leaves, flower quality, fresh weight, and dry weight at the level of 1%, and with chlorophyll and water content, there is a positive and significant correlation at the level of 5% was observed.

Growing tomatoes under 70% red + 30% blue light without sunlight with a light intensity of 15,000 lux was shown by Li et al. (2017) to increase total carbohydrates and starch, as well as sucrose accumulation. Hao et al. (2016) found that red-blue supplementary LED light at a ratio of 1:1 with an intensity of 4700 lux increased the number of flower buds as well as the amount of carbohydrates, which is consistent with the current study.

In the interaction effect of light treatment with cultivar, the longest vase life was observed in the Allstar cultivar in LED night day 4000 lux, which is 44% compared with control. In the

Dolcevita cultivar, the longest vase life was observed in LED day 4000 lux, which is a 133% increase compared with control (Table 7). The vase life index has a positive correlation with the number of buds, chlorophyll b, total chlorophyll, carotenoids, and anthocyanins at the 1% level, as well as with stem diameter, flower quality, greenness, fresh weight, dry weight, flower water content, chlorophyll A positive correlation was observed at the 5% level.

Horticultural products have a limited shelf life after harvesting, which is due to factors such as weight loss, aging, loss of strength, softening of tissue corruption, and so on. Supplemental lighting during plant growth can increase vase life by assisting in the storage of sugars and carbohydrates (Hasperue et al., 2016). The use of LED light has a significant impact on the life of horticultural products after harvest. In its most basic form, artificial LED lights can help with more photosynthesis assimilate and more food storage (Dayani et al., 2018). According to Samuoliene et al. (2012), in a study of the effect of red LED light on the changes in photochemical content in lettuce leaves, it was discovered that exposing lettuce plants to LED light during cultivation increased the production of ascorbic acid, which has antioxidant properties and reduces the activity of radicals. In the same study, the carbohydrate content and antioxidant capacity of the lettuce plant increased under the LED light treatment, which had a positive correlation with the increase in vase life after harvesting the plant (Samuoliene et al., 2012).

A study found that using a 1000 lux LED supplemental light increased the vase life of cabbage after harvesting and delayed the yellowing of its inflorescences (Hasperue et al., 2016). It was able to increase the vase life of the plant after harvesting by 30% more than the Allstar control and 23% more than the Dolsovita control in the vase life light intensity index of 4000 lux, which is consistent with the research of Pettersen et al. (2007) who reported that increasing the exposure time to the plant increased the longevity of flowers on potted roses.

Table 7. Interaction effect of LED light regimes and rose cultivar on some flower characteristics.

Cultivar	Light	Chlorophyll b of leaves (mg/g FW)	Total Chlorophyll of leaves (mg/g FW)	Carotenoids of leaves (mg/g FW)	Anthocyanin of petal (µmol/g)	Carbohydrate of leaves (mg/g)	Vase life (day)	Speed of flower bud opening (mm/day)	Flower water content (%)
	Control	0.20e	0.73bc	0.17cde	227.68bcd	124.05de	9.00de	2.021c	81.21b
	Night 2000	0.21de	0.77bc	0.18cde	283.84ab	148.00d	11.00c	2.22bc	82.90ab
	Night 4000	0.25ab	0.87b	0.20bc	310.81a	160.50a	11.66c	2.29bc	82.73ab
	Day 2000	0.24bc	0.93b	0.23b	322.73a	152.69d	12.33b	2.73a	83.24a
	Day 4000	0.32a	1.19a	0.28a	307.58a	163.23cd	12.33b	2.82a	82.65ab
Allstar	Night Day 2000	0.19efg	0.73bc	0.15e	258.29abc	159.25d	11.66c	2.82a	83.11a
	Night Day 4000	0.29a	0.99b	0.23b	258.89abc	157.30d	13.00a	2.76a	82.45ab
	Dark 2000	0.20e	0.65d	0.15e	163.43de	125.706de	10.00d	2.16c	81.84b
	Dark 4000	0.22cd	0.78bc	0.19cd	194.75cd	129.75de	9.66de	2.73a	82.00ab
	Control	0.19efg	0.70cd	0.16e	13.99g	74.14f	5.00e	1.79f	82.02ab
	Night 2000	0.21de	0.66d	0.14e	29.83g	155.40d	10.00d	2.61abc	83.38a
	Night 4000	0.22cd	0.83b	0.19cd	21.21g	168.18abc	11.33c	2.27bc	83.17a
Dolcevita	Day 2000	0.17g	0.64d	0.15e	19.80g	163.37cd	11.66c	2.87a	83.40a
	Day 4000	0.24bc	0.87b	0.21bc	24.34g	176.30ab	11.66c	2.64abc	83.46a
	Night Day 2000	0.26ab	1.01a	0.26a	17.37g	167.19bc	10.66d	2.94a	83.11a
	Night Day 4000	0.27a	1.01a	0.25a	22.32g	179.84a	8.33de	2.59bcd	83.49a
	Dark 2000	0.19efg	0.63d	0.13e	11.52g	138.91d	10.00d	3.00a	82.60ab
	Dark 4000	0.21de	0.79bc	0.18cde	12.13g	147.63d	11.33c	2.05ef	82.30ab

In each column, the averages that have at least one letter in common do not have a significant difference at the 5% probability level based on the least significant difference (LSD) test.

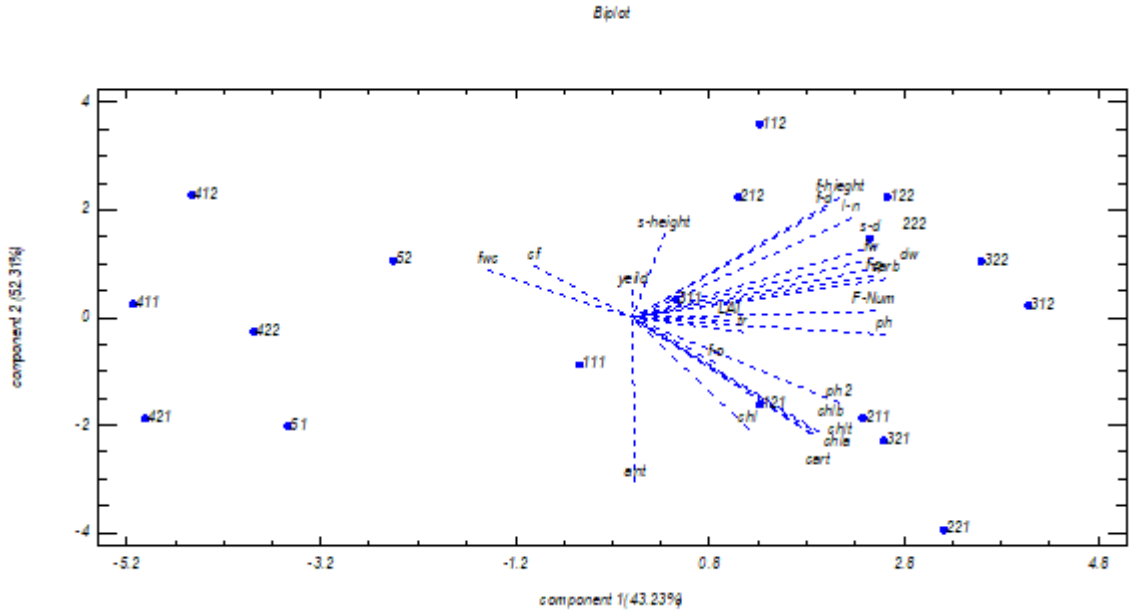

Fig. 3. The PCA analysis of the effect of LED on some characteristics of Rose.
Bud number (F-Nun), Mean harvest weekly (yield), Cut flower height (S-height), Bud diameter (F-d), Stem diameter (S-d), Number of leaves (l-n), Flower quality (f-q), Greenness (Chl), Chlorophyll fluorescence (cf), Fresh weight (fw), Dry weight (dw), Flower water content (fwc), Vase life 1 day (ph), Vase life 2 day (ph2), Chlorophyll a (Chla), Chlorophyll b (Chlb), Total chlorophyll (Chlt), Carotenoid (Cart), Anthocyanin (ant), Leaf surface (LA1), Thrips pest (tr), Speed of flower bud opening (f-o), Carbohydrate of leaves (Carb). N2 Allstar (111), N4 Allstar (121), D2 Allstar (211), D4 Allstar (221), DN2 Allstar (311), DN4 Allstar (321), T2 Allstar (411), T4 Allstar (421), Control1 Allstar (51), N2 Dolcevita (112), N4 Dolcevita (122), D2 Dolcevita (212), D4 Dolcevita (222), ND2 Dolcevita (312), ND4 Dolcevita (322), T2 Dolcevita (412), T4 Dolcevita (422), Control 11 Dolcevita (52).

In the interaction effect of light treatment with the cultivar, the Allstar cultivar had the fastest flower opening in LED day 4000 lux, which was 40% faster than the control (Table 7).

Flower water content was highest in LED day 2000 lux in Allstar and in night day 4000 lux in Dolcevita cultivar (Table 7).

According to PCA the best light intensity for increasing growth parameters and pigment content with 4000 lux in both cultivars and the best LED regime was 24 hours on (with sunlight), 24 hours light (without sunlight) in both cultivars improve the majority of flowers characteristics (Fig. 3).

CONCLUSION

Conclusively, chlorophyll parameters such as chlorophyll a, b, total, carotenoid, chlorophyll fluorescence and also the average number of flower harvests per week improved in LED day 4000 lux. The light intensity of 4000 lux had a better effect on the vase life of roses in both cultivars. Allstar showed the longest vase life in the treatment of LED night day 4000 lux, and Dolcevita showed the longest vase life in LED day 4000 lux treatment. In general, according to the information obtained in this research, it is recommended to use 4000 lux LED supplemental light during the day to increase the quantity and quality of roses in low light seasons (autumn and winter).

Conflict of interest

To the best of our knowledge, the named authors have no conflict of interest, financial or otherwise.

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Molecular identification of fungal species infesting tomato (*Lycopersicon esculentum*) at postharvest phase in Kwanar Gafan, Kano State

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ABSTRACT

Purpose: Fungal infections have become the most common problem to cause postharvest loss in tomato enterprise across Nigeria. Morphology-based identification of fungal species usually provides the inconclusive results with several species remain unidentified. Molecular identification method was used to supplement morphology-based techniques to identify the fungal species. **Research Method:** A Deoxyribonucleic Acid (DNA) through 5.8S-ITS (Internal Transcribed Spacer) region of the ribosomal DNA (rDNA) was used to identify 180 infested tomato specimens. The samples were collected from farm, retail and wholesale points in Kwanar Gafan located at Kura LGA of Kano State. **Findings:** Eight haplotypes have been detected from the total fungal specimens examined, A 65.6% of fungal specimens from wholesales and farms constituted haplotype-1 and identified as *Pichia kudriavzevii* (with 99.54% similarity) as BLAST in National Centre for Biotechnology Information (NCBI) database. The remaining seven haplotypes were exclusively found in the retailing points and largely constitutes *Aspergillus* spp., *Mucor fragilis*, *Russula atroglauca, Ganoderma* sp., *Alternaria* spp., *Exserohilum rostratum*, *Colletotrichum boninense*, *Naganishia* sp. and *Cladosporium* spp. **Research limitations:** Further research on molecular identification from other parts of kano is required for better understanding the fungi associated with postharvest loss in the state. **Originality/Value:** *Pichia kudriavzevii* is a single and only dominant fungal species that infest tomatoes in both farms and wholesale points (65.6%). However, 34.4% of the diverse fungal species have been found in retailing points which is related to the rapid infestation of tomatoes.

INTRODUCTION

Postharvest loss of fruits and vegetables is significant in developing countries where lack of appropriate storage facilities are the main challenges (Kassa & Senay, 2019). As a result, highly perishable fruits and vegetables undergo the greatest proportion of postharvest losses. Almost half of all fruits and vegetables produced are lost and wasted along the agri-food supply chain (Tiong et al., 2018).

Tomato (*Lycopersicon esculentum*) belongs to the family Solanaceae which is regarded as one of the most important, cultivated and consumed vegetable in the world (Lamidi et al., 2020; Maurya et al., 2022). Monte et al. (2013) reported that about 146 million tons of fresh tomato fruits are produced annually throughout the world. Tomatoes are consumed in large quantities directly in salads, cooked into soups or processed into juice, ketchup, whole peeled tomato and paste (Adedeji et al., 2006; Shankara et al., 2015). Tomatoes are rich in vitamins (particularly A and C), minerals, sugars, essential amino acids, iron, dietary fibres and phosphorus (Ayendiji & Adeniyi, 2011). It also contains high amount of β-carotene, vitamin C and lycopene, a carotenoid with antioxidant properties (Britt & Kristin, 2011; Dandago et al., 2018; Rani & Khetarpaul, 2009). In Nigeria, tomato fruits consumption accounts for about 18% of the daily consumption of vegetables (Lamidi et al., 2020). Nigeria is the second largest producer of tomatoes in Africa and 13th largest in the world (FAOSTAT, 2014). In Nigeria, tomatoes are majorly produced in the Northern part and conveyed to the other parts of the country using different types of vehicles (Etebu et al., 2013). Owing to lack of information on appropriate postharvest technology, packaging and storage conditions, the fruits not only lose their quality but also encounter substantial postharvest loss. Kutama et al. (2007) showed that the estimated postharvest loss of tomatoes in Nigeria to be around 60% resulting in a serious economic loss (Dandago et al., 2017). Kitinoja et al. (2019) reported postharvest loss in tomatoes of 1-18% in India, 10-40% in Nigeria, and 50-60% in Rwanda. These losses are particularly higher at the wholesale and retail stages (Aghadi et al., 2019).

Postharvest loss often develops on wounds, bruised tissues and during fruit softening. Therefore, sound tomatoes can be inoculated by plant pathogens via cross contamination. Tomato fruits are susceptible to numerous decays from field through postharvest handling. Fungi are the most important pathogens infecting tomatoes causing important losses during harvesting, transportation and storage (Dandago et al., 2018). The sources can be diseased fruits, dirty harvest containers and poorly sanitized handling systems. Fungal decays of tomatoes include black rot (*Alternaria alternata*), fusarium rot (*Fusarium* spp.), grey mould rot (*Botrytis cinerea*), mucor rot (*Mucor muledo*), Rhizopus rot (*Rhizopus stolonifer* and *Rhizopus oryzae*) and watery soft rot (*Sclerotonia minor* and *S. sclerotiorum*) (Sargent & Moretti, 2004).

Therefore, it is necessary to identify these microscopic pathogens especially those affecting humans to reduce the risk of contamination and future infection. In fact, there was a limited knowledge regarding the stage (harvest, retail and wholesale) at which the fungal contamination of tomato begins. Also, little is known concerning the fungal species affecting tomatoes at each stage. Therefore, understanding the susceptible stage of contamination and species involved would be a milestone for aiding the stakeholders to take the appropriate management decisions and strategies.

Conventional cultivation and microscopic identification have been widely practiced for fungal studies based on mycelia (colour, size and shape) and morphological characteristics (Hasan & Zanuddin, 2020). Earlier studies on isolation and identification of postharvest fungi from tomato fruits particularly in Nigeria were based on conventional methods (Dandago et al., 2018; Kutama et al., 2007; Mailafia et al., 2017; Yahaya & Ahmad, 2008; Yusuf & Okunsanya, 2007). These methods are challenging especially for *Alternaria* spp. and could misled the final

conclusions (Gherbawy et al., 2018). Alternatively, molecular identification has been recently used and provides a higher resolution of the fungal species of vegetable fruits including tomato (Akbar et al., 2018; Gherbawy et al., 2018). Therefore, the aim of this research is to use molecular techniques to identify postharvest fungi associated with tomato fruits decay at three different handling stages in Kura axis of Kano State.

MATERIALS AND METHODS

Source of materials and sample collection

Sixty tomato fruits (UC18) were collected from three different level of handling units (farm, whole sale and retail) located at Kwanar Gafan of Kano State, each from Unit (20 samples per farm × 3), Unit-2 (20 samples per retail-point × 3) and Unit-3 (20 samples per wholesale-points × 3) collected from November 2020 (Fig. 1). In total, One Hundred and Eighty (180) rotten tomato samples were randomly collected from nine sub-units. Tomato fruits samples were rinsed with sterile distilled water and were immediately arranged in the sterilized containers, labelled appropriately, and transported to Food Microbiology Laboratory of Kano University of Science and Technology for fungal isolation.

Isolation and identification of fungi

The pathogens from the tomato samples were isolated and identified via the method adopted by Mustapha and Yahaya (2006). A small dissect (4 mm thick) of the infected part of the tomato was cut with a sterilized scalpel and placed aseptically on a sterilized and dried Potato Dextrose Agar (PDA). The inoculated petri dishes were incubated at room temperature for a period of 5 days. The colonies on each plate were counted and recorded. Each type of fungal colony was sub-cultured onto fresh medium PDA to obtain pure culture in PDA slants. The fungal isolates were identified using the illustrated genera of imperfect fungi using Collins and Lyne (2004). All glass wares and working environment were properly cleaned and sterilized throughout the experimental period to avoid any external contaminants.

Molecular identification

DNA extraction, polymerase chain reaction (PCR) amplification and Sequencing of ITS region

Genomic DNA was extracted from cultures (purified fungi) using a modified protocol of plant genomic DNA kit (TIANGEN BIOTECH CO., LTD, Beijing, China). The 5.8S-ITS region of the ribosomal rDNA was amplified by PCR with universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCGG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Liu et al., 2007). The PCR was performed using Veriti 96 Well Thermal Cycler (Applied Biosystem, California, USA) in 25 µL reaction volume containing 18.2 µL sterile distilled water, 2.5 µL Taq buffer, 2.0 µL dNTP Mix (2.5 mM), 0.5 µL of each primer (10 µM), 0.3 µL of 5 unit/µL Taq polymerase (TaKaRa) and 1 µL template DNA (1-50 ng/µL). The PCR started with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 60 s and then a final extension at 72 °C for 5 min.

The success of the PCR amplification was checked through 1.5% of agarose gel in 1.0 × TBE using FloroSafe DNA Stain. The expected sizes of the PCR products were confirmed by including 2 µL 100-bp DNA ladder in the gel run (Elpis Biotech, Daejeon, Korea). Electrophoresis was run at 100 volts for 1 hour, photographed and visualized using Luminescent Image Analyzer LAS-1000plus v.2.0 (Fuji Photo Film Co. Ltd., Tokyo, Japan). Successful amplificons were sequenced by inqaba biotec.

Data analysis

The ITS sequences (current study) were aligned and edited using ClustalW multiple sequence alignment program in MEGA v.7 (Kumar et al., 2016). DnaSP v.6 software was used to determine the variable sites and haplotypes among the sequences (Rozas et al., 2017). To trace the identity of fungal species, the detected haplotypes in the present study were queried using basic local alignment search tool (BLAST) in National Centre for Biotechnology Information (NCBI) nucleotide database. The highest species matching had been identified to a sequence similarity of at least > 92% to avoid false positives. Fungal haplotypes obtained in the current study were used to construct a phylogenetic tree using Maximum Likelihood method in MEGA v.7 to analyse the relationship.

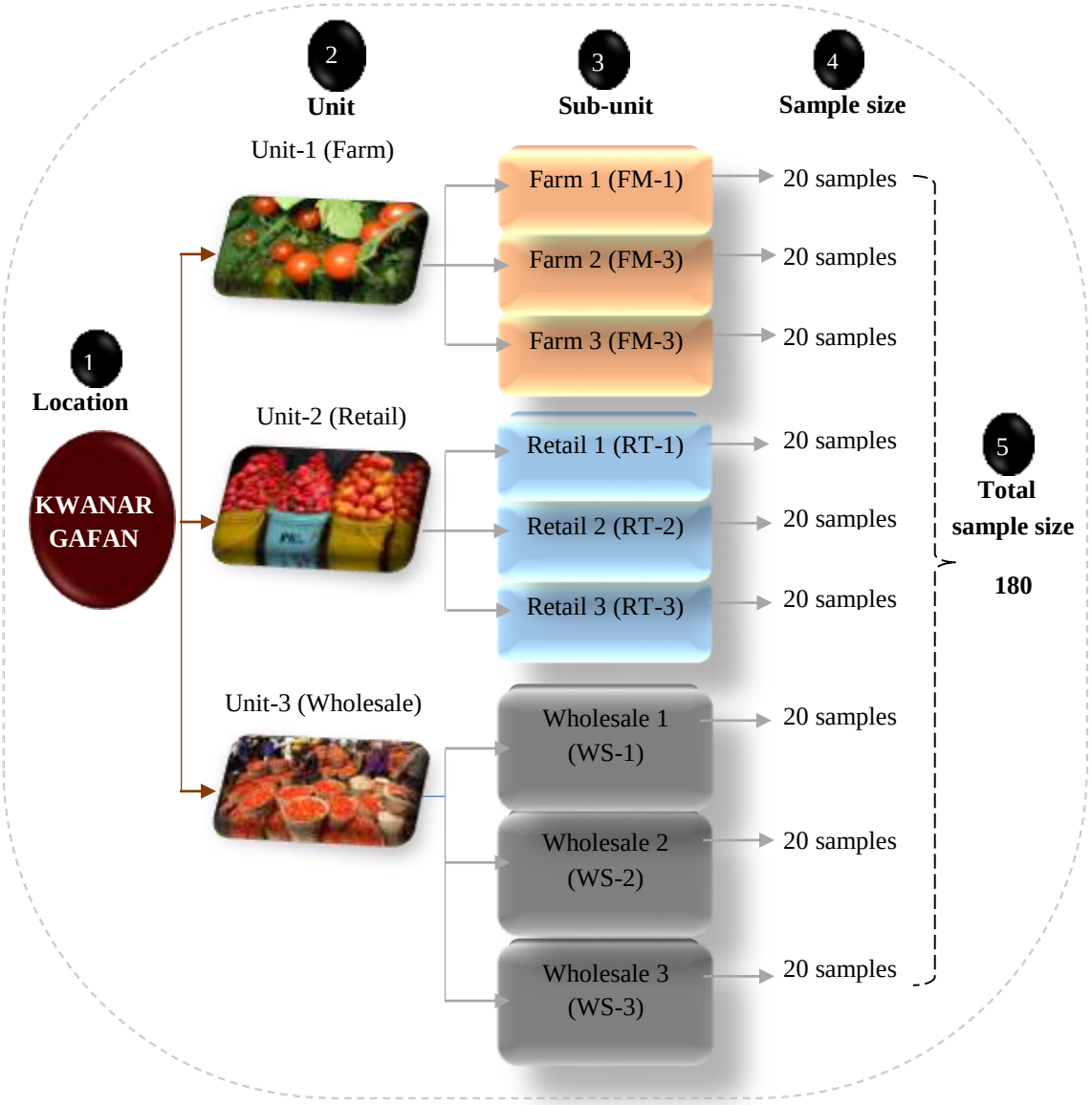

Fig. 1. Description of infected tomatoes sampling location, units, sub-units, sample sizes and total sample sizes used in current study.

RESULTS

Fungal isolates and haplotypes

A total of 13 unidentified fungi were successfully isolated from 180 tomato samples collected in the current study (Fig. 2). The sequences of 5.8S-ITS region of the ribosomal rDNA generated have a total sequence length of 502 base pairs after deleting low quality nucleotides of both primers ends. The sequences yielded eight haplotypes that are highly distinct and distributed (Table 1). Entire samples from farm and wholesale made-up Hap-1 (accept two samples from WS-3). Other haplotypes (Hap-2 to Hap-8) were largely from to the retail units. The BLAST search results for eight haplotype sequences match displayed high percentage identity with various sequences of fungal species as shown in Table 2. Hap-1, Hap-3 and Hap-4 showed the highest sequence similarity with only one NCBI sequence each. While the remaining haplotypes matched with more than one NCBI sequences with the same % similarity.

Table 1. Fungal haplotypes and their percentage distribution isolated from tomatoes in Kwanar Gafan.

	Sampling units										
	Farm			Retail			Wholesale				
Haplotype	FM-1	FM-2	FM-3	RT-1	RT-2	RT-3	WS-1	WS-2	WS-3	N	%N
Hap-1	20	20	20	—	—	—	20	20	18	118.00	65.60
Hap-2	—	—	—	4	7	9	—	—	—	20.00	11.10
Hap-3	—	—	—	3	2	1	—	—	—	6.00	3.30
Hap-4	—	—	—	6	4	1	—	—	—	11.00	6.10
Hap-5	—	—	—	2	1	5	—	—	2	10.00	5.60
Hap-6	—	—	—	3	2	1	—	—	—	6.00	3.30
Hap-7	—	—	—	1	2	1	—	—	—	7.00	3.90
Hap-8	—	—	—	1	2	2	—	—	—	6.00	3.30
Total	20	20	20	20	20	20	20	20	20	180.00	100.00%

N: number of samples.

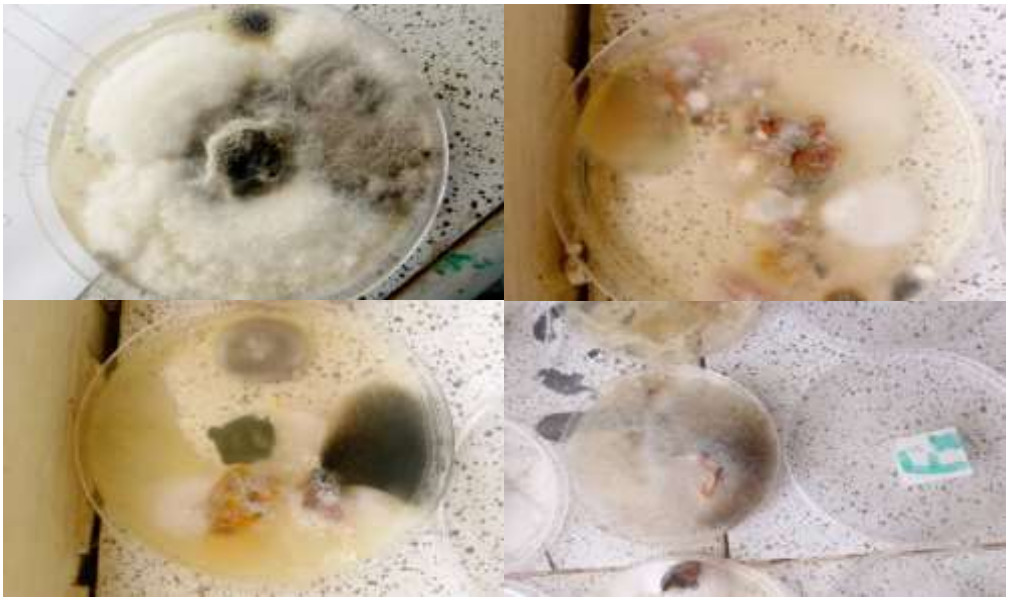

Fig. 2. Varieties of unidentified cultures of fungi.

Table 2. Haplotype sequence BLAST showing different percentage identity between fungal species obtained in the current study and those from the NCBI.

Hap. No.	Acces. No.	Organism	% I.D	Reference
Hap-1 ∞	MN310532	*Pichia kudriavzevii*	99.54%	(*Vu et al., 2016)
Hap-2 ‡	MT529928	*Aspergillus flavus*	99.80%	(*Becker 2022)
	ON819577	*Aspergillus tamari*	99.80%	(*Niu, 2022)
Hap-3 ‡	KY345406	*Mucor fragilis*	93.30%	(*Villanueva-Ibanez et al., 2016)
Hap-4 ‡	MW850413	*Russula atroglauca*	92.50%	(*Shi, 2021)
Hap-5 †	ON208264	*Aspergillus niger*	90.23%	(*Lu et al., 2022)
	MK530080	*Aspergillus welwitschiae*	90.23%	(*Sanjotha et al., 2019)
Hap-6 ‡	MT534034	*Ganoderma* sp.	99.60%	(*Singh and Kumar, 2020)
	MN856333	*Alternaria eichhorniae*	99.60%	(*Feng, 2019)
	MN599626	*Exserohilum rostratum*	99.60%	(*Chand and Korra, 2019)
	MF495402	*Colletotrichum boninense*	99.60%	(*Mejia et al., 2017)
	MK793207	*Alternaria citri*	99.60%	(*Yarahmadi et al., 2019)
	MK158222	*Alternaria brassicicola*	99.60%	(*Gill and Vasundhara, 2018)
	OK011826	*Alternaria alstroemeriae*	99.60%	(*Lei et al., 2021)
Hap-7 ‡	MK518426	*Alternaria tenuissima*	99.39%	(*Kiranmayee et al., 2019)
	MK226308	*Alternaria tomato*	99.39%	(Poudel et al., 2018)
	MH560053	*Alternaria burnsii*	99.39%	(Poudel et al., 2018)
	KX156938	*Alternaria alternata*	99.39%	(*Gowrishankari, et al., 2016)
	KU639594	*Alternaria longipes*	99.39%	(*Gowrishankari, et al., 2016)
	HM204456	*Alternaria porri*	99.39%	(*UdayaShankar et al., 2010)
	HM003687	*Alternaria arborescens*	99.39%	(*Visalakchi et al., 2010)
Hap-8 ‡	ON712254	*Cladosporium crousii*	100%	(*Nageen et al., 2023)
	MT529231	*Cladosporium ramotenellum*	100%	(*Li, 2020)
	MT378422	*Cladosporium colombiae*	100%	(*Yue, 2020)
	MT378416	*Cladosporium austroafricanum*	100%	(*Yue, 2020)
	MT367262	*Cladosporium cladosporioides*	100%	(*Shi, 2020)
	MN947589	*Cladosporium coralloides*	100%	(*Poli and Varese, 2020)
	MN886551	*Cladosporium pseudocladosporioides*	100%	(*Choi, and Lee, 2019)
	MN857898	*Cladosporium anthropophilum*	100%	(*Silva-Rojas et al., 2019)

* Direct submission; ‡ farms and wholesale; ∞ retail; † retail and wholesale.

DISCUSSION

Tomato is regarded as an essential vegetable cultivated for human consumption (Sibomana et al., 2015). In Nigeria, the tomato farming and marketing activities are largely controlled and practiced in northern part of the country (Etebu et al., 2013). Tomato farming and enterprises provides huge support and helps the locals for their daily needs as well as boosting the economy of the country. Despite these benefits, the enterprise usually suffers the great losses from the harvest to retails and wholesales units, largely caused by fungal infections. To address the problem, we analysed the scenario in Kwanar Gafan by identifying the fungal species involved throughout the units using molecular identification method. Kwanar Gafan is in Garun Malam LGA of Kano State, < 7 km away from giant tomato processing factory own by Dangote Farms Limited Kadawa. We chose the area to conduct this study as its highly recognised for large tomato production and marketing centre in the state. Recently, the local tomato farmers in the area were benefited from the World Bank grant supported to boost the tomato production through Agro-Processing Productivity Enhancement and Livelihood Improvement Support (APPEALS) Project (2018-2022).

Identified fungal species

Eight haplotypes (Hap-1 to Hap-8) obtained in current study had clearly indicated that Kwanar Gafan have a diverse fungal species responsible for infesting the tomatoes after harvesting. Phylogenetic relationship using a Maximum Likelihood between the haplotypes had confirmed the huge fungal species diversity in the current study area (Fig. 3). Only one haplotype (Hap-1) was shared between the samples obtained from farms and wholesale units. It is possible to have a common haplotype between them, since most wholesalers bought the entire ripped tomato in the farm (at point of harvest), harvest and filled the baskets directly within the farm. Based on BLAST, Hap-1 was identified as *Pichia kudriavzevii* with highest percentage similarities of 99.54% (Table 1). Application of *P. kudriavzevii* have been recently reported for retarding the fungal decay rate, delay the colour change, and weight loss of different fruits including tomato (Liu et al., 2020). Appearance of *P. kudriavzevii* alone in farms and wholesale units may likely inhibit the growth of other fungal species. The *P. kudriavzevii* could possibly be a primary fungal species that strike tomato fruits immediately after harvest in the farm and wholesale units.

Aspergillus spp., *Mucor fragilis*, *Russula atroglauca*, *Ganoderma* sp., *Alternaria* spp., *Exserohilum rostratum*, *Colletotrichum boninense*, *Naganishia* sp. and *Cladosporium* spp. were the main fungal species identified are contributed to the deterioration of tomato fruits in the retail units studied. These species were previously reported for damaging tomato fruits from different markets in Nigeria (Etebu et al., 2013; Sinno et al., 2020). The number of fungal species identified in the retail unit was higher compared to the farms and wholesale units. Figure 4 described the general factors responsible for having more fungal species in retail section. Fully ripe tomatoes are commonly found in retail points which are more susceptible to be infected by fungal species. Also, tomatoes stay longer during postharvest period in retail units compare to farms and wholesale units. Therefore, tendencies of more fungal effect would be higher in retail units. For wholesale purpose sometimes, tomatoes were harvested partially ripped to decrease fungal activities as the fruit may be transported for more than 700 km without cooling facilities aid. In addition, fungal activities at farms may be reduced due to a residual effect of pesticides used during the production period. Tomatoes are packaged in separate baskets in both farms and wholesale points. Unlike retail points where multiple fruits were mixed which may result to the rapid transfer of fungal species from other fruits (Fig. 5).

Continuous usage of traditional weaved baskets and unclean surfaces where tomatoes are kept in retail units may also promote the infestation of tomato fruits (Etebu et al., 2013; Kutama et al., 2007). For decades, it has been reported that the microbes responsible for tomato spoilage originates or initiated from the contaminated baskets used for storing tomatoes (Snowdon, 1991). Lack of cooling conditions in most tomato retailing units favours the growth and development of fungal species especially during the period of hot season. Few years ago, a study was conducted in Kura Kano state to investigate the effects of packaging and storage conditions on storage life and quality of tomatoes (Dandago et al., 2017). The findings revealed that storing tomato in cool place (not freezing) and packaging in kraft paper bag was a better combination for maintaining the fruit quality and extending the storage period. However, most retailers could not maintain such conditions as it requires energy and extra storage facilities.

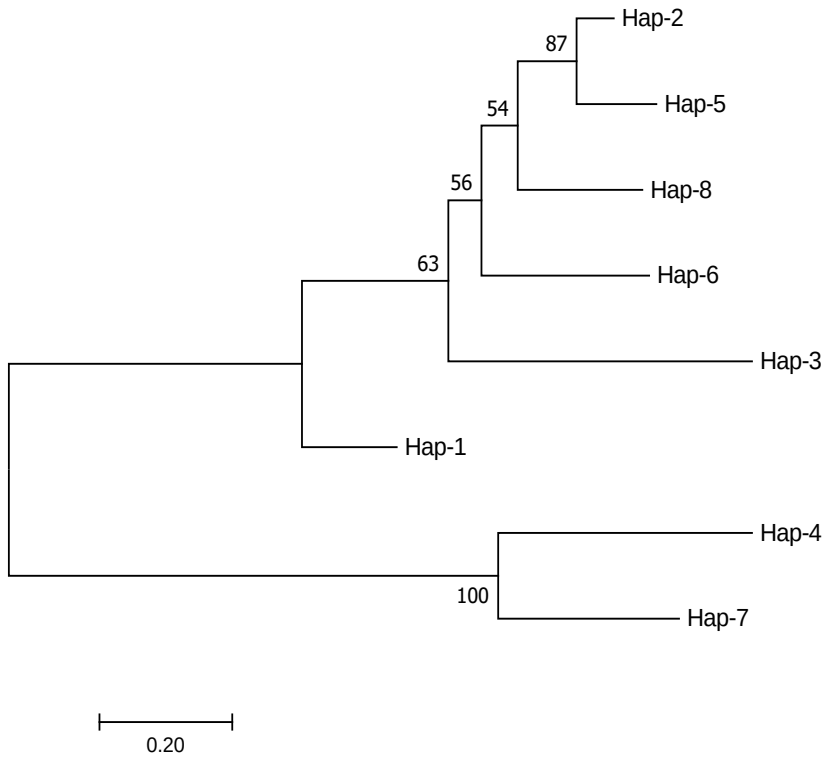

Fig. 3. Molecular Phylogenetic analysis inferred by Maximum Likelihood method based on the Kimura 2-parameter model of eight fungal haplotypes discovered from infested tomatoes collected in Kwanar Gafan.

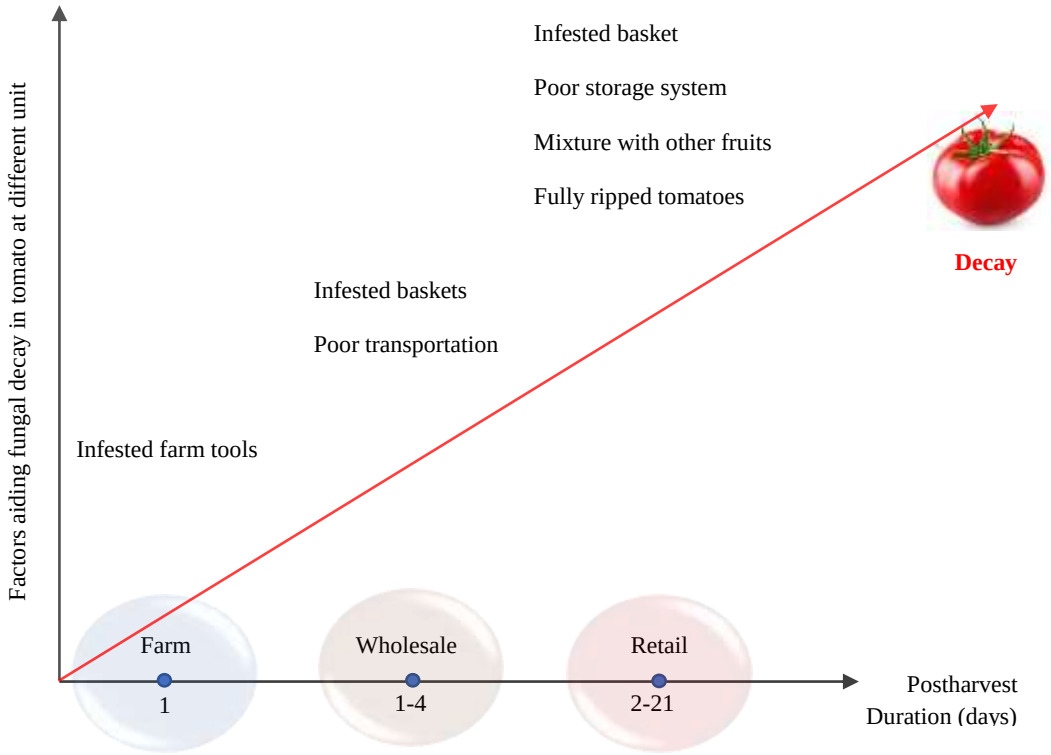

Fig. 4. Increased fungal decay of tomato in farm, wholesale, and retail with an increase of postharvest duration and factors aiding the fungal species.

Fig. 5. Possibility of fungal transfer in a retail unit where several vegetables are mixed together A, tomatoes; B, cucumber; C, lemon; D, cabbage; E, okra; F, pepper; G, hot pepper; H, onion.

CONCLUSION

Molecular identification was successfully used in identifying the fungal species collected from farms, wholesale, and retail units. In current study, *Pichia kudriavzevii* was a major fungal species identified in tomato samples collected from both farms and wholesale units. In retail units however, eight fungal genera were identified from retail unit collected. Large number of fungal species identified in retail units was attributed to several factors which includes long duration of tomatoes in retail market and poor storage conditions.

Conflict of interest

The authors declare that there is no conflict of interest.

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The effect of using peracetic acid in the processing terminal to reduce microbial contamination of pistachio

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ABSTRACT

Purpose: Pistachio is a crusial agricultural product in Iran, but its contamination with various micro-organisms can cause problems in production, consumption, and export. Peracetic acid is an antimicrobial substance that can eliminate a wide range of micro-organisms quickly. This study aimed to evaluate the effectiveness of peracetic acid in reducing microbial contamination of pistachios in processing terminals. **Research method:** Harvested pistachios were treated with concentrations of 0 (control), 1, and 2% peracetic acid at the washing basin of the terminal. Then microbial growth, bacterial and fungal population, lipid percentage, and peroxide number of pistachio kernels were evaluated at 0 and 6 months after treatment. **Findings:** The results showed that both 1 and 2% peracetic acid inhibited bacterial and fungal growth by about 100% and reduced microbial flora growth by more than 90%. As there was no significant difference between 1 and 2% peracetic acid, the final recommendation is to use 1% concentration. After 6 months, the contamination level increased by about 5%, possibly due to storage conditions and contamination in subsequent stages. Different concentrations of peracetic acid did not have a significant effect on lipid percentage or peroxide number of pistachio kernels. **Research limitations:** As the treatment time with peracetic acid in the washing basin is uncontrollable, this factor has been eliminated in this research. **Originality/Value:** The study confirms the importance of using peracetic acid in the terminals to reduce and control pistachio contamination without producing harmful by-products. Economic evaluation also showed that using 1% peracetic acid for disinfecting pistachios in processing terminals is cost-effective.

INTRODUCTION

Iran and the United States of America are the largest pistachio (*Pistacia vera* L.) producing countries in the world, holding nearly 90% of the global pistachio market (FAO, 2021). Microbial contamination is one of the most important factors that have affected the pistachio market in recent years. In 1997, the European Union returned all pistachios exported from Iran due to the aflatoxin level of 11-400 ppb (Bui-Klimke et al., 2014). Some countries have also established laws restricting the allowable levels of aflatoxin in foodstuffs. In 2003, the European Union defined a maximum limit of 4 ppb for aflatoxin, which was changed to 10 ppb in 2009 (Wu, 2007; Bui-Klimke et al., 2014, European Food Safety Authority, 2020). However, the product quality seems to be varying among countries. Iranian pistachios contain an average of 54 ppb aflatoxin, while most pistachios of the United States have levels below the European Union standard of 10 ppb (Bui-Klimke et al., 2014). Despite such standards, the global pistachio market has changed and Iran's pistachio exports, which higher contamination had compared to American pistachios, decreased to European and other countries with higher aflatoxin limits at a lower price (Wu, 2007; Bui-Klimke et al., 2014). In this regard, Abdolahi Ezzatabadi (2010) showed that removing aflatoxin from Iranian pistachios increased their price by up to 26% and would benefit producers.

Peracetic acid ($C_2H_4O_3$), also known as peroxyacetic acid or percidine, is an antimicrobial substance obtained from the combination of hydrogen peroxide and acetic acid (Moghassem Hamidi et al., 2021; Lin et al., 2023). This compound, in addition to its environmental advantages, is capable of affecting a wide range of micro-organisms (Gehr et al., 2003; Pietrysiak et al., 2019; Suurnäkki et al., 2020). Due to its antibacterial, antiviral, and antifungal properties, peracetic acid has gained significant attention in recent years (Kitis, 2004; Garg et al., 2018; Lin et al., 2023). Another reason for the increased importance of this disinfectant compared to others such as chlorine dioxide is the absence of harmful by-products (Huang et al., 2022; Danielewicz, 2023; Pant et al., 2023). Peracetic acid rapidly decomposes into oxygen and acetic acid when in contact with organic compounds, ultimately resulting in water and carbon dioxide (Zhao et al., 2008; Pant et al., 2023). This compound is widely used in cheese and dairy processing plants, food processing equipment, as a disinfectant for various beverages, as well as for nuts and grains (Joshi et al., 2013; Thomas et al., 2016; Zoellner et al., 2018; EnviroTech, 2021; Pant et al., 2023).

Considering the importance of pistachio health in exports and its economic value, the use of peracetic acid as a safe disinfectant can be significant. As review of literature shows there is no report so far regarding the effect of peracetic acid on pistachios under terminal conditions. Therefore, in this study, the antimicrobial effect of peracetic acid in pistachio processing terminals was evaluated in terms of reducing microbial contamination and improving product quality.

MATERIALS AND METHODS

P. vera cv. Fandoghi were harvested from a commercial garden in September 2021 and then transferred to Hejri processing terminal located in Rafsanjan city, Kerman province, Iran. After peeling, pistachio nuts were disinfected with 0 (control), 1, and 2% peracetic acid in washing basin of the processing terminal. In fact, different concentrations of peracetic acid (formulated by Barafza Keshavarz Pars Company) were prepared inside a 1000-liter tank. The terminal washing basin was filled with these concentrations and the other processes were carried out according to the usual procedure. As the treatment time with peracetic acid in the washing basin is not controllable, the time factor has been eliminated in this research.

A portion of the treated product was transferred to the laboratory, and 100 pistachios were selected in three repetitions and placed in one-liter flasks. Then, 500 ml of sterile distilled water containing a concentration of 2 in 1000 peptone (as a nitrogen source) was added, and the flasks were shaken for 2 h on a shaker (150 rpm) at room temperature (Moradi et al., 2014). Suspensions with dilutions of 10^{-3} were prepared, and 100 µl were cultured in petri dishes (8 cm diameter) containing Malt Extract Agar (MA) medium (HiMedia^{TM}, India, 35 g l^{-1}) for fungal growth, Nutrient Agar (NA) medium (HiMedia^{TM}, India, 28 g l^{-1}) for bacterial growth, and Potato Dextrose Agar (PDA) medium (HiMedia^{TM}, India, 39 g L^{-1}) with pH=5.6 for microbial population growth. It should be noted that suspensions prepared from untreated pistachios (zero concentration of peracetic acid) were cultured as control in MA, NA and PDA culture media. Then, the petri dishes obtained from the above steps were placed in the darkness at the temperature of 28 °C. After 48 h, the number of grown colonies was examined and counted. The percentage of inhibition by peracetic acid was calculated based on the number of grown colonies in each treatment compared to the control using the following formula (1):

$$\text{Inhibition (\%)} = \frac{\text{number of colonies under control condition} - \text{number of colonies under different peracetic acid treatments}}{\text{number of colonies under control condition}} \times 100 \quad (1)$$

For the extraction of pistachio oil, dried kernels were ground carefully and mixed with 120 ml *n*-hexane. After 24 h, the mixture was passed through a filter to separate the oil and hexane from the pistachio residue. Then, *n*-hexane was separated using a rotary device (model RV8, Germany) (Kaviani et al., 2015) and the percentage of obtained oil was evaluated by the following formula (2):

$$\text{Lipid content (\%)} = (\text{initial weight of sample} - \text{final weight of sample}) \times 100 \quad (2)$$

In order to evaluate the peroxide value, 5 g of extracted oil was mixed with 25 ml of the solvent consisted of acetic acid and chloroform in a ratio of 3:2. After 5 min, 1 ml of saturated potassium iodide solution was also added. The mixture was placed in a dark place for 1 min. Thereafter, 25 ml of distilled water and 0.5 ml of 1% starch indicator were added to it, and titration continues until the blue color of the solution disappears using a normal sodium thiosulfate solution. The peroxide value was calculated using the following equation (3):

$$\text{Peroxide value (meq kg}^{-1}) = \frac{1000 \times \text{normality of sodium thiosulfate} \times \text{used amount of sodium thiosulfate (ml)}}{\text{sample weight (g)}} \quad (3)$$

It should be noted that microbial contamination, lipid percentage, and peroxide value were also evaluated for disinfected pistachio nuts that were stored 6 months at room condition (the temperature of 20-25 °C and relative humidity of 30-50%).

The economic analysis of the cost of this pistachio disinfection method (using peracetic acid) at the processing terminal was conducted according to partial budgeting method (Soleiman et al., 2010). Partial budgeting is a basic method designed to evaluate economic consequences of partial changes in farm or orchard activities. This method is based on the logic that a small change in farm activity structure reduces some costs and benefits while simultaneously increasing other costs and benefits. The net economic effect of a change is equal to the sum of positive economic effects minus the sum of negative effects (Table 1). In other words, partial budgeting method is not designed to express the total costs and benefits of the entire farm, but rather aims to express the net increase or decrease in farm income.

In this study, using data extracted from the technical section of the project, costs were calculated and using the results of Abdolahi Ezzatabadi's study (2010), benefits were

evaluated in two scenarios of using and not using peracetic acid. Net benefits were calculated using Table 1. This research was conducted as a completely randomized design with 3 repetitions. Statistical analysis of data was performed using SPSS software, and Dunken's multiple range tests was used at a significance level of 5% to compare means.

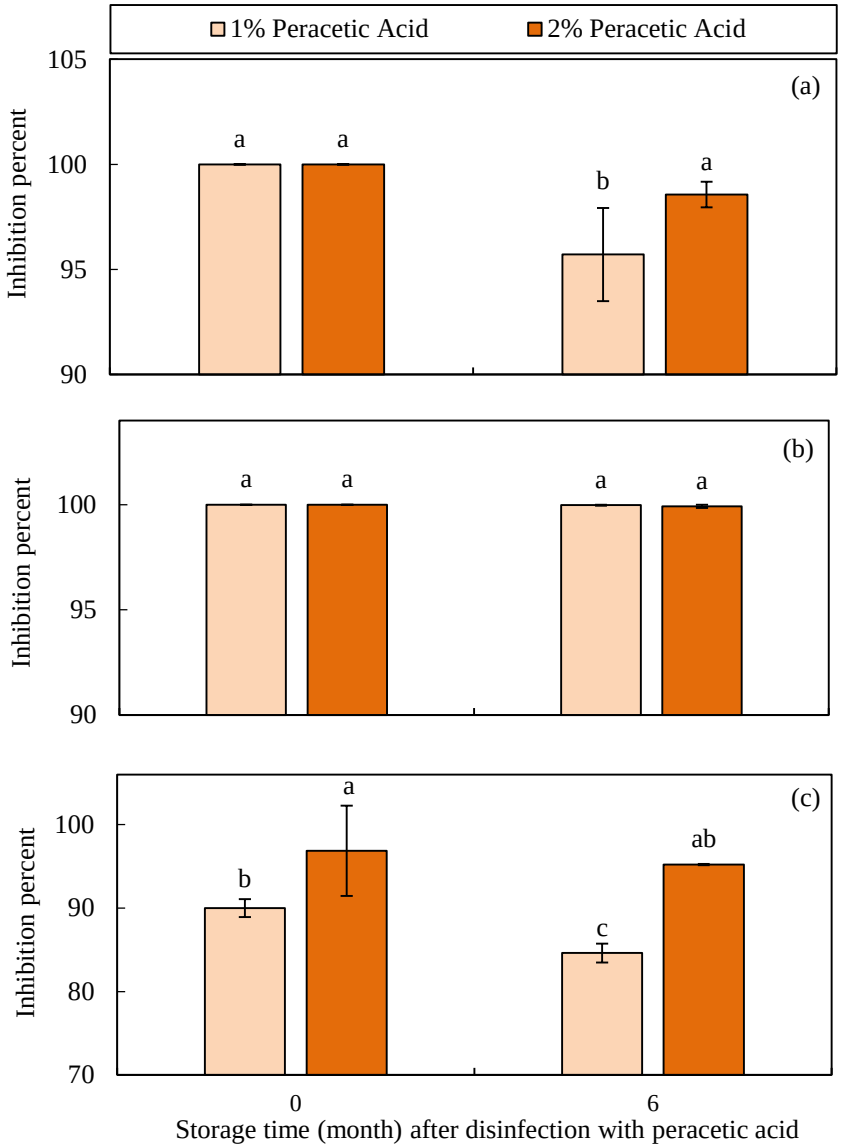

Fig. 1. The effects of 1 and 2% concentrations of peracetic acid and the duration (0 and 6 months) after treatment on the growth inhibition percent of bacteria (a), fungi (b), and microbial flora (c) contaminating pistachio nuts, respectively in NA, MA, and PDA culture media. The values were evaluated relative to the control (without peracetic acid), and columns with common letters do not have a significant difference according to the Duncan test at a 5% level.

Table 1. Partial Budgeting Map (Soleiman et al., 2010).

Costs	Benefits
A- Added costs: Costs incurred in alternative options that do not exist in the current situation.	C- Added revenues: Revenues in alternative options that do not exist in the current situation.
B- Reduced revenues: Revenues in the current situation that do not exist in the alternative option.	D- Reduced costs: Costs in the current situation that do not exist in the alternative option.
Total cost: A + B	Total benefits: C + D
Net profit: C + D - A - B	

Partial budgeting: Comparing the current situation (not using acid plastic) with the alternative option (using peracetic acid).

RESULTS AND DISCUSSION

The effect of 1 and 2% concentrations of peracetic acid and storage period (0 and 6 months) after treatment on the inhibition percentage of the growth of bacteria, fungi, and microbial flora of pistachio seeds compared to the control is shown in Figure 1. As observed, disinfecting pistachios with 1 and 2% peracetic acid causes approximately 100% reduction in bacterial growth compared to the control (without peracetic acid) at time zero (Fig. 1a). At this stage of the experiment, no significant difference was observed between the concentrations of 1 and 2% of peracetic acid. However, when bacterial growth was investigated at 6 months post peracetic acid treatment, a significant difference between 1 and 2% concentrations was observed, with an inhibition rate of approximately 96% in the 1% peracetic acid (Fig. 1a).

The concentrations of 1 and 2% peracetic acid limit fungal contaminants of pistachios by approximately 100%, and the results remained consistent even after six months (Fig. 1b). These Concentrations also caused a reduction in microbial flora contamination by approximately 90% and 96%, respectively, compared to the control (without peracetic acid) (Fig. 1c). Six months after peracetic acid treatment, the inhibition rate for microbial flora of pistachios treated with 1 and 2% peracetic acid reached approximately 85% and 95%, respectively (Fig. 1c). The results of this study demonstrated that the use of 1 and 2% peracetic acid in the washing basin of pistachio processing terminals can effectively inhibit the growth of bacteria and fungi by up to 100% and reduce the overall microbial flora by more than 90%. The increase in pistachio contamination after six months may be due to storage conditions and subsequent contamination.

In a study conducted by Hasani et al. (2020), two disinfection methods were evaluated for inactivating the important foodborne pathogens of *Salmonella* or *Listeria monocytogenes* on de-shelled pistachios. One method was based on the combination of peracetic acid-ethanol sanitizer (PAA-ethanol), and the other involved an Advanced Oxidation Process (AOP) utilizing UV-C, ozone, and hydrogen peroxide simultaneously. Disabling *Salmonella* in pistachios using 2.5 ‰ (part per thousand), PAA-ethanol was significantly more effective than the AOP method. *L. monocytogenes* had a higher sensitivity to hydrogen peroxide and AOP, so it can be eliminated from the samples. They showed that these two disinfection methods are used to reduce *Salmonella* and *L. monocytogenes* on pistachios, although their efficacy depends on the pathogen and product type (Hasani et al., 2020).

Pao et al. (2006) treated almonds contaminated with *Salmonella* with water, acetic acid, citric acid, acidified sodium hypochlorite, peroxyacetic acid, and a combination of citric, hydrochloric, and phosphoric acids. Their results showed that these acids caused a reduction in the bacterial population contaminating almonds. Furthermore, increasing the application number (1 to 3 times) and the concentration of acid treatments resulted in increased effectiveness and further reduction in the population of pathogenic bacteria (Pao et al., 2006).

Research on the effect of peracetic acid on bacterial growth has shown that this disinfectant compound delays bacterial growth and also causes destruction of bacterial cell walls (Wang et al., 2020; Liu et al., 2023).

Spraying peracetic acid on fresh strawberry cuts showed that its concentration (1-240 mg l^{-1}) and spraying duration (11-138s) did not have any effect on fruit quality characteristics (Méndez-Galarraga et al., 2019). On the other hand, this method led to a reduction in contaminating micro-organisms such as molds, yeasts, and bacteria.

The effect of using different concentrations of peracetic acid (0 as control, 1, and 2%) in the washing basin of the processing terminal and the duration after treatment (0 and 6 months) on the lipid content of pistachio kernels is shown in Figure 2. As observed, the amount of lipid present in pistachio kernels is approximately 30-35%, and treatment with different concentrations of peracetic acid does not have a significant effect on the lipid content at both time points (Fig. 2).

Figure 3 illustrates the effect of different concentrations of peracetic acid (0 as control, 1, and 2%) and the duration after treatment (0 and 6 months) on the peroxide value of pistachio kernels. As observed, different concentrations of peracetic acid do not have a significant effect on the peroxide value at a level of 5%.

Excluding the effect of peracetic acid disinfectant on lipid content and peroxide value in pistachio kernels can be important for preserving product quality and increasing shelf life (Tavakolipour, 2015; Akhavan-Mahdavi et al., 2023). In another study, the effect of chlorine dioxide disinfectant on green walnuts was investigated, but it did not cause any changes in the peroxide value during storage period (Jiang et al., 2015).

Ribeiro et al. (2020) investigated the disinfectant effect of different concentrations (20, 80, and 140 $mg.l^{-1}$) peracetic acid on Brazil nuts for 2, 8.5, and 15 min. Their results showed that the optimal sanitizing condition was 15 min treatment with 140 $mg.l^{-1}$ peracetic acid. It was demonstrated that the used concentrations of peracetic acid have no effect on aflatoxins, despite their effectiveness in controlling fungi. Peraceic acid treatment did not have any sensorial properties of the products (Ribeiro et al., 2020).

Since the use of peracetic acid almost eliminates the contamination of pistachio by fungi, bacteria, and microbial flora, the likelihood of incompatibility between Iranian exported pistachio samples and global health standards, especially those set by the European Union, has decreased and even reached zero. This issue leads to a decrease in the number of returned Iranian pistachio samples from global markets, resulting in an increase in the price of pistachio at the Iranian producer level. According to Abdolahi Ezzatabadi (2010), removing aflatoxin from Iranian pistachios increases their price at the produce level by 26%. Based on current prices (spring 2023), the average price per kilogram of pistachios (different varieties with various qualities) at the producer level is around five million Rials. If we multiply this number by 0.26, it will amount to 1,300,000 Rials. Considering that currently there are no other costs for disinfecting pistachios at terminals, 1,300,000 Rials per kilogram is considered as the only benefit of using this disinfectant.

The costs of using peracetic acid include the price of purchasing this material itself, and due to the automation of the machines in the terminals, there is no need for the labor. Since the washing basins are already present in the pistachio processing terminals, no additional costs were included in this regard. Considering that the final research recommendation for the use of peracetic acid is 1% concentration, the cost of using this type of acid was also taken into account in the economic evaluation. For the final application, one liter of 1% peracetic acid is required to disinfect one kilogram of pistachios, with a price of 77,600 Rials per liter. Therefore, the cost of using peracetic acid is equivalent to 77,600 Rials per kilogram. Since the use of peracetic acid does not eliminate any pre-existing benefits, the costs associated with

using this material are only additional costs resulting from its application. The summary of benefits and costs resulting from the use of peracetic acid is provided in Table 2. As shown, the net profit resulting from peracetic acid in pistachio processing terminals is positive and equivalent to 1,222,400 rials per kg of pistachios. In other words, its use is economically viable.

Table 2. Partial budgeting for the application of acid plastic for pistachio disinfection in processing terminals.

Costs	Benefits
A- Additional Costs: 77,600	C- Additional Revenues: 1,300,000
B- Reduced Revenues: 0	D- Reduced Costs: 0
Total Costs: 77,600	Total Benefits: 1,300,000
Net Profit: 1,222,400	

(unit: Rials per kilogram of pistachios).

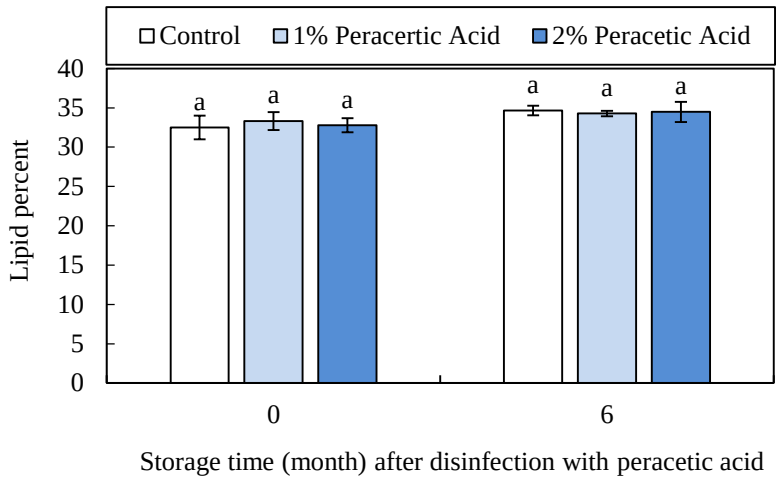

Fig. 2. The effect of using concentrations of 0 (control), 1, and 2% peracetic acid in the terminal washing basin and the duration after treatment (0 and 6 months) on lipid content of pistachio kernels. Columns with common letters do not show a significant difference according to the Duncan test at a 5% level.

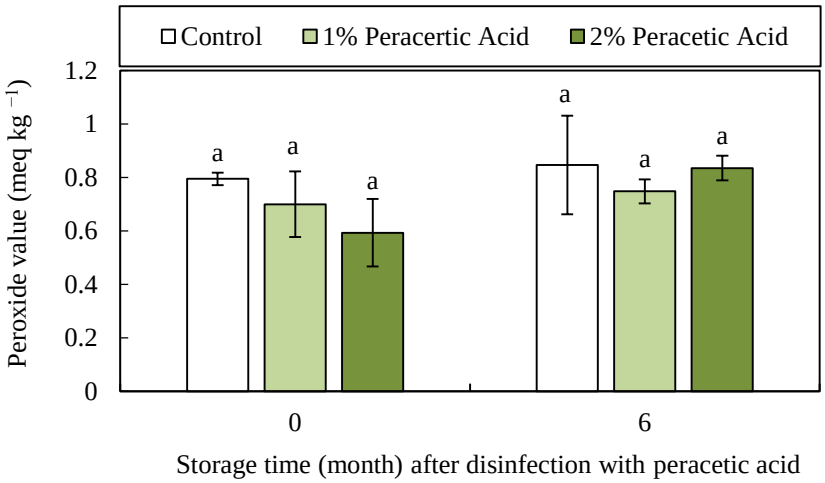

Fig. 3. The effect of using concentrations of 0 (control), 1, and 2% peracetic acid in the terminal washing basin and the duration after treatment (0 and 6 months) on the peroxide value (meq kg^{-1}) of pistachio kernels. Columns with common letters do not show a significant difference according to the Duncan test at a 5% level.

CONCLUSION

Microbial contaminations are among the most important factors that affect the quality of pistachios and restrict pistachio exports. Peracetic acid is a compound that, besides not producing harmful by-products and having environmental benefits, is capable of eliminating a wide range of micro-organisms in the shortest possible time, which gives it an advantage over other disinfectants. The results of this study showed that the use of 1 and 2% concentrations of peracetic acid in the washing basins of pistachio processing terminals can inhibit the growth of bacteria and fungi by up to 100% and reduce the general microbial flora by more than 90%. The significant reduction of microbial population (fungi and bacteria) contaminating pistachios, along with not producing harmful by-products, can confirm the importance of using peracetic acid disinfectant to reduce and control pistachio contamination. As there was not any significant difference between 1 and 2% peracetic acid in controlling microbial contamination of pistachios, the final recommendation is the treatment with 1% peracetic acid. In addition, economic evaluation also showed that the use of 1% peracetic acid in pistachio processing terminals for disinfection is cost-effective.

Conflict of interest

The authors have no conflict of interest to report.

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Molecular markers associated with male-sterile cytoplasms and male-fertility restorer locus in onion (*Allium cepa*): a review

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ABSTRACT

Purpose: CMS hybrid seed production systems are employed effectively for onion. Cytoplasmic male sterility (CMS) has long been used to economically produce hybrids that harness growth vigor through heterosis. Three types of onion CMS (CMS-S, CMS-R, and CMS-T) have been used in hybrid onion breeding. **Findings:** The sources of onion CMS, their maintainer plants and fertility restorer lines can be distinguished by markers, saving times spent on crop establishment and avoiding the complex of phenotypic screening. Numerous molecular markers especially PCR markers associated with male-sterile (CMS-S and CMS-T) cytoplasms, male-fertile normal (N) cytoplasm and nuclear-male- fertility restorers (*Ms* locus) were developed. These simple PCR markers are valuable tools for the marker-assisted selection of segregating individuals in onion F1 hybrid breeding programs. The present review reveals practical utility and functional effectiveness in the MAS of male-sterile cytoplasm types with nuclear-fertility-restorer locus. **Limitations:** the environment effect especially temperature may cause male-sterility instability and deviations in segregation ratios for male sterility. Also, maximum exploitation of molecular markers linked to *Ms/ms* and CMS-S, -T genes aid in the recovery of male-sterile traits requires to a perfect linkage disequilibrium that must be investigated further. **Directions for future research:** Furthermore, this information could highly have paved the way for hybrid onion development by applicating of the molecular findings to identify onion male sterility, maintainer and male fertility restorer lines.

INTRODUCTION

F1 hybrids have been used in onion breeding for the past 50 years in the United States, Europe and Japan (Brewster, 2008). Hybrid-onion (*Allium cepa*) seed became economically feasible using sources of CMS. The production of hybrid seed through the CMS system necessitates a male-sterile line (A-line), a male-sterility maintainer line (B-line, male fertile) and male-fertility restorer line (C-, or R-line, male fertile) (Goldman et al., 2002). Male sterility in onion is conditioned by the interaction of the male-sterile cytoplasm with the homozygous recessive genotype at one nuclear locus (*msms*) (A-line) (Yu & Kim, 2021) that all genes responsible for CMS have been existed in mitochondrial genomes (Fauron et al., 1990; Budar et al., 2003; Hanson & Bentolila, 2004; Knoop, 2004). CMS-associated genes often consist on the partial sequences of known mitochondrial genes and unknown sequences (Chen & Liu, 2014; Hanson & Bentolia, 2004; Chen et al., 2017). In most cases, multiple recombination events involving known mitochondrial genes as well as sequences of unknown origin create new open reading frames (ORF) associated with cytoplasmic male sterility in higher plants (Chen & Liu, 2014; Tuteja et al., 2013). These CMS-associated ORFs have been used for producing molecular markers to identify the type of CMS (Khrustaleva et al., 2023). The maintainer line (B-line) of the male-sterile line is produced by crossing normal fertile (N) cytoplasm with the homozygous recessive genotype at one nuclear locus (*msms*). Thus, A- and B-lines are isogenic lines, with the difference only for male sterility trait. Likewise, the male-sterile plants were maintained and reproduced by mating with maintainer plants (Jones & Clarke, 1943; Jones & Davis, 1944). The restorer of fertility (C/R line) governed by single dominant (Ms) allele at the nuclear locus and normal cytoplasm (Manjunathagowda, 2021). This review summarizes the molecular mechanism of male sterility and restoration of male fertility, depicts the identification of male sterility systems through molecular markers that could accelerate breeding of F1 hybrids and production of pure hybrid seed.

CMS cytoplasm

To date, three different types of CMS and fertility restoration systems, have been described and utilized for F1 hybrid cultivar development in onion, termed CMS-S, was first identified during 1925 in the cultivar 'Italian-Red' (Jones & Emsweller, 1936), CMS-R or T-like was identified in 'Rijnsburger' onion (Banga & Petiet, 1958) and CMS-T, traced in the variety 'Jaune paille des Vertus' (Berninger, 1965). The CMS-S is conditioned by the sterility inducing cytoplasm (S) and the single nuclear in its recessive condition (*msms*) with restorer gene *Ms/ms* (Jones & Emsweller, 1936; Jones & Clarke, 1943). The CMS-T is influenced from three independently segregating loci. The male-fertility restoration of CMS-T cytoplasm had been controlled by three Rf genes, one independent gene (a) and two complementary genes (b and c) that revealed a complex nature of inheritance (Schweisguth, 1973).

The discovery of these CMS, paved the way for hybrid onion development, besides uniformity in size, shape, color and maturity and high yields (Khar & Saini, 2016). At present, S cytoplasm due of its stability under diverse environmental conditions and simple monogenic inheritance, is the most common source of CMS used to produce hybrid onion seed and made it more popular among breeders around the world (Havey, 2000). T cytoplasm has been used extensively to produce hybrid onions in European countries, although its use is less common than S cytoplasm (Havey, 1994). The T-cytoplasm had originated from N-cytoplasm through point mutation in the mitochondrial genome. R cytoplasm is very close to N and T cytoplasms and is distantly related to S cytoplasm, and is widely used commercially (Havey & Kim, 2021). In a study with evaluating of onion breeding lines from commercial entities to distinguish sources of onion CMS, their results reveal that T cytoplasm is rarely used

commercially to produce hybrid-onion seed, and both S cytoplasm and "T-like" cytoplasm are widely used (Havey & Kim, 2021). HRM analysis (Khrustaleva et al., 2023) showed that the most and the least source of CMS, was S-cytoplasm and R-cytoplasm, respectively and the proportion of T-cytoplasm among the analyzed onion breeding lines was 20.5%.

Marker assistant selection (MAS) of male sterility, maintainer and restorer lines in onion

Identification of CMS types and *Ms* locus are key steps in onion F1 hybrid breeding. However, since onion is a biennial crop, it takes 4–8 years to identify cytoplasm types and *Ms* alleles using progeny tests (Khrustaleva et al., 2023). Thus, molecular markers can greatly accelerate breeding processes by replacing time-consuming and laborious progeny tests. The conventional method of hybrid development is less efficient as compared to molecular marker assisted methods for identification of S, T, N cytoplasm, maintainer lines and fertility restorer genes with high heterosis. Thus, tightly linked nuclear markers at the *Ms* Locus and CMS genes would allow for molecular-assisted segregation analysis and facilitate the processing of F1 breeding and development. In order to implement reliable marker assisted selection systems for CMS types and the restorer-of-fertility locus (*Ms*) in onions (*Allium cepa* L.), simple PCR based codominant markers linked to the *Ms* Locus were developed (Bang et al., 2011).

Development of polymorphic markers linked to the cytoplasms

Restriction-enzyme analyses of the chloroplast (cpDNA) and mitochondrial (mtDNA) DNAs of normal (N) fertile, S-, and T-cytoplasms have demonstrated fragment size differences (de Courcel et al., 1989; Holford et al., 1991; Havey, 1993; Satoh et al., 1993). de Courcel et al. (1989) delineated four cytoplasmic groups (M I through M4) based on *BamHI* digests of the mtDNA. Restriction enzyme digests of both the mtDNA and cpDNA distinguished S-cytoplasm from the M-cytoplasmic groups (de Courcel et al., 1989). Holford et al. (1991) were able to distinguish among N-, S-, and T-cytoplasms with *BamHI* and *HindIII* digests of mtDNA and among N- and S-cytoplasms with *EcoRI*, *HindIII*, and *XbaI* digests of cpDNA. Havey (1993) after digesting genomic DNA with 15 restriction enzymes and probing with a complete set of chloroplast clones identified five polymorphisms between N- and S-cytoplasms. The complete DNA isolations from single plants, restriction-enzyme digestions, blotting, and hybridizations was time-consuming. For the facilitation of marker-assisted selection and the acceleration of onion hybrids breeding, the Polymerase chain reaction (PCR) would allow a quick and confident identification of the cytoplasm of individual plants. The first published PCR-marker for cytoplasm types in onion amplifies (Havey, 1995) demonstrated that there is a 100-bp insertion in the IGS in (N)-cytoplasm, resulting in a bigger amplicon of corresponding size. Then, further variation within the (M) cytoplasmic group was found and reported for the identification of onion cytotypes (Sato, 1998; Engelke et al., 2003; Kim et al., 2009). Second PCR-marker anchors in the upstream region to the mitochondrial gene *cob* were reported (Sato, 1998). According to the author, the (S)-cytoplasm contains an insertion in this region, which is homologous to the chloroplast *orf1708* of *Nicotiana tabacum*, and which can be used to anchor a (S)-specific primer. Sato (1998) suggested a second primer which should be (N)-specific, and a common antisense primer. Then Engelke et al. (2003) developed a PCR-marker, *orfA501* that distinguished all the three known cytoplasms in the onion, both male sterility inducing cytoplasms, CMS-(S) and CMS-(T), from the normal cytoplasm in onion (*Allium cepa*). The PCR RFLP marker was located in a chloroplast *psbA* gene amplicon could distinguish male-fertile (N) and male-sterile (S) cytoplasm in onions (Cho et al., 2006). Kim et al. (2009) designated a new marker,

orf725. RT-PCR results showed that *orf725* was not transcribed in normal cytoplasm. Meanwhile, the normal *coxI* gene, which is essential for normal mitochondrial function, was not expressed in CMS-S cytoplasm. However, both *orf725* and *coxI* were transcribed in CMS-T cytoplasm. Von Kohn et al. (2013) checked the size of two types of cytoplasm (N and S) and found that the size difference was primarily due to small indels in intergenic regions and a deletion in the *accD* gene of N-cytoplasmic onion. According that result, they designated a new marker, *accD*.

This marker along with *cob* and *MK* were used in detecting of the cytoplasm types of onion Indian germplasm (Khar & Saini, 2016). It was observed that accD should be used more for cytoplasm determination because of the relative simplicity of two primers and its visualization (Khar & Saini, 2016). Dehghani et al. (2021), identified the of *Ms* locus and cytoplasmic types in 123 onion accessions (three populations, male sterile lines and maintainer lines) by three cytoplasmic markers *cob, accD* and *MK* and four nuclear molecular markers (*OPT, PsaO, Jnurf-13 and AcSKP1*). The two markers *accd* and *cob* detected the type of cytoplasm as well, with 100% male sterility for male sterile lines and 100% fertility for maintainer lines. Also, T-type cytoplasm could be distinguished by the *MK* marker.

Development of linked molecular markers to the maintainer lines and the restorer of fertility

In the production of hybrid-onion seed, *Ms/ms* alleles were used in maintainer (B) lines of male sterility (are used to create male-sterile (A) lines) and restorer-of-Fertility (R/C) lines. As a result, identifying restorer (N_{MsMs}) and maintainer (N_{msms}) lines is critical for onion hybrid development (Manjunathagowda, 2021). However, molecular markers capable of distinguishing genotypes at a nuclear locus (*Ms*) are crucial for breeders to save time and effort. Few molecular markers for allelic selection of the nuclear *Ms* Locus have been reported (Gökçe et al., 2002; Martin et al., 2005). For the facilitation of marker-assisted selection, *Ms* locus-specific simple PCR co-dominant markers were created (Bang et al., 2011; Huo et al., 2012; Bang et al., 2013; Kim et al., 2019). Two markers OPT and PSAO were designed by Bang et al. (2011). A simple PCR marker for OPT was developed by designing a primer pairs on the flanking regions of the 108-bp indel which is created by two tandem repeats. The OPT marker was tightly linked to the *Ms* Locus at a distance of 1.5 cM (Bang et al., 2011). Despite the low distance between the OPT marker and Ms locus (1.5 cM), the researcher studies (Khar & Saini, 2016, Dehghani et al., 2021) did not show significant linkage disequilibrium between this marker and the Ms locus, indicating that crossing-over contiguous to the Ms locus mostly occurred throughout the history of onion breeding. Yu and Kim (2021) stated that the discrepancy between genotypes of molecular markers tagging the Ms locus and phenotypes of male-fertility in Indian and Brazilian onion germplasm, is probably because of the presence of the Ms2 locus that needs more studies. Also, it is likely that the relatively low expressivity of the Ms2 locus caused unstable male-sterility (Kim et al., 2019). PSAO marker was designed using tandem repeats (14 and 39 bp) based on the photosystem-I-subunit-O (*PsaO*) gene, which was isolated by genome walking of EST-RFLP probe, which was linked to the *Ms* locus at a istance of 6.5 cM (Bang et al., 2011). A PCR-based marker (WHR240) related to the *AcPME* gene was designed and validated in six different onion lines, and it successfully identified male fertility restorer lines (Huo et al., 2012). Two markers (DNF-566 and RNS-357) from the AFLP markers linked to the *ms* allele might be used to distinguish *MsMs*, *Msms* or *msms* allelic phenotypes among varieties, F1 hybrids and the OP population (Yang et al., 2013). A more reliable simple PCR marker (jnurf13) linked to the *Ms* Locus was constructed using a 12-bp InDel sequence and 5.5 kb flanking sequences. The male-fertility phenotypes of all studied breeding lines were perfectly

matched with marker genotypes (Kim, 2014). Another marker such as ACms.1100 (Bang et al., 2013) and CAPS markers (jnurf05, jnurf06, jnurf10, jnurf17) (Park et al., 2013), having tight linkage with *Ms* Locus, and these markers are of co-dominant nature, thus effectively differentiate the dominant from recessive alleles. The markers linked to cytoplasm types and *Ms* Genotypes and validation of identified markers in different studies are shown in Table 1 and Table 2, respectively.

Table 1. Marker assistant selection of male-sterile cytoplasmic and *Ms* Locus lines for breeding of F_1 hybrid onion.

The Marker based on genes	Method	Cytoplasm's/male-fertility loci	Reference
A two-step HRM marker system with both *cox1* and *orf725* genes	High-resolution melting (HRM) analysis	Identification of the N-, S-, R- and T-cytoplasms	(Khrustaleva et al., 2023)
Ms2 locus was likely to be positioned at the end of chromosome 2	High-resolution melting (HRM) analysis based on single nucleotide polymorphisms (SNPs) detected by RNA-Seq	*Ms* locus	(Yu & kim, 2023)
AcPMS1 gene	PCR	*Ms* locus	(Kim & Kim, 2021)
molecular marker (AcCN) (two markers at the Ms loci and one cytoplasm marker)	multiplex-PCR	CMS and *Ms* locus	(Liu et al., 2019)
100 breeding lines of Onbreetech Corp., Haenam, Korea seed company were employed using CAPS markers	PCR	CMS-S, -T and N-cytoplasm and Ms locus	(Kim & Kim 2015)
301 plants of F2 and F3 populations developed from H6 male-fertile line and 506L male-sterile line using CAPS marker	PCR	Ms locus	(Bang et al., 2013)
	PCR	Ms locus	(Yang et al., 2013)
F2 populations developed from H6 male-fertile line and 506L male- sterile line using OPT and PSAO marker	PCR	Ms locus	(Bang et al., 2011)
176 breeding lines and cultivars using *orf725* marker	PCR	CMS-S, -T and fertile N-cytoplasm	(Kim et al., 2009)
orfA501	PCR	CMS-S, -T and fertile N-cytoplasm	(Engelke et al., 2003)
W202B (*Nmsms)*, W202A (*S msms*) and the S-cytoplasmic male-fertile	PCR	CMS-S and fertile N-cytoplasm	(Sato, 1998)

Table 2. Validation of identified markers in different studies.

Cytoplasm's/male-fertility loci	Genetic material	Reference
cytotype and *Ms* locus	Cytotype markers, *accD*, and *MKFR* and for *Ms* locus identification, PCR markers *AcPMS1* and *AcSKP1* in Indian breeding lines, variety and hybrids	(Khar et al., 2022)
CMS-S, -T and fertile N-cytoplasm/ Ms locus	Three cytoplasmic markers *cob, accD* and *MK* and four nuclear molecular markers (*OPT, PsaO, Jnurf-13 and AcSKP1*) on 123 onion accessions (three populations, male sterile lines and maintainer lines)	(Dehghani et al., 2021)
CMS-S, -T and fertile N-cytoplasm	OPV onion genetic stock of Punjab province using *orf725* markers	(Ahmad et al., 2020)
CMS-S	OPV onion genotypes using *orf725* marker	(Manjunathagowda & Anjanappa, 2020)
CMS Y and *Ms* locus	S1 segregating population was produced from a single plant selected from PI273626 (containing cytotype Y)	(Kim et al., 2019)
CMS-S and -T and fertile N-cytoplasm	Brazilian onion germplasm using *orf725* marker	(Ferreira & Santos, 2018)
CMS-S, -T and fertile N-cytoplasm	5' *cob*/*orfA501* and *orf725* markers deployed in 59 genotypes of the Embrapa Onion Germplasm Bank for MAS of cytoplasms	(Ferreira et al., 2017)
CMS-S, -T and fertile N-cytoplasm/ Ms locus	Five cytoplasmic (5'cob, orfA501, orf725, IGS and cob-type 2) and four nuclear markers (jnurf13, isotig34671_610, isotig30856_1351 and isotig29186_1830)	(Gazendam et al., 2018)
CMS-S and fertile N-cytoplasm	Open-pollinated populations of onion varieties Punjab Naroya, Punjab Selection and Punjab White using '*cob*' marker for cytotype	(Malik et al., 2017)
CMS-S, -T and fertile N-cytoplasm/ Ms locus	Three cytoplasmic markers *cob, accD* and *MK* and four nuclear molecular markers (*OPT, PsaO, Jnurf-13 and AcSKP1*)	(Khar & Saini, 2016)

CONCLUSION

F1 hybrid cultivars are popular due to more bulb uniformity and higher productive potential for heterosis. The sources of onion CMS, their maintainer plants and fertility restorer lines can be distinguished by markers, saving times spent on crop establishment and avoiding the complex of phenotypic screening. Cytoplasmic determinations in onion have been greatly simplified by molecular markers in the mitochondrial and chloroplast DNAs distinguishing N and S cytoplasms (von Kohn et al., 2013). Marker-assisted selection of the PCR markers in determining types of S/N and T cytoplasm and *Ms* locus were reported in Indian onion germplasm (Khar & Saini, 2016) and Brazilian germplasm (Ferreira et al., 2017; Ferreira et al., 2018) and Iranian germplasm (Dehghani et al., 2021). Although these molecular markers do not shorten the generation time of onion, they represent a more sensible use of resources because only plants with known cytoplasms are used for crossing (Havey, 1995). The development of F1 hybrids in onion heterosis breeding was made possible by the use of PCR markers that linked cytoplasms and restorer-of- fertility genes. Many onions in North

America are three-way hybrids. An example is the hybrid 'Spartan Banner 80,' which was developed and released jointly by the USDA and Michigan Agricultural Experiment Station. Another three-way hybrid developed by Dr. Peterson was 'Sweet Sandwich,' from the cross (MSU5718A × MSU8155B) × MSU826B (Havey, 2018).

Two important factors in successful deployment of a CMS breeding pipeline are CMS stability and sterility and fertility transition. Recently, three proposed strategies for the genetic control of sterility and fertility transition to engineer "on–off" switches include gene editing or RNA interference of MSH1, design of Rfs, and mt-targeted gene editing of CMS-associated ORFs in the CMS line (Xu et al., 2022). Recent attempts have been made to develop restorer lines using wide hybridization (Yu et al., 2020) and genetic transformation (Li et al., 2021).

The cp-cytotype RFLP marker (Havey, 1995), cob gene-specific marker (Sato, 1998), orfA501 gene marker (Engelke et al., 2003), the psbA gene marker (Cho et al., 2006), orf725 gene-specific marker (Kim et al., 2009), atp6 gene CAPS marker, atp1 gene, cob gene and cox2 gene-specific markers (Kim & Yoon, 2010), petN gene-specific marker (Kim & Kim, 2015; Kim et al., 2015) and mtDNA CAPS marker (Kim & Kim, 2019) were able to differentiate CMS-S, CMS-T and normal (N) cytoplasm in a mixed population. These cytoplasmic markers, coupled with single nucleotide polymorphisms (SNPs) tightly linked to the nuclear Ms locus (Havey, 2013), can be used to select individual plants using high-throughput genotyping platforms to aid in the development of male-fertile maintainer (N msms) and restorer lines (N/S MsMs) lines for the production of hybrid onion seed (von Kohn et al., 2013). The restorer-of-fertility locus distinguishes through SSCP marker (McCallum et al., 2001), RFLP marker (Gökçe et al., 2002), OPT and PSAO gene-specific markers (Bang et al., 2011), AcPME gene (Huo et al., 2012), CAPS marker (Bang et al., 2013), SCAR marker (Yang et al., 2013), InDel marker (Kim, 2014), AcSKP1 (Huo et al., 2015) and AcPMS1 genes-specific markers (Kim et al., 2015). Liu et al. (2019) designed a multiplex-PCR marker, AcCN that could detect cytoplasm types and the nuclear locus in a single PCR experiment.

Conflict of interest

Author has no conflict of interest, financial or otherwise to declare.

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Putrescine and IBA enhanced the adventitious root formation in Damask rose (*Rosa × damascena* Mill.) under *in vivo* and *in vitro* conditions

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ABSTRACT

Purpose: To investigate the effects of Putrescine and Indole-3-Butyric Acid (IBA) on the adventitious rooting of micro-cuttings and semi-hardwood cutting of *Rosa damascena,* this study was conducted under both *in vitro* and *in vivo* conditions. **Research Method:** The rooting of micro-cuttings was induced on the basal MS medium supplemented with five concentrations (0, 0.25, 0.5, 1 and 2 mg/L) of IBA and putrescine. *In vivo* experiment, putrescine and IBA at five concentrations (0, 0.25, 0.5, 1 and 2 g/L) were applied on semi-hardwood damask cuttings, while a downward wounding was created by a sharp blade on the bases of cutting as another treatment. **Findings:** Data showed significant variations in the root number and root length for *in vitro* and *in vivo* cuttings treated with different concentrations of putrescine and IBA. The obtained results revealed that presence of putrescine and IBA in both conditions enhanced root formation, as significantly improved the number of roots and root length in each explant. Under *in vitro* conditions, the maximum root length and root number were observed on the MS medium supplemented with 1 mg/l IBA+1 mg/l putrescine. **Research limitations:** No limitations were found. **Originality/Value:** The present study highlighted the role of putrescine and IBA in the adventitious rooting of *R. damascena*, under both *in vitro* and *in vivo* situations.

INTRODUCTION

One of important species of Rosaceae family is *Rosa* × *damascena* Mill., commonly referred to as Damask rose (Huxley, 1992). Damask rose is considered as an important medicinal and ornamental plant cultivated world-widely. Today, some of scented and fragrant roses are cultivated in Bulgaria, France, Italy, Turkey, Iran, Morocco, USA, and India for the production of essential oil extracted from their flowers (Rusanov et al., 2005; Mirzaei et al., 2016). In Iran, Damask rose is also cultivated to produce rose water, dried buds and petals, which are mostly used as flavoring agent in syrup, tea and food products (Mirzaei et al., 2016).

The asexual propagation methods for roses could be pointed to cutting, budding, grafting, stenting and root grafting (Salehi & Khosh-Khui, 1997; Azadi et al., 2013). Further, *in vitro* propagation has become very popular for some genotypes; this method is commonly applied for the large-scale plant multiplication of roses in some areas of the world (Pati et al., 2010). Although, plants' rapid clonal multiplication is regarded as an important feature and advantage of micro-propagation; but considering the rooting of micro-cuttings would be evaluated under *in vitro* condition (Goel et al., 2018). Vegetative propagation can be effective to get true-to-type plants, especially in superior rose cultivars, considering that commercial varieties are commonly very heterozygous and polyploid (Nasri et al., 2015). Adventitious root formation as an important step in cuttings should be considered from an economic point of view in the rose industry (Nguyen et al., 2020).

The adventitious rooting process has been reported to be under the influence of many factors (De Klerk, 1996), range from genotype to the endogenous levels of some significant biochemical molecules including plant growth regulators, as well as environmental conditions (Kibbler et al., 2004; Kumar, 1996). Plants produce natural auxin in their apical meristems, in the tips of the growing stem, root, fresh shoots as well as buds (Galvan-Ampudia et al., 2020). Despite synthesis of auxin in plants, nowadays, the synthetic auxin is exogenously applied for rapidly and simultaneously inducing root formation, and even preventing cuttings death (Štefančič et al., 2007; Kasim & Rayya, 2009). In roses, auxins, particularly IBA (Indole-3-Butyric Acid), were commonly used to accelerate the adventitious root formation in some cultivars in both *in vitro* and *in vivo* conditions (Ahmadi, 2012; Rather & Tsewang Tamchos, 2017; Nguyen et al., 2020; El-Banna et al., 2023).

Polyamines, as organic compounds, have important role in plant growth and developmental processes. They are involved in cellular processes such as cell proliferation, differentiation, root formation, apoptosis, senescence, as well as fruit development and ripening., Moreover they can be involved in plant's tolerance or resistance against abiotic or biotic stresses (Kusano et al., 2008; Liu et al., 2015; Pang et al., 2007). Putrescine was applied in both *in vitro* and *in vivo* conditions resulted in increasing the root formation and root length in pear, apple (M9 rootstock, clone P3), olive, hazelnut, GF677 (*Prunus amygdalus*× *Prunus persica*), and *Panax ginseng* (Rugini et al., 1993; Cristofori et al., 2010; Denaxa et al., 2014; Kordzadeh & Sarikhani, 2021; Wu et al., 2021).

The adventitious root induction as a complex process is regulated by diverse environmental and endogenous factors (Bellini et al., 2014; Díaz-Sala, 2014; Druege et al., 2016). Adventitious roots are induced through such stresses as wounding, flooding, nutrient deprivation, or etiolation (Steffens & Rasmussen, 2016). Improving effects of wounding could be related to increasing the Jasmonate compounds which play a prominent role in plant physiological behavior (Schilmiller & Howe, 2005).

In this study, the effects of IBA and putrescine concentrations were investigated on rooting of Damask rose cutting in both *in vitro* and *in vivo* conditions. Also, in this research,

the impact of wounding on the formation of the adventitious root of cuttings under *in vivo* condition was evaluated.

MATERIALS AND METHODS

Plant materials

Healthy and vigorous shoots were cut from the upper regions of the 3-4-year-old damask shrubs cultivated in Meybod County, Yazd, Iran. The micro-cuttings (single-node cuttings), containing an axillary bud of *Rosa damascena* Mill., were prepared, with a size around 1-2 cm (Fig. 1A). For *in vivo* experiment, semi-hardwood cuttings were prepared with a mean of 15-20 cm length and 0.5-1 cm diameter; containing five to six nodes in December 2021. The trials were carried out in two parts (*in vitro* and *in vivo*) as a factorial based on a completely random design with three replications at Ardakan University.

In vitro root induction

The explants were first rinsed with sterile distilled water three times, and then transferred to the sterilization solution containing 0.05% citric acid and 0.1% mercuric chloride for 4 min. Finally, the explants were rinsed in 0.05% citric acid solution three times. The explants were continuously shaken during the sterilization applying mentioned washing steps (Khamushi et al., 2019). Explants were transferred into glass bottle (10-cm height and 6-cm diameter) containing MS (Murashige & Skoog, 1962) culture media (Fig. 1B). The containers (at least three explants in each jar) were kept in growth chamber with 16:8 h light/dark period. The temperature was adjusted at 18 ± 2 °C for dark and 23 ± 2 °C for light periods with the photosynthetic photon flux density (PPFD) of 34–40 µmol m^{-2} s^{-1}. The MS basal culture media were supplemented with 8.5 g l^{-1} Agar agar and 30 g l^{-1} sucrose. The culture media pH was adjusted to 5.7, and the culture media were subjected to autoclaving for 15 min at 121 °C.

Rooting was induced in micro-cuttings on a basal MS medium supplemented with Indole-3-Butyric Acid (IBA) and putrescine at 0, 0.25, 0.5, 1 and 2 mg l^{-1} concentrations. Recording data was done after 50 days for rooting parameters such as percentage of rooted cuttings (%), length of roots, the number of roots and shoots per explants, and leaves chlorophyll index. Each treatment included three replicates of three explants.

After a 6- week period for rooting, the *in vitro* rooted micro-cuttings were removed from the culture medium, washed with autoclaved distilled water for the elimination of any agar traces and then transferred to the plastic pots contained sterilized perlite and cocopeat (1:1) (Fig. 1D). Covering of pots by clear plastic cups in an invert position, the rooted cuttings were protected from water stress by keeping relative humidity at high level (Fig. 1E). After 2-week period, the cover cups were slightly punctured and the removal of cups from the pots was done every couple of hours, at the third week. Following 4 weeks, the plantlets were transferred to the controlled condition in greenhouse for the purpose of acclimatization and hardening; then they were transferred to the outdoor condition.

In vivo root induction

In this experiment after preparing the cuttings, they were treated by various levels of IBA and putrescine and wounding in a factorial design.

Five levels of IBA and putrescine concentrations were applied on cuttings, each at concentrations of 0, 0.25, 0.5, 1, and 2 g/l. At first, half of the cuttings were wounded by developing a 1-cm incision along the axis into the cutting's basal end; the other half was not wounded (control). Then basal ends of the cuttings were inserted in Benomyl (20%) for a

period of 60 seconds to prevent fungal diseases and then immediately inserted in the mentioned concentrations of IBA and putrescine for a period of 60 seconds; finally, cuttings were potted by inserting them in sandy medium. Each factorial combination of IBA, putrescine, and wounding treatment was subjected to testing on 15 cuttings, 5 in each of the three blocks, totally 150 cuttings. Each experimental unit consisted of three cuttings and cuttings were planted in plastic pots containing sand as a rooting medium. Pots were then placed on benches in polyethylene greenhouse. Semi-controlled greenhouse was set at 20 ± 5 °C and 11 and 13 h light/dark, respectively. The pots were irrigated daily. After 8 weeks, the percentage of rooting, number of roots per cutting, root and shoot length, number of leaves per cutting, as well as chlorophyll index was evaluated. Leaf chlorophyll index was measured using Chlorophyll Content Meter (Minolta SPAD CCM-200).

Statistical analysis

The data was subjected to analysis in SPSS 15.0 software by conducting the two-way analysis of variance (ANOVA) which was followed by Duncan multiple range test (DMRT) to compare the means ($p \leq 0.05$). The results were represented as the average of the replications ± standard error (SE). The Pearson's correlation coefficients were obtained between vegetative growth parameters, as well as *in vitro* and *in vivo* conditions, for the comparison of the possible impacts of each of the factors on the rooting of *R. damascene* cuttings.

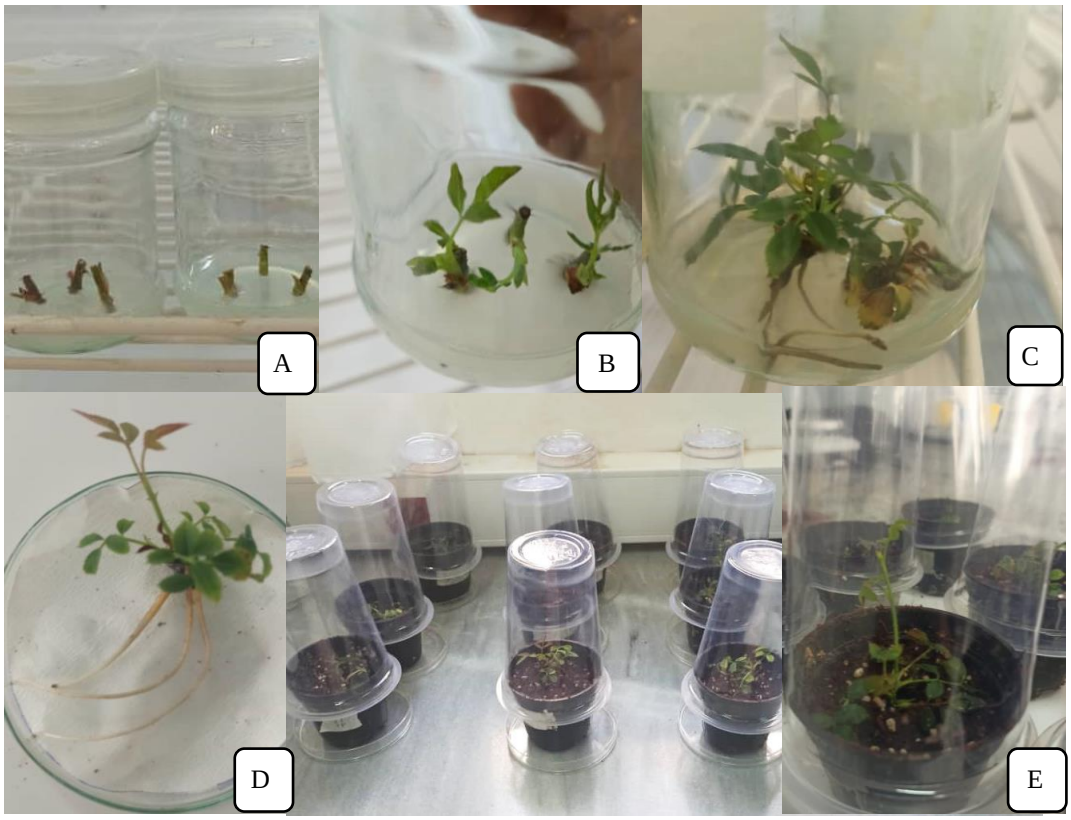

Fig. 1. *In vitro* propagation of *R. damascena* using micro-cuttings A) *in vitro* culture of nodal explants in glass jars, B) Shoot proliferation of nodal explants which were incubated in the MS medium + 0.5 mg/l IBA + 0.5 mg/l putrescine after 3 weeks, C) Root induction on the MS medium 0.5 mg/l IBA + 0.5 mg/l putrescine after a period of 6 weeks, D) Rooted micro-cutting ready for transferring to pot, and E) Acclimatized plantlets after 7 weeks under controlled conditions of growth chamber.

RESULTS

In vitro root formation

Statistical analysis of the obtained data for root number, root length, chlorophyll index, leaf number, and shoot length displayed significant differences between various concentrations of IBA and putrescine at p = 0.01, whereas no remarkable differences (at p = 0.05) were observed between the concentrations of putrescine for root length characteristic.

Roots began to appear from the explants about seven days following the culture on a basal MS medium which was supplemented with different concentrations of IBA and putrescine. A fully developed root system was then observed from all explants within 30 days in culture. Simultaneously new shoots and leaves were emerged on micro-cutting.

Adventitious roots were formed *in vitro* in all treatments (Fig. 1C, D). The roots were regenerated at the base of the shoot, which is sometimes associated with the formation of callus (Fig. 1C, D). Despite this, significant differences in the number as well as the length of the formed roots were found (Tables 1 and 2). The average total root number ranged from 2 for 2 mg/l IBA + 0.5 mg/l putrescine to 5 for 1 mg/l IBA + 1 mg/l putrescine and 2 mg/l IBA + 0.25 mg/l putrescine.

The average total root length was in the range of 2.33 to 12.83 cm for the control and 1 mg/l IBA + 0 mg/l putrescine, respectively (Fig. 1C). Parameters, root number as well as the average total root length also displayed considerable differences between different concentrations of IBA and putrescine.

The shoot length was significantly ($P \leq 0.05$) affected by diverse concentrations of IBA and putrescine (Table 2). The shoot length ranged from 2.58 cm for the control treatment to 7.90 cm for 1 mg/l IBA + 1 mg/l putrescine (Table 2).

The chlorophyll index was significantly ($P \leq 0.05$) affected by diverse concentrations of IBA and putrescine (Table 1), as it varied from 23.73 SPAD for 1 mg/l IBA + 0.25 mg/l putrescine treatment to 38.91 SPAD for 1 mg/l IBA + 2 mg/l putrescine (Table 2).

The MS media supplemented with 1 mg/l IBA + 1 and 2 mg/l putrescine showed the maximum leaf number per micro-cutting (8.67 ± 0.87) when compared to other treatments (Table 2), while it was 1.73 times higher than control. All growth indices, including root number, root length, shoot length and leaf number, were raised with the increasing of IBA and putrescine concentration in the culture medium to 1 mg/l; however, further increase in IBA and putrescine concentrations (> 1.0 mg/l) led to the decrease of these indices in micro-cuttings. The results of Pearson's correlation showed a significant correlation between most studied traits; the highest positive correlation was established between the shoot length and root length (Table 3).

Table 1. Analysis of variance of the effect of IBA and Putrescine on growth traits of *R. damascena* micro-cutting under *in vitro* culture.

Sources of variation	Df	Root number	Root length	Chlorophyll index	Leaf number	Shoot length
IBA	4	4.19**	154.62**	21.56**	13.75**	14.16**
Putrescine	4	0.94^{ns}	34.94**	15.93**	5.96**	2.36**
IBA× Putrescine	16	2.04**	7.44**	25.49**	2.31*	1.89**
Error	50	0.56	0.64	0.35	1.13	0.47
C.V		18.38	12.49	2.09	15.80	8.68

^{ns}, *, **, non-significant or significant at $p < 0.05$, or 0.01, respectively.

Table 2. The impact of diverse concentrations of IBA and putrescine on the rooting of *R. damascena* micro-cuttings.

IBA (mg/l)	Putrescine (mg/l)	Root length (cm)	Root number	Leaf number	Shoot length (cm)	Chlorophyll index (SPAD)
0	0	2.33i±0.17[X]	3.00[cde]±0.58	5.00[c]±0.00	2.58[i]±0.31	27.40[f-i]±0.06
	0.25	2.67[ghi]±0.32	2.67 [de]±0.33	5.00[c]±0.00	3.15[h]±0.03	26.90[h-k]±0.10
	0.5	2.53[ghi]±0.32	2.67 [de] ±0.67	5.00[c]±0.00	4.13[g]±0.09	26.70[kl]±0.12
	1	5.27[def]±0.15	3.33[b-e]±0.33	8.33[ab]±0.67	4.29[fg]±0.15	25.70[l]±0.30
	2	4.90[def]±0.10	2.67 [de] ±0.33	5.66[c]±0.67	4.10[g]±0.06	26.30[jkl]±0.50
0.25	0	2.47[hi]±0.41	2.67 [de] ±0.33	5.67 [c]±0.67	3.27[h]±0.09	27.67[fgh]±0.22
	0.25	3.63[fgh]±0.47	3.33[b-e] ±0.33	5.00[c]±0.00	2.62[i]±0.05	26.43[i-l]±0.09
	0.5	4.13[ef]±0.47	3.00[cde] ±0.58	5.00[c]±0.00	3.36[h]±0.09	27.13[g-j]±0.09
	1	3.60[fgh]±0.57	3.67[a-d]±0.33	7.00[abc]±0.58	3.38[h]±0.07	29.73[bc]±0.15
	2	6.70[cde]±0.35	2.67 [de] ±0.33	5.67 [c]±0.67	4.03[g]±0.07	25.50[l]±0.10
0.5	0	4.10[fg]±0.30	3.67[a-d] ±0.33	5.00[c]±0.00	4.29[fg]±0.04	27.33 [f-i]±.018
	0.25	2.67[ghi]±0.52	3.33[b-e] ±0.67	5.67 [c]±0.67	4.52[efg]±0.12	28.27[ef]±0.15
	0.5	4.60[def]±0.21	3.67[a-d] ±0.33	6.33[bc]±0.67	4.48 [fg]±0.04	28.73[def]±0.15
	1	11.00[abc]±0.58	3.33[b-e] ±0.67	7.00 [abc]±1.15	4.40 [fg]±0.31	28.23[ef]±0.43
	2	7.60[bcd]±0.08	3.00[cde]±0.58	5.67 [c]±0.67	4.02[g]±0.19	29.60[cd]±0.31
1	0	12.83[a]±0.17	4.50[ab]±0.29	8.33[ab]±0.67	5.57[bc]±0.19	30.67[b]±0.09
	0.25	8.73[abc]±0.15	2.67[de]±0.33	6.33 [bc]±0.67	4.67[fg]±0.12	23.73[m]±0.32
	0.5	10.03[abc]±0.58	4.50[ab] ±0.27	8.00[ab]±0.58	5.17[cde]±0.33	30.70[bc]±0.19
	1	12.50[ab]±0.29	5.00[a]±0.58	8.67[a]±0.88	7.90[a]±0.50	24.27[m]±0.43
	2	14.16 [a]±0.44	4.50[ab]±0.29	8.67 [a]±0.88	5.27[cd]±0.33	38.17[a]±0.60
2	0	7.16[cd]±0.83	4.33[abc]±0.33	5.00[c]±0.00	4.31[fg]±0.53	30.37[bc]±0.88
	0.25	7.00 [cd]±0.00	5.00[a] ±0.00	7.00 [abc]±0.58	5.80[bc]±0.36	30.00[bc]±0.58
	0.5	4.67 [def]±0.33	2.00[e]±0.00	7.00 [abc]±0.58	6.15[b]±0.09	28.06[efg]±0.52
	1	7.67 [bcd]±0.88	2.33[de]±0.33	5.67 [c]±0.67	4.85[def]±0.14	28.17[ef]±0.07
	2	7.00 [cd]±0.58	2.33[de]±0.33	6.67[abc] ±0.88	4.27 [fg]±0.15	28.10[efg]±0.00

[x] Mean separation within columns for each factor by the Duncan multiple range test (DMRT) at the 5% significance level; n=3.

Table 3. Pearson's correlation coefficients of the studied traits of *R. damascena* under *in vitro* culture conditions.

Parameters	Root number	Root length	Chlorophyll	Leaf number	Shoot length
Root number	-	-	-	-	-
Root length	0.451[**]	-	-	-	-
Chlorophyll	0.369 [**]	0.393 [**]	-	-	-
Leaf number	0.368 [**]	0.606 [**]	0.270 [*]	-	-
Shoot length	0.386[**]	0.607 [**]	0.109	0.543 [**]	-

[ns], [*], [**], non-significant or significant at $p < 0.05$, or 0.01, respectively.

In vivo adventitious root formation

Statistical analysis conducted on the data of root number, root length, chlorophyll index, leaf number and shoot length displayed significant differences between different concentrations of IBA and putrescine, as well as wounding, at p = 0.01, whereas no significant differences (at p = 0.05) existed between the wounding treatment for the shoot length and IBA concentrations for leaf number (Table 4).

Fig. 2. *In vivo* adventitious root formation in of *R. damascena* cuttings after the 7-week culture in the rooting medium (Some treatments showed here). A) 0.25 mg/l IBA + 0.25 mg/l Putrescine, B) 0.25 mg/l IBA + 0.25 mg/l Putrescine + wounding, C) 1 mg/l IBA + 0.25 mg/l Putrescine, D) 1 mg/l IBA + 0.25 mg/l Putrescine + wounding, E) 1 mg/l IBA + 1 mg/l Putrescine, F) 1 mg/l IBA + 1 mg/l Putrescine + wounding, G) *in vivo* culture of cutting in greenhouse on the first day of experiment, and H) Cutting in greenhouse after 5 weeks.

In vivo adventitious root formation was investigated in the greenhouse condition (Fig. 2). Under such conditions, all cuttings were able to form roots (100% root formation), though to different extents (Fig. 2A-H). The *in vivo* root number ranged from 1 to 9.67, and the root length varied from 2.00 to 32.83 cm. The significantly higher number of roots per cutting (9.67) and root length (32.83 cm) was observed in wounding cuttings treated with 1 g/l IBA + 0.5 g/l putrescine and 2 g/l IBA + 0.25 g/l putrescine respectively, the same IBA and putrescine concentration led to the higher number of roots (6.67) and root length (23.76 cm) in non-wounding cuttings (Fig. 3A, B). Further increase of IBA and putrescine concentrations did not increase the number of roots per cutting, as well as root length (Fig. 3A, B). The presence of wounding, as compared to non-wounding cuttings, increased the root length and root number (Fig. 2A-F, Fig. 3B). Shoots length was also affected by the IBA and putrescine presence, as well as wounding of cuttings. Maximum shoot length equal to 14.33 cm was observed on 1 g/l IBA + 2 g/l putrescine in wounding cuttings (Fig. 3C). The chlorophyll index was significantly ($P \leq 0.05$) under the influence of various levels of IBA, putrescine and wounding (Table 4). The chlorophyll index varied from 35.17 SPAD for 2g/l IBA + 0.25 g/l putrescine treatment to 48.00 SPAD for 0.5 g/l IBA + 0.25 g/l putrescine (Fig. 3D). Our data showed that wounding increased the root length and root number as compared to non-wounding cuttings. There is a paradoxical report on the effect of wounding on the rooting ability of cutting which were grown under *in vivo* conditions (Fig. 2).

It should be explained that the most vigorous plant having the highest leaf number was found in the wounding cuttings (Fig. 4). The leaves maximum number (8.33) was obtained in treated cuttings with 1 g/l putrescine (without IBA) (Fig. 4).

The results of the Pearson's correlation coefficient showed that in *in vivo* condition, there was a correlation between the roots number and other traits at the probability level of $P < 0.05$ and 0.01. The highest positive correlation was obtained between the root length and root number ($r = 0.832$); however, there was a weak negative correlation between chlorophyll index and two traits of root number as well as root length (Table 5).

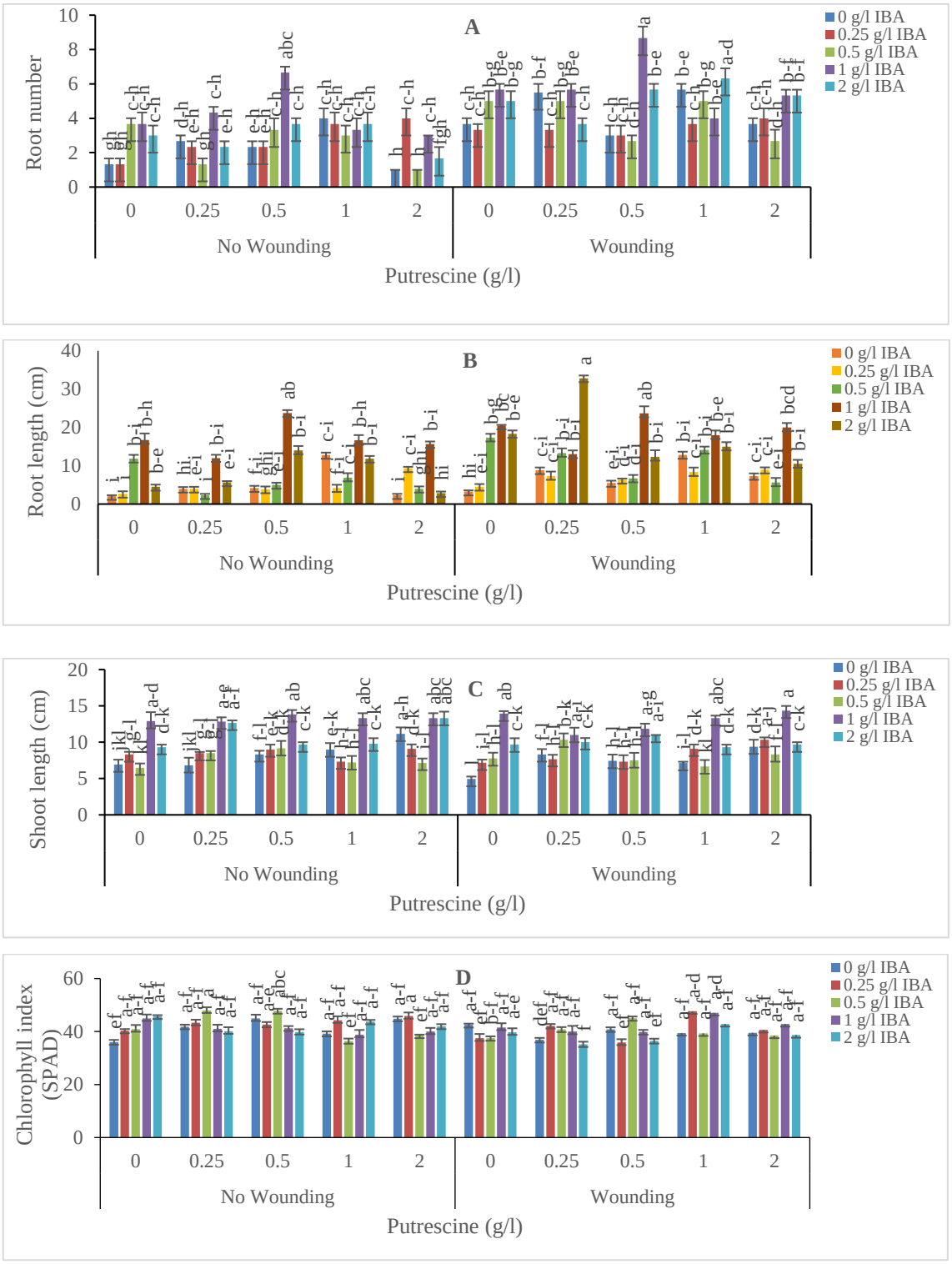

Fig. 3. *In vivo* rooting responses of *Rosa damascena* cuttings to the triple effects of wounding, IBA and putrescine treatments. A) Roots mean number per in vivo shoot, B) The average total in vivo root length per shoot, C) The average total *in vivo* shoot length, and D) Chlorophyll index. Vertical bars show means ± S.E. (n=3). Means accompanied by diverse letters are significantly different ($P < 0.05$) according to the Duncan multiple range test (DMRT) at the significance level of 5%.

Table 4. Analysis of variance related to the impact of IBA, putrescine and wounding on the growth traits cuttings of *R. damascena* in *in vivo* condition.

Sources of variation	df	Root number	Root length	Chlorophyll index	Leaf number	Shoot length
IBA (a)	4	18.89**	267.76**	23.51**	2.78ns	146.13**
Putrescine (b)	4	4.71**	30.95**	10.23*	11.11**	14.60**
Wounding (c)	1	95.12**	185.52**	234.95**	11.84**	5.12^{ns}
a × b	16	7.04**	35.24**	63.39**	4.87**	4.36**
a × c	4	1.99**	5.56**	57.67**	11.11^{ns}	2.90^{ns}
b × c	4	2.39**	10.52**	117.46**	3.05^{ns}	1.22^{ns}
a × b × c	16	1.38*	7.90**	48.99**	2.30*	4.85**
Error	100	0.67	2.44	4.16	1.67	1.46
C.V	-	18.09	11.89	18.89	18.94	10.65

^{ns}, *, **, non-significant or significant at $p < 0.05$, or 0.01, respectively.

Table 5. Pearson's correlation coefficients of the studied traits of *R. damascena* under *in vitro* culture conditions.

	Root number	Root length	Chlorophyll	Leaf number	Shoot length
Root number	-	-	-	-	-
Root length	0.832 **	-	-	-	-
Chlorophyll	- 0.179 *	-0.127	-	-	-
Leaf number	0.309 **	0.318 **	0.012		-
Shoot length	0.177 *	0.363 **	0.081	0.038	-

^{ns}, *, **, non-significant or significant at $p < 0.05$, or 0.01, respectively.

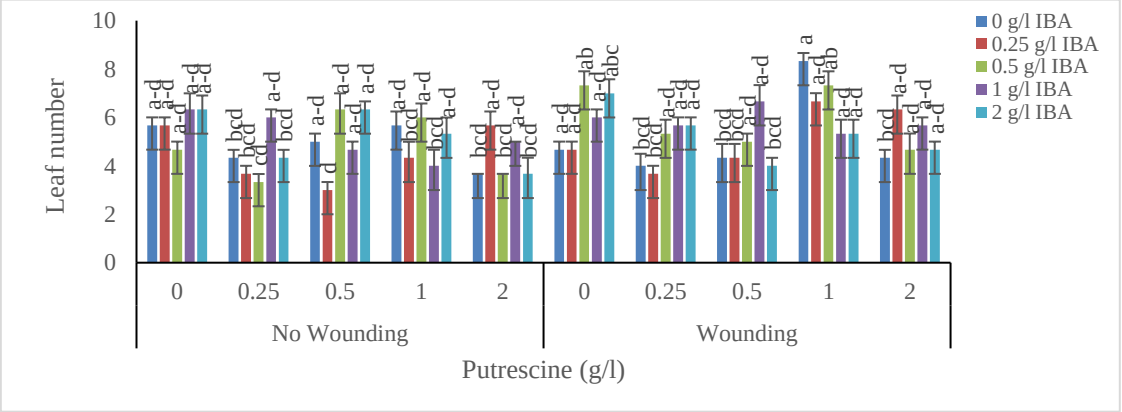

Fig. 4. *In vivo* rooting responses of *Rosa damascena* cuttings to the triple effects of wounding, IBA and putrescine treatments on the leaves mean number per *in vivo* shoot. Vertical bars show means ± S.E. (n=3).

DISCUSSION

In the present study, the impacts of IBA, and putrescine on the rooting of cutting and micro-cuttings of *R. damascena* under *in vivo* and *in vitro* culture conditions were investigated. Moreover, the effect of vertical wounding on the basal part of cutting on rooting characteristics was evaluated under *in vivo* condition. Adventitious rooting can be regarded as an important step of micro-propagation and the success of commercial micro-propagation systems depends on the rooting efficacy of micro-cuttings and quality of roots (Goel et al., 2018). The simplest and most widely employed method of growing roses is using stem cuttings (Anderson & Woods, 1999). Emerging adventitious roots in stem cuttings depends on various aspects such as species and cultivar, growth season, the cutting wood condition, type of cuttings and other factors (Hartmann et al., 2002). Adventitious rooting is influenced by

endogenous as well as exogenous items (De Klerk et al., 1999; Kumar, 1996). The clear role of auxin that is naturally produced in plant organs has been well documented in formation of root. Nowadays, commercial synthetic auxin is used as rooting accelerating agent and inhibiting cuttings death (Kasim & Rayya, 2009). The beneficial role of auxin, especially IBA, in adventitious root induction has been shown in a considerable number of studies (Han et al., 2009; Tchinda et al., 2013; Camellia et al., 2009; El-Banna et al., 2023). In the first experiment of this research study, rooting characteristics of micro-cuttings were evaluated on the MS medium supplemented with diverse concentrations of IBA or putrescine under *in vitro* culture conditions. Although induction of the root was done on all of the combinations which included the basal MS medium, the maximum number of roots per micro-cuttings and root length were observed on the medium which was supplemented with 1 mg/l IBA + 1 mg/l putrescine and 2 mg/l IBA + 0.25 mg/l putrescine (Table 3). The use of proper auxins at the optimum concentration has been considered as an important factor contributing to the roots l putrescine. IBA can be regarded as the most popular auxin that has been commonly applied in stimulating the rooting of cuttings in diverse plant species (Hartmann et al., 2002). The high rooting induction efficiency of IBA could be attributed to its reported lower toxicity as well as the ability to enhance the endogenous IAA levels (Han et al., 2009). The advantageous effect of IBA on micro-cuttings or micro-shoots rooting has been well established before (Kumar et al., 2001; Goel et al., 2018).

In both in *vitro* and *in vivo* experiments, adventitious roots were regenerated at the base of micro-cuttings or the cutting after four to five weeks. Previous studies have addressed roses rooting have concentrated on either *in vitro* or *in vivo* rooting comparisons (Pati et al., 2010; Rather & Tsewang Tamchos, 2017; Nguyen et al., 2020).

Nguyen et al. (2020) displayed that most rose genotypes formed roots partly under both *in vitro* and *in vivo* conditions; however, rooting happened at higher rates *in vitro*, rather than *in vivo*. Our data showed the rooting of all micro-cutting and cuttings under *in vitro* and *in vivo* culture conditions.

Prior researches indicated that the medium without any plant growth regulators could be suitable for *in vitro* rooting of *Rosa* × *hybrida* cultivars (Ibrahim & Debergh, 2001); however, our study indicated that the presence of IBA and putrescine would be essential for enhancing the root length and root number of *R. damascena*, as poor rooting was found in PGR-free or low concentrations of them in the medium. IBA at various concentrations has been effective on *in vitro* rooting of other rose species (Misra & Chakrabarty, 2009; Akhtar et al., 2016). These synthetic auxins could serve as synergistic agents with IAA to serve as the natural auxin in plants (Ambros et al., 2016).

According to the results, treatment of cuttings with IBA alone or in combination with putrescine resulted in the maximum root number or root length. Nasri et al. (2015) investigated the impact of a quick dip (for the 20s) of IBA on the rooting of 12 wild genotypes of *R. damascena*, showing that the highest rooting was recorded with the quick dip of shoots in 1 g/l IBA.

As the results showed, wounding increased the root length and root number of cuttings. Immediately following cutting, wound response signaling pathways start at the cutting base (Creelman et al., 1992; Schilmiller & Howe, 2005), with a quick rise of jasmonic acid levels that peaked 30 min following cutting (Ahkami et al., 2009; Rasmussen et al., 2015). This soaring peak of jasmonic acid could be correlated with the formation of adventitious root (Ahkami et al., 2009; Rasmussen et al., 2015).

In this work, the improvement in root development appeared to be related to the increase of putrescine, which was in agreement with the findings of González-Hernández et al. (2022) on tomatoes. The role of polyamines in various crucial physiological processes has been

documented very well. These processes encompass cell division and differentiation, growth regulation, gene expression and overall plant survival (Chen et al., 2019). Moreover, the effect of IAA and putrescine concentrations in the induction of the root has been demonstrated by Tonon et al. (2001) on *Fraxinus angustifolia* plant, which seems that polyamine catabolism could play a significant role in root formation as well as elongation.

CONCLUSION

In this research, based on our hypothesis, applying simultaneously IBA and putrescine was done on Damask rose cutting, using different concentrations of IBA and putrescine under both *in vitro* and *in vivo* culture conditions, as well as vertical scarring on the base of cutting. Great differences in rooting traits between IBA and putrescine treatments were observed in both conditions. Adventitious roots were formed in both situations in all treatments, though to different extents. The highest total root number was observed in 1 mg/l IBA + 1 mg/l putrescine and 2 mg/l IBA + 0.25 mg/l putrescine applied *in vitro* on micro-cuttings and in 1 g/l IBA + 0.5 g/l putrescine applied on wounded cuttings under *in vivo* culture condition. In both conditions, all growth indices, which included root number, root length, shoot length and leaf number, were increased with the rise of IBA and putrescine to 1 mg/l; however, further increase in IBA and putrescine concentrations (> 1.0 mg/l) led to a decrease of these indices in both situations. Under *in vivo* condition, all growth parameters were better in wounded cuttings. This study revealed that IBA, putrescine and wounding could be effective in the rooting of *R. damascene* under *in vitro* and *in vivo* situations.

Conflict of interest

The authors declare that there is no conflict of interest.

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Improvement of friable callus induction of *Crocus sativus* L. and establishment of a cell suspension culture system with high biomass

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ABSTRACT

Purpose: This study aims to explore the potential of in vitro culture as a method for scaling up the production of saffron based medicinal compounds, the most expensive spice renowned. Emphasis is placed on the critical role of friable callus (FC) formation as a prerequisite for successful suspension culture. **Research method:** The research primarily investigates FC formation, focusing on the impact of varying strengths of Murashige and Skoog (MS) medium as well as combinations of NAA or 2,4-D and BA or Kin on compact callus. Subsequently, the study involves supplementing the MS medium with different concentrations of 2,4-D, kin, zeatin, glutamine, sucrose, and nitrogen to establish a cell suspension culture. **Findings:** The highest FC yield was achieved on a solid medium containing 2,4-D (1 mg l^{-1})+Kin (0.2 mg l^{-1}), resulting in a fresh weight (FW) of 0.413 g. Furthermore, MS combined with 2,4-D (1 mg l^{-1})+Kin (0.2 mg l^{-1})+glutamine (10 mg l^{-1}), as well as MS+2,4-D (0.5 mg l^{-1})+zeatin (0.3 mg l^{-1})+glutamine (10 mg l^{-1}), demonstrated the highest FW under suspension conditions. The study also identified that 30 g l^{-1} sucrose and 30 µM were optimal for inducing maximum FW. **Research limitations:** Cell biomass is influenced by several factors that should to be optimized. **Originality/Value:** This research concludes that a cell suspension system holds promise for rapidly generating sufficient cell biomass to produce valuable secondary metabolites within a limited timeframe and space. Notably, the system successfully increased biomass from 0.2 to 1.2 g, underscoring its potential for efficient saffron-based product development.

INTRODUCTION

In contemporary research, there is a growing interest in identifying alternative methods that can effectively reduce costs and enhance the efficiency of producing secondary metabolites. One such successful method is the use of plant Cell Suspension Culture (CSC), as highlighted by Pant (2014). CSC has demonstrated the capability to generate natural compounds at the rates comparable to, or even higher than the intact plants, presenting a significant advantage in terms of quantity (Ziaratnia et al., 2009). A noteworthy benefit of CSC is its ability to ensure the continuous production of valuable compounds, unaffected by geographical or seasonal constraints, thus distinguishing it from conventional field cultivation practices (Rao & Ravishankar, 2002).

To establish an effective CSC system, the formation of Friable Callus (FC) with homogeneous cells is imperative (Mustafa et al., 2011). Friability, a morphological characteristic of callus, attributes to loosely aggregated cells with lower density (Souza et al., 2014). Successful callogenesis relies on appropriate explant selection, optimization of medium compositions, and determining the type and concentrations of Plant Growth Regulators (PGRs). Among various chemical factors influencing FC production, hormonal combinations and basal medium composition are identified as crucial contributors (Thacker et al., 2018). Additionally, the type of explant is reported to play a significant role in callus initiation (Dar et al., 2021).

Several studies have reported FC production in various plants. For instance, Keng et al. (2010) successfully produced FC from leaf-derived explants of *Artemisia annua* by culturing them on MS medium supplemented with benzyl adenine (BA) (0.5 mg l-1) and naphthalene acetic acid (NAA) (0.5 mg l-1). Furthermore, Dar et al. (2021) demonstrated the significant impact of different PGR concentrations and combinations on callus induction and maintenance from leaf and root explants of *Atropa acuminate*. Notably, leaf explants exhibited a higher production of friable calli, and reducing NAA levels in the medium resulted in significantly higher friable callus production.

In a CSC system, the consideration of secondary metabolite production becomes high when biomass reaches its maximum level. To achieve this, the improvement of callogenesis, FC production, and subsequent optimization of CSC must be undertaken to attain desired levels of biomass and secondary metabolites. Given the plant species-specific responses to CSC conditions, factors such as PGR type and level, organic and inorganic medium compositions (e.g., nitrogen and phosphate), and sucrose concentrations should be systematically assessed (Murthy et al., 2014; Thacker et al., 2018). Notably, scientists have highlighted the influential role of medium constituents, PGRs, and nitrogen sources in optimizing the callus formation and cell culture of various plant species, such as *Dracocephalum polychaetum*, *D. kotschyi*, and *Artemisia. annua* (Taghizadeh et al., 2020; Keng et al., 2010).

Apart to optimizing medium constituents, several reports reveal the efficacy of adding organic materials, including amino acids like glutamine, in stimulating growth across different plant cells, such as *Juniperus excelsa*, *Catharanthus roseous*, *Hyoscyamus muticus*, and *Glycine max* (Shanjani 2003; Scragg et al., 1990; Basu & Chand 1998; Gueven & Knorr 2011).

Despite numerous reports on saffron corm callogenesis and cell culture optimization, particularly in studies by Verma et al. (2016), Moshtaghi (2020), Taghizadeh et al. (2020), Amini et al. (2022), and Ramandi et al. (2022), the production of saffron friable callus a crucial step for successful cell suspension culture remains unexplored. Therefore, this study was designed to investigate the optimization of solid MS medium components, PGR types,

and concentrations for friable callus production. The ultimate goal is to establish a robust CSC system that enhances saffron cell biomass production.

MATERIALS AND METHODS

This study was conducted at the Research Institute of Food Science and Technology (RIFST), Mashhad, Iran, from 2020 to 2022. Since friable calli production is an imperative step to establish a successful cell suspension culture; therefore, in the first step, the possibility of FC production from the corm-derived compact cells was evaluated in the solid medium by changing in PGRs types and concentrations. The second experiment was focused on optimizing CSC conditions to increase cell biomass. The latter experiment investigated the effect of different factors, such as medium constituent strengths, sucrose, PGRs, and glutamine at different levels. All media components were purchased from Sigma (Japan) and Merck (USA) companies.

Plant materials

In current study, mature corms of saffron were used as plant material for callus induction. The disinfection steps of corms were carried out according to the method of Ziaratnia and Amini (2021). The callus was prepared from a preliminary experiment in which different PGRs at several concentrations were used for higher callus induction. Among all treatments, a combination of NAA and BA (NAA, 8 mg l^{-1} and BA, 1 mg l^{-1}) was the best regarding fresh weight and reddish colour (data not published), but the calli were compact. Therefore, the compact calli from this treatment were selected as an explant for further investigations in this study.

FC induction on solid medium

To produce friable calli and desirable biomass, the compact corm derived-callus from solid MS medium was sub-cultured on MS medium with lower levels of PGRs than those in callus induction. Media were supplemented with different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) or NAA (0.5 and 1 mg l^{-1}) as auxins and Kin or BA (0.2, 0.5 and 1 mg l^{-1}) as cytokinins (Table 1).

Table 1. Plant growth regulators treatments for friable callus formation in solid MS medium.

Treatment	2,4-D (mg l^{-1})	Kin (mg l^{-1})	BA (mg l^{-1})	Treatment	NAA (mg l^{-1})	Kin (mg l^{-1})	BA (mg l^{-1})
E1	0.5	0.2	-	F1	0.5	0.2	-
E2	0.5	0.5	-	F2	0.5	0.5	-
E3	0.5	1	-	F3	0.5	1	-
E4	1	0.2	-	F4	1	0.2	-
E5	1	0.5	-	F5	1	0.5	-
E6	1	1	-	F6	1	1	-
E7	0.5	-	0.2	F7	0.5	-	0.2
E8	0.5	-	0.5	F8	0.5	-	0.5
E9	0.5	-	1	F 9	0.5	-	1
E10	1	-	0.2	F10	1	-	0.2
E11	1	-	0.5	F11	1	-	0.5
E12	1	-	1	F12	1	-	1

The medium pH was adjusted to 5.7, and then solidified by 0.7% agar before autoclaving at 121°C for 15 minutes. Cultures were incubated at 22±2 °C in dark conditions. After four weeks, all treatments were statistically analysed based on cell fresh weight and the percentage of organogenesis or embryogenesis. The friability and yellow to red-coloured intensities of the calli were evaluated visually. The friability was determined as an easily friable callus with no apparent regenerated organ. Treatments with desirable friability with no organogenesis and higher cell biomass were used to establish and optimize CSC in the next experiments.

CSC establishment and adaptation

In this part, four-week-old FC with yellow colour related to the superior treatments from the previous experiment was pooled together and used as initial cells for the establishment of CSC. To make homogeneity and adapt the cells to the new conditions, 0.5 g of the pooled cells were inoculated into 100 ml Erlenmeyer flask containing 20 ml liquid MS medium supplemented with an optimum concentration of 2,4-D and kin (0.5 and 0.2 mg l^{-1} respectively). Cultures were then placed on an orbital shaker at 110 rpm at 20±2 ºC in dark conditions for four weeks.

CSC medium optimization by changing PGRs type and glutamine concentration

This part evaluated the effect of MS medium (Full MS, ½ MS), PGRs type (2,4-D, Kinetin, Zeatin), and glutamine at different levels. Table 2 shows the different treatments prepared in this part of the experiment.

At first, 0.2 g adapted cells were inoculated into the 100 ml flasks containing 20 ml liquid MS in different strengths with a content of 3.0 % sucrose and MS vitamins. Thereafter, flasks were placed on an orbital shaker as mentioned before. For analysis of biomass accumulation, data was recorded as fresh and dry weight (g) every week for nine weeks.

CSC optimization by changing the levels of sucrose and nitrogen

In this part, different levels of sucrose and nitrogen were examined to reach the higher cell biomass (Table 3). MS medium supplemented with 2,4-D (0.5 mg l^{-1}), Zeatin (0.3 mg l^{-1}), and Glutamine (10 mg l^{-1}) selected from the previous experiment were similar for all treatments. Treated factors were evaluated based on the rate of cell growth over nine weeks.

Table 2. Different treatments for cell suspension culture optimization of saffron (*Crocus sativus* L.).

Treatment	MS strength	2,4-D (mg l^{-1})	Kin (mg l^{-1})	Zeatin (mg l^{-1})	Glutamine (mg l^{-1})
A1	Full	1	0.2	-	10
A2	Full	1	0.2	-	-
A3	Half	1	0.2	-	10
A4	Half	0.5	-	0.3	10
A5	Full	0.5	-	0.3	10
A6	Full	0.5	-	0.3	-

*Nitrogen sources were NH_4NO_3 and KNO_3.

Table 3. Different levels of sucrose and nitrogen in saffron (*Crocus sativus* L.) cell suspension culture.

Treatment	Sucrose (g l^{-1}) and Nitrogen (µM)
B1	Sucrose (30) + Nitrogen (30)
B2	Sucrose (30) + Nitrogen (60)
B3	Sucrose (30) + Nitrogen (90)
B4	Sucrose (60) + Nitrogen (30)
B5	Sucrose (60) + Nitrogen (60)
B6	Sucrose (60) + Nitrogen (90)

*Nitrogen sources were NH_4NO_3 and KNO_3.

Statistical analysis

The traits of cell fresh weight and the percentage of organogenesis or embryogenesis (in FC induction on solid medium experiment), cell fresh and dry weight (in CSC medium optimization by changing PGRs type and glutamine concentration experiment) and rate of cell growth in CSC optimization by changing the levels of sucrose and nitrogen experiment, were statistically evaluated using a Completely Randomized Design (CRD) with three replications. Data were analyzed with JMP software version 11. Mean comparisons were made using Duncan's new multiple range test and the significant differences among means were shown at 5%. Graphs were drawn with Excel software.

RESULTS AND DISCUSSION

FC formation on solid medium

Analysis of variance showed that there were significant differences among the PGRs treatments on FC formation. Figure 1 shows the results of the fresh callus weight in MS medium. The highest FC formation (0.413 g) was observed in E4, in which 2,4-D and Kin were at 1 and 0.2 mg l^{-1}, respectively. Based on the results, the maximum concentration of 2,4-D with the minimum amount of Kin is a more effective combination in FC formation. Interestingly, the callus in all treatments was red with no obvious differences (Fig. 2).

The observed percentage of callogenesis and organogenesis (Fig. 3) revealed that E1, E4, F8, and F10 had the highest (100 %), while E7 and F3 had the lowest rate (11.11 %). The maximum frequency of organogenesis (100%) was observed in F5 with NAA (1.0 mg l^{-1}) and Kin (0.5 mg l^{-1}) (Fig. 3 and 4).

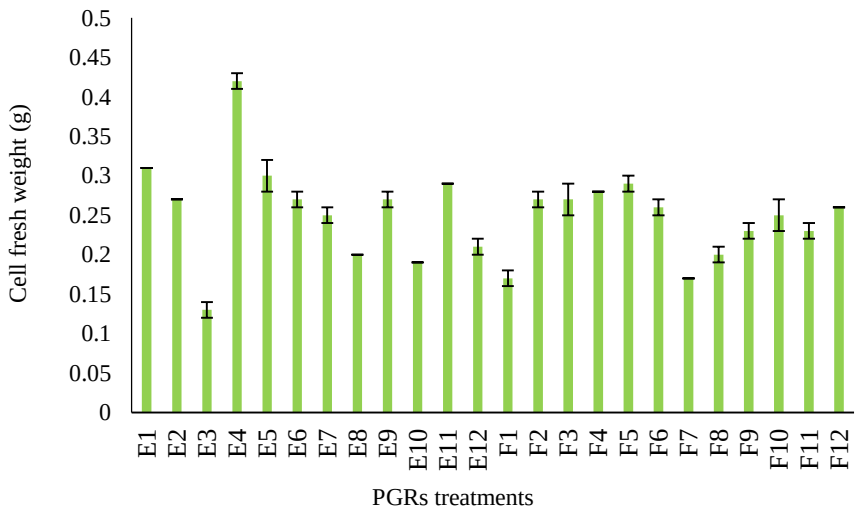

Fig. 1. Effect of different PGRs treatments in solid MS medium on friable callus fresh weight of saffron (*Crocus sativus* L.) ($p \leq 0.05$).

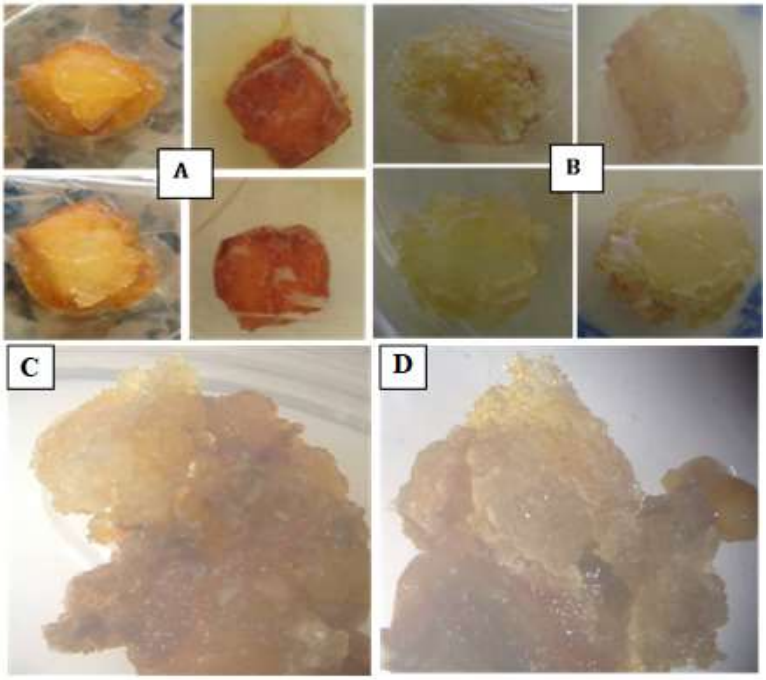

Fig. 2. Compact and friable cells derived from saffron (*Crocus sativus* L.) corm. A) Compact callus (after 2 weeks), B) Compact callus (after 4 weeks), C) Friable cells, Light-red to yellowish, and D) Friable cells, Light-red to whitish.

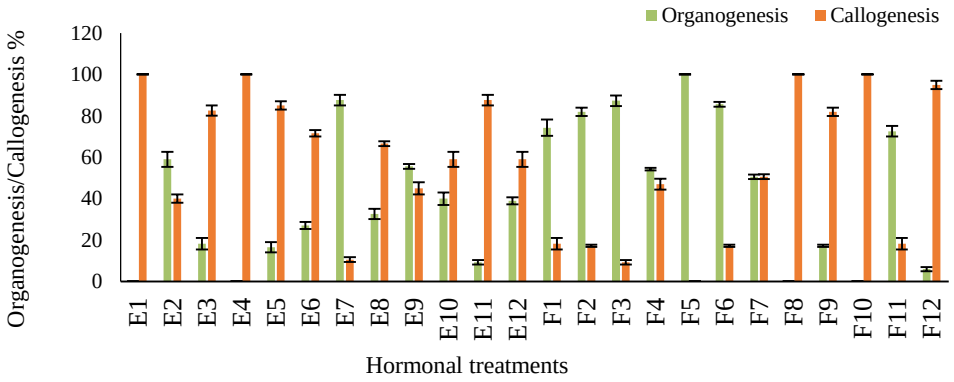

Fig. 3. Effect of different PGRs treatments on saffron corms (*Crocus sativus* L.) organogenesis and callogenesis.

As mentioned, there is no report on FC formation on *C. sativus* corms derived-explants. thus, the results presented here are compared with the findings from other plant species. Several researchers reported different optimized formulas for the formation of fine and small cell aggregates, including NAA and BA (0.25 mg l^{-1}) (Keng et al., 2010), 2,4-D and kin (1 and 1.5 mg l^{-1}) (Osman et al., 2016), or 2,4-D and BA (2 and 0.5 mg l^{-1}) (dos Santos et al., 2017). Although in most of the reports, the type of cytokinin used in FC formation was different, 2,4-D was the dominant type of auxin. It was also revealed that, 2,4-D at lower concentrations than 2 mg l^{-1} is the best concentration for induction of loose cells. It also supports this study's results that 2,4-D at 1 mg l^{-1} was found to be the best for FC formation. Although the results of this study confirmed the efficiency of PGRs on FC formation of saffron, in some cases, these are different, particularly in the types and concentrations of PGRs, which can be attributed to the use of different plant species and organs.

Fig. 4. Organogenesis on corm-derived callus of saffron (*Crocus sativus* L.).

Optimization of medium and CSC establishment

The results of CSC optimization revealed that the medium, PGRs, and glutamine levels could significantly influence cell fresh and dry weight averages. Generally, the sigmoid growth curve was observed in all treatments (Fig. 5). Cells grew vigorously in A1 and A5 treatments. These two media produced the maximum cell fresh and dry weight over nine weeks. All treatments were in a lag phase over the first week. Treatments A1 and A5 started the exponential phase from the second week, while other treatments remained in lag phase until the end of the third week. Cell growth in A1 and A5 in the week of 4 to 6 went to the linear phase. At this stage, the fresh cells' weight raised from 0.42 to 0.77 g in A1 and from 0.42 to 0.75 g in A5. Grown cells in these two treatments reached their maximum weight (0.8 g) in the seventh week. In the week of 7 and 8, their weight remained stable (stationary phase) and finally, in the ninth week, cells entered the death phase, and a decrease in fresh (0.66 g) and dry (0.062 g) weight was observed. Accordingly, the end of the linear phase was in the sixth week (Fig. 5). Therefore, the end of the sixth week is the best time to subculture cells. In the establishment of CSC, A1 and A5, containing the same amount of glutamine, showed a statistically similar effect, while there were different in types and levels of hormones. Although A2 and A6 had similar hormonal combinations to A1 and A5, without glutamine, their fresh and dry weights were significantly lower than A1 and A5 during the tested period. It suggested that hormones alone cannot play a decisive role in cell biomass increment. It means glutamine could be suitable organic nitrogen for saffron cell growth in a CSC system.

Based on the obtained results in this study, A5 containing 2,4-D (0.5 mg l^{-1}), Zeatin (0.3 mg l^{-1}) and glutamine (10 mg l^{-1}) was chosen as a superior treatment for the next experiment as it showed maximum biomass growth. In the second part of the CSC optimization experiment, which was studying different levels of sucrose and nitrogen, it was found that B1 containing sucrose (30 g l^{-1}) and Nitrogen (30 µM) was the best for saffron cell growth (Fig 6). Although in the first week, the highest growth was observed in B5 (0.35 g) and B4 (0.34 g), during the week of 3 to 4, the maximum fresh and dry weight was found to be B1 (1.08 g) and B4 (0.95 g). In the sixth week, B4 entered to the diminishing growth phase, while B1 was remained in the linear phase. The later treatment reached to the maximum fresh weight (1.2 g) in the week of 7. The growth curves of the cells in B1, B2, and B4 show a sigmoid pattern that follow lag, exponential, linear, deceleration, stationery and decline phases. Indeed, the end of exponential phase, similar to the previous experiment, was at the end of sixth week (Fig 6), which is the appropriate time to subculture of the cells or elicitation. In this part, as the same as FC experiment, cells were in maximum biomass level at the week of 7 to 9 and entered to the death stage afterward. This identical sigmoid growth was not visible in other treatments (B3, B5, B6), as their cells growth were very limited.

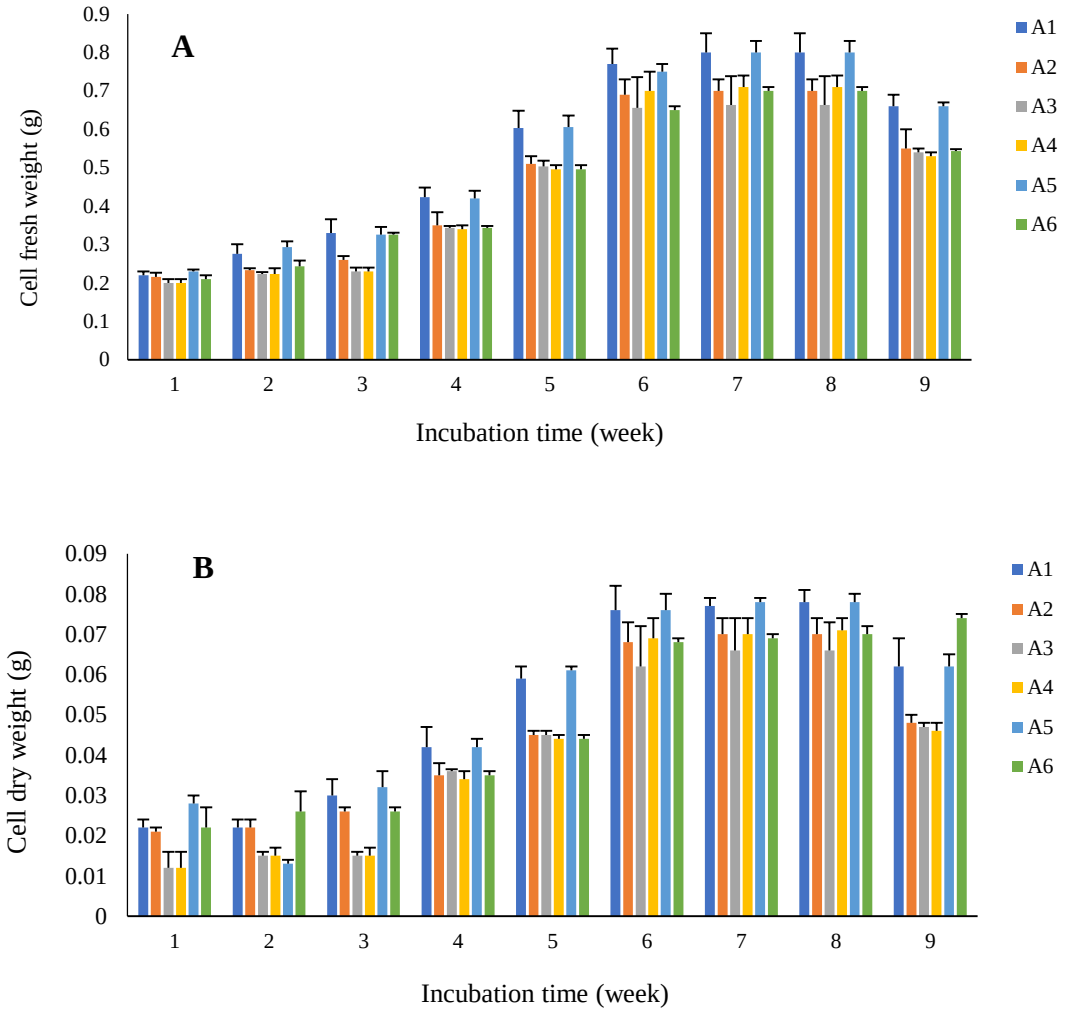

Fig. 5. Effect of different treatments (A1, A2, A3, A4, A5, A6) on weight of saffron (*Crocus sativus* L.) cells in suspension culture system over 9 weeks. A) Cell fresh weight, B) Cell dry weight.

Scientists suggested that PGRs composition, mineral salts, and carbon sources can influence plant cell growth in suspension cultures (Knobloch & Berlin, 1980). Their results revealed that the optimal mineral salts in the medium and hormonal concentration/ratio for cell growth in various cultivars of *Cannabis sativa* are different. Indeed, the type of plant species and the type of cultivar play a decisive role in the selection of medium components (Thacker et al., 2018). The same results have also been reported by Jamil et al. (2018) in which, the establishment of CSC of mangosteen (*Garcinia mangostana*) was found to be faster in the lower concentration of growth regulators than those applied in callus induction. They used MS medium supplemented with 2,4-D and BAP at 1 mg l^{-1} for FC induction while it was 0.5 mg l^{-1} for the establishment of CSC. PGRs concentration reduction to achieve maximum biomass growth during the transfer of cells from solid to liquid medium was also clearly observed in the present study. Notably, in all the research mentioned above, the basal medium used for different plants was MS, which can be concluded that MS is desirable with its macro and micro elements for FC formation. These findings confirm the result of this study in which MS was found to be more suitable than ½ MS.

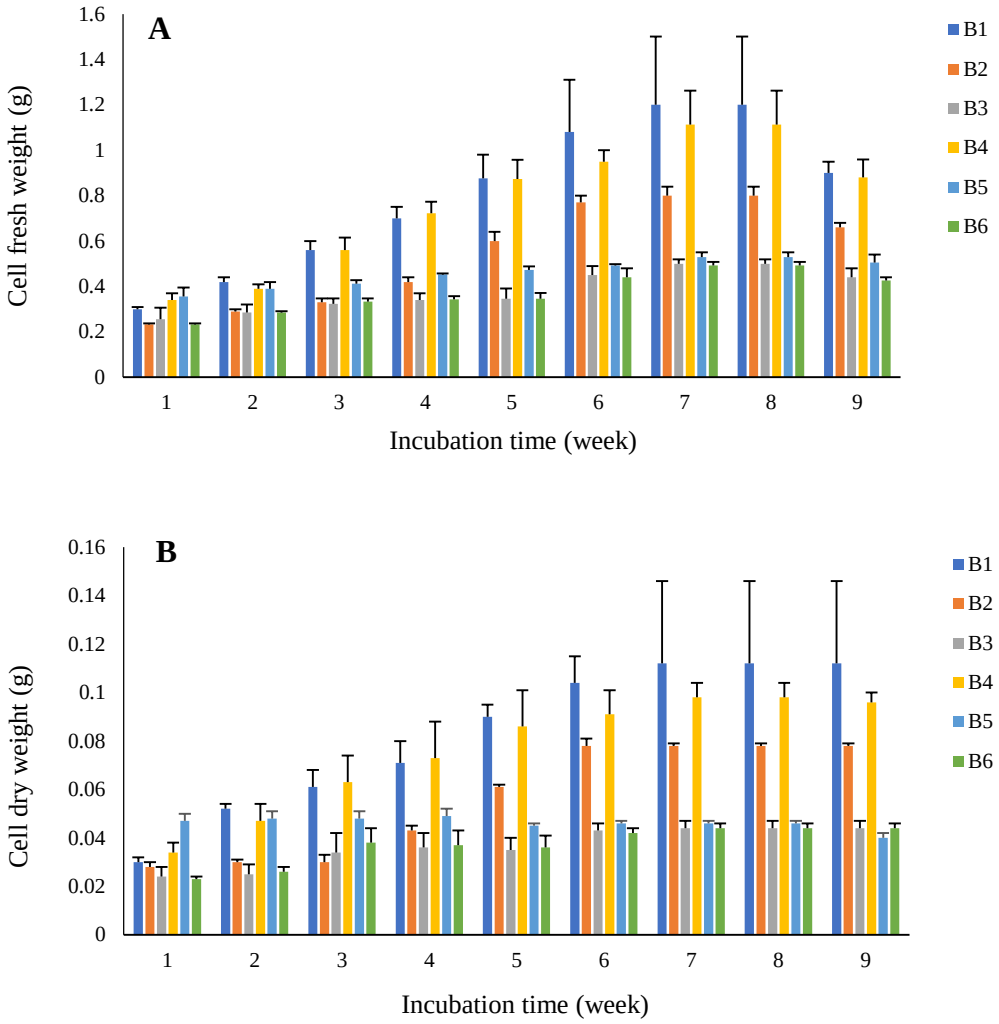

Fig. 6. Effect of different concentrations of sucrose and nitrogen in different treatments (B1, B2, B3, B4, B5, B6) on weight of saffron (*Crocus sativus* L.) cells in suspension culture system over 9 weeks. A) Cell fresh weight, B) Cell dry weight.

Indeed, glutamine and glutamate are the major sources of endogenous amino acids which supply nitrogen for the biosynthesis of nitrogenous compounds such as nucleic acid and proteins in plant cells (Hamasaki et al., 2005). Although the mechanism of glutamine influence on *in vitro* cultures is poorly understood, it is extensively used as a complimentary or sole source of organic nitrogen in plant tissue culture (Marques et al., 2017). This compound is a reduced source of nitrogen that is energetically less costly to assimilate than nitrate or ammonium (Leustek& Kirby 1988). Leustek and Kirby (1988) results clearly indicated that the application of glutamine in protoplast culture acts as a stimulant for cell growth. Hamasaki et al. (2005) found that glutamine, through stimulating the endogenous indole acetic acid (IAA) and isopentenyl adenine (iP), can evoke competence for *in vitro* organogenesis in explants. This study also found that glutamine could increase cell biomass caused by division stimulation.

CONCLUSION

Several factors can influence on increasing efficiency of CSC in order to have higher biomass. Apparently, FC formation is essential to establishing a homogenous CSC system. The results of this study demonstrated that saffron, in addition to the FC formation, as an initial plant material, the optimization of the medium can also increase biomass. It was also found that full-strength MS supplemented with a lower concentration of PGRs has a great role in FC formation. The superior PGRs combination was found to be 2,4-D and kin (1 and 0.2 mg l^{-1}) which showed the most effective role in the FC formation. On the other hand, a protocol was developed to establish an efficient saffron CSC with higher biomass. In this protocol, an improved MS medium was developed, in which the total nitrogen was reduced to 30 µM and supplemented with 2,4-D (0.5 mg l^{-1}), zeatin (0.2 mg l^{-1}) and glutamine (10 mg l^{-1}). This protocol could successfully increase fresh cell weight from 0.2 to 1.2 g. In addition, it is suggested to investigate the production of saffron metabolites under CSC conditions.

Conflict of interest

The authors declare no conflict of interest to report.

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Effects of climatic conditions on the physical and extensional properties of pomegranate fruit peel in Malas-e-Saveh and Yousefkhani cultivars

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ABSTRACT

Purpose: This research was carried out to investigate the physical and extensional properties of fruit peel in two commercially grown pomegranate cultivars across three distinct climatic conditions in Iran. **Research method**: Two pomegranate cultivars were examined in a factorial experiment in frame of completely randomized design across three producing regions, including Saveh, Sari, and Aliabad. **Findings**: The findings revealed that Yousefkhani had a higher crown diameter compared to Malas-e-Saveh. Among the regions studied, Saveh and Aliabad produced fruits with the highest and lowest fruit weight and percentage of membranous septum, respectively. Additionally, Sari and Aliabad, which have humid weather conditions, produced fruits with a lower crown diameter and membranous septum thickness compared to Saveh, which has dry weather conditions. Furthermore, the highest percentage of moisture in the fleshy mesocarp and peel and the lowest percentage of moisture in membranous septum were found in fruits cultivated in Sari and Aliabad, respectively. The results also showed that Malas-e-Saveh had the highest peel deformation in transversal oriented samples. Yousefkhani had a higher extension force compared to Malas-e-Saveh, while no significant difference was observed among regions or between the two sampling directions in these properties. **Research limitations**: None were found to report. **Originality/Value**: Based on the findings of this study, it can be concluded that most pomegranate fruit characteristics were significantly influenced by cultivar and growing region. Yousefkhani exhibited a thicker peel, a higher percentage of fleshy mesocarp, and greater tolerance to extension force compared to Malas-e-Saveh.

INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruits and can grow in diverse agro-climatic conditions, ranging from tropical to sub-tropical regions (Zaouay et al., 2020). It is believed to have originated in Iran and central Asian countries (Ashrafi et al., 2023). The fruit develops from the ovary and is a fleshy berry (Singh et al., 2020). Pomegranate fruits are typically globular or somewhat flattened, with a diameter ranging from 5 to 12 cm, and are characterized by a thick tubular calyx. The calyx remains attached to the fruit until maturity, which is a distinguishing feature of pomegranates. The skin, peel, or exocarp of the fruit plays a vital role in supporting the internal cell layers. It serves as an essential component that provides a protective barrier against water loss and potential pathogen infiltration (Macnee et al., 2020). Within the fruit, the multiovule chambers (locules) are separated by membranous walls (septa) and fleshy mesocarp. The chambers are filled with many seeds (arils) and organized in a non-symmetrical way, with the lower part of the fruit containing 2 to 3 chambers, while the upper part typically includes 6 to 9 chambers (Holland et al., 2009).

In pomegranates, the occurrence of cracking can be attributed to variations in the growth rate between the fruit peel and flesh, as well as the pressure exerted by the rapidly expanding arils on the stretched peel (Ginzberg & Stern, 2019). Fruit cracking in pomegranates is primarily caused by deficiencies in nutrients such as calcium and boron during the early stages of fruit development, drastic fluctuations in day and night temperatures, irregular watering regimes during fruit ripening, and prolonged dry periods followed by heavy rains or irrigation (Galindo et al., 2014; Ghanbarpour et al., 2019; Fraschetti et al., 2023). Excessive water during the developmental stage is a prominent environmental stress factor that contributes to fruit cracking. This water excess can disrupt the balance of essential cations, such as calcium (Ca), which is crucial for maintaining the structural integrity of the fruit (Santos et al., 2023). Approximately 65% of the pomegranate cultivar Shishe-Kab experiences cracking (Hamedi Sarkomi et al., 2019). The mechanical properties of the cuticle can have both positive and negative effects on disorders such as fruit splitting, fungal pathogen penetration, and pest infestation (Dominguez et al., 2011). Additionally, changes in peel properties and fruit volume are believed to be involved in fruit cracking (Joshi et al., 2021). Saei et al. (2012) reported significant differences in many physical properties between resistant and sensitive cultivars to fruit cracking. Their results indicated that characteristics such as fruit volume, fruit shape, total arils weight, and peel weight/fruit weight had significant correlations with fruit cracking. Fruit cracking can also be attributed to environmental and nutritional factors, high-temperature daytime and nighttime travel, and the hardening of the fruit peel during a prolonged dry period, followed by a sudden expansion in the volume of the inner part of the fruit after rain or heavy irrigation (Fraschetti et al., 2023). Parvizi et al. (2014) showed that fruit cracking was enhanced with an increase in mean relative humidity and a decrease in mean air temperature during the four-day period before each measurement of fruit cracking.

Environmental conditions have a significant influence on fruit quality (Schwartz et al., 2009). Pomegranate is a subtropical plant, and suboptimal climate conditions during fruit development can lead to physiological skin disorders, such as cracking. A study conducted in 2010 investigated the physical, chemical, and sensory characteristics of pomegranate fruits from the Glavaš cultivar grown at nine different locations in western Herzegovina. The study revealed that location, agricultural practices, and microclimate had some influence on the same cultivar (Gadže et al., 2012). Understanding the properties of fruit peels and how they are affected by different climatic conditions can help identify the best management practices

needed in orchards and breeding programs. Previous research has shown that the Malas-e-Saveh pomegranate cultivar is sensitive to cracking, while the Yousefkhani cultivar is resistant (Saei et al., 2014). However, limited information is available regarding the impact of climatic conditions on the properties of pomegranate fruit peel. Therefore, this research was carried out to investigate the influence of climatic conditions on the physical and extensional properties of fruit peel in pomegranate cultivars Malas-e-Saveh (referred to as Malas in this paper) as a sensitive cultivar to cracking and Yousefkhani as a resistant cultivar to cracking, grown in three different climatic conditions in Iran.

MATERIALS AND METHODS

The study was conducted at Gorgan University of Agricultural Sciences and Natural Resources using a factorial arrangement in a completely randomized design with three replications.

Fruit material

To investigate the effect of climatic conditions on fruit peel, two pomegranate cultivars grown in three producing regions were examined. The producing regions included Saveh (1076 m above sea level, latitude 35.02 N, and longitude 50.19 E) in Markazi province, Sari (-13 m above sea level, latitude 36.40 N, and longitude 53.08 E) in Mazandaran province, and Aliabad Katool (63 m above sea level, latitude 36.58 N, and longitude 54.43 E) in Golestan provinces. Fruit samples were harvested at the stage of commercial maturity based on native growers' experience and immediately transported to the laboratory.

Physical analysis

Individual fruit weight was determined using a digital balance with an accuracy of 0.01 g. The length and diameter of the fruit crown were measured using digital calipers with an accuracy of 0.01 mm. Different parts of the fruits (Fig. 1A), including membranous septum (Fig. 1B), fleshy mesocarp (Fig. 1C), and the peel (with crown) (Fig. 1D) were manually separated from the arils (Fig. 1E) for further measurements. Peel and membranous septum thickness were measured at the equatorial zone of the fruits using digital calipers. Moisture percentage was determined by drying 10 g of the peel, fleshy mesocarp, and membranous septum in an oven at 70°C until a constant weight was reached.

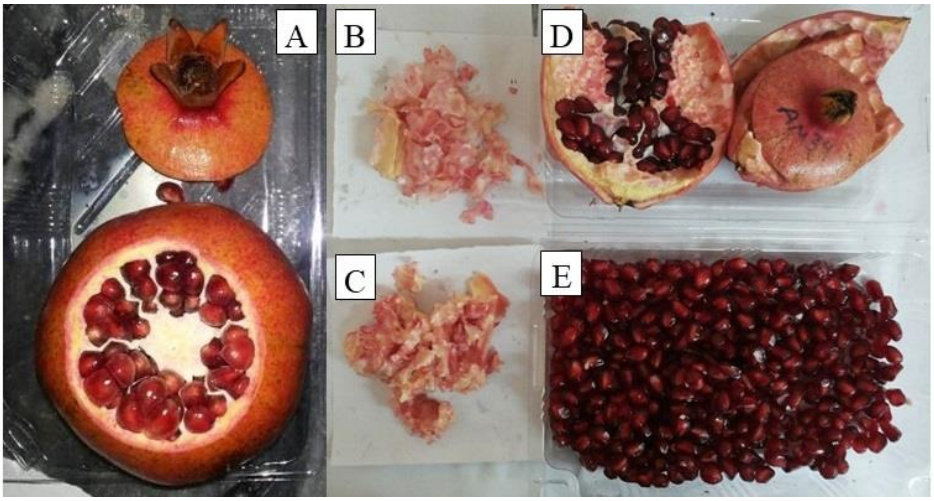

Fig. 1. Different parts of a pomegranate fruit in Malas cultivar (A), membranous septum (B), fleshy mesocarp (C), peel (with crown) (D), and arils (E).

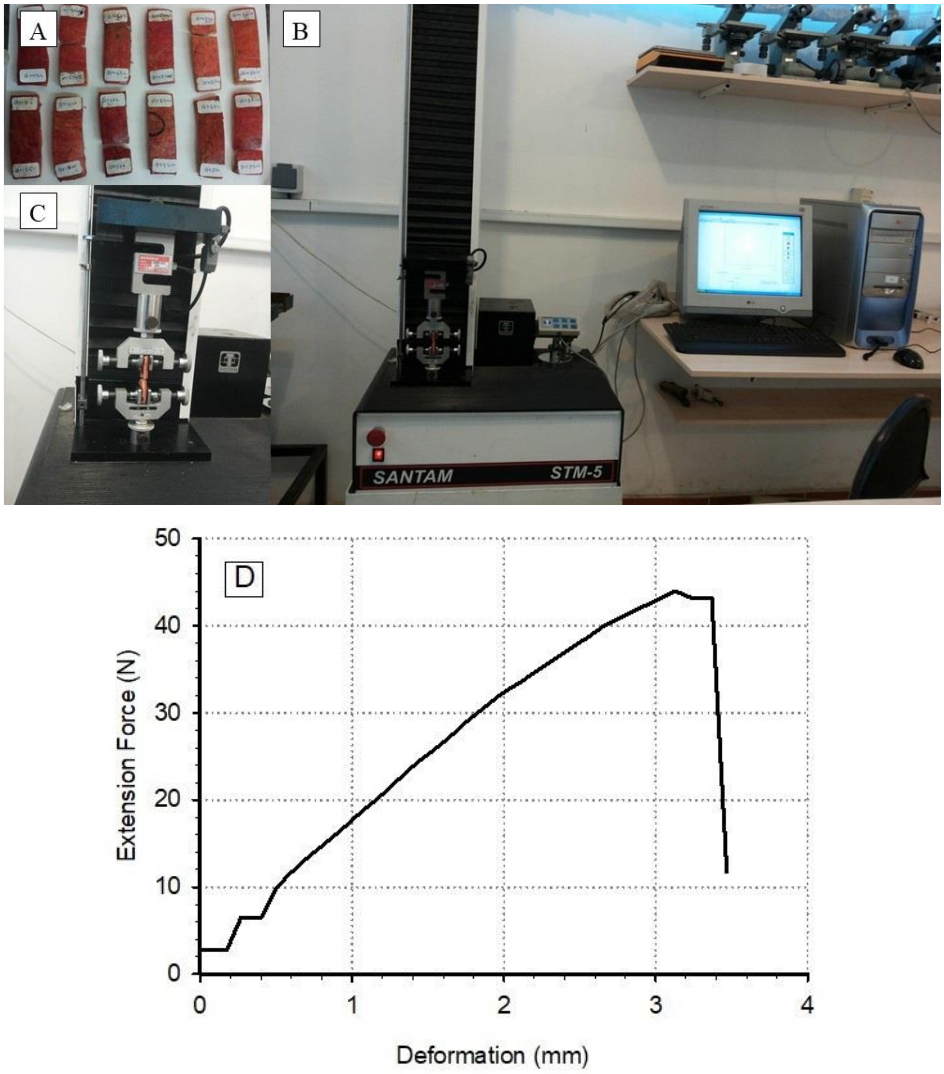

Fig. 2. Peel samples (A), Universal Testing Machine (STM-5, Santam Co.) (B and C), and force-deformation curve (D) of pomegranate fruits under extension test.

Extensional analysis

To determine the fruit's peel extension force and deformation, two peel samples (Fig. 2A) were prepared in the longitudinal and transversal directions from each fruit. The samples were taken from parts of the peel with uniform thickness but without any cracks and were rectangular, measuring 60 mm in length and 20 mm in width. An extension test was conducted using a Universal Testing Machine (Model: STM-5, Santam Co., Iran) (Fig. 2B and C) at a displacement rate of 30 mm/min. The extension force and deformations of the samples, as well as the force-deformation curve (Fig. 2D), were recorded in Excel software.

Statistical analysis

The data obtained from this study were analyzed statistically using JMP software, version 8. Numeric and percentage data were properly transformed before analysis, if necessary. The comparison of means was performed using the least significant difference (LSD) test.

RESULTS AND DISCUSSION

Physical properties

The analysis of variance results (Table 1) indicate that the interaction effect of cultivar and growth region was significant for crown length/diameter, peel percentage, peel thickness, and the number of septa at a 5% probability level. It was also significant for crown length and the percentage of fleshy mesocarp at a 0.1% probability level. However, the interaction effect was not significant for fruit weight, crown diameter, thickness and percentage of membranous septum, peel moisture percentage, fleshy mesocarp moisture percentage, and membranous septum moisture percentage. The main effect of cultivar was significant for crown diameter at a 1% probability level and for crown length, peels percentage and thickness, and the percentage of fleshy mesocarp at a 0.1% probability level. The main effect of growth region was significant for crown diameter at a 5% probability level, peel thickness at a 1% probability level, and for fruit weight, crown length/diameter, percentage of peel, number of membranous septa, thickness and percentage of membranous septum, percentage of fleshy mesocarp, and the percentage of peel moisture, fleshy mesocarp moisture, and membranous septum moisture at a 0.1% probability level.

Table 1. The analysis of variance of the effects of cultivar and growing region on some physical properties of pomegranate fruit.

Sources of variation	df	Mean of squares						
		Fruit weight	Crown length	Crown diameter	Crown L/D	Peel (%)	Peel thickness	Number of septa
Cultivar	1	0.044 ns	284.978 ***	64.078 **	0.078 ns	0.035 ***	7.006 ***	0.178 ns
Region	2	144455.6 ***	244.174 ***	33.010 *	0.693 ***	0.25 ***	4.211 **	7.511 ***
Cultivar × Region	2	8908.9 ns	44.907 ***	6.506 ns	0.236 *	0.00061 *	2.843 *	2.178 *
Error		5655.5	6.11	7.374	0.054	0.022	0.608	0.543
		Membranous septum (%)	Membranous septum thickness	Fleshy mesocarp (%)	Peel moisture	Fleshy mesocarp moisture	Membranous septum moisture	
Cultivar	1	0.371 ns	3.043 ns	35.01 ***	6.135 ns	32.873 ns	2.48 ns	
Region	2	4.357 ***	7.276 ***	31.539 ***	268.55 ***	91.916 ***	1581.8 ***	
Cultivar × Region	2	0.978 ns	3.634 ns	13.748 ***	2.583 ns	23.119 ns	108.718 ns	
Error		0.364	0.383	1.247	3.825	10.545	63.546	

***, **, *: Significant difference at 0.1%, 1% and 5% probability level, respectively. ns: no significant difference.

Table 2. The interaction effects of cultivar and growing region on some physical properties of pomegranate fruit.

Cultivars and regions	Crown length (mm)	Crown L/D	Peel (%)	Peel thickness (mm)	Number of septa	Fleshy mesocarp (%)
Malas - Sari	25.51c	1.51ab	28.37c	2.48d	7.33b	6.85d
Malas - Aliabad	22.22d	1.37bc	28.69c	2.86cd	6.67c	8.11c
Malas - Saveh	23.88cd	1.27c	32.19b	3.62ab	7.40b	8.96b
Yousefkhani - Sari	31.15a	1.66a	35.39a	3.75a	8.00a	9.21b
Yousefkhani - Aliabad	23.08d	1.23c	29.89bc	3.13bc	6.67c	7.85c
Yousefkhani - Saveh	28.06b	1.44b	36.11a	3.77a	7.00bc	10.60a
Cultivar × Region	P=0.001	P=0.015	P=0.034	P=0.012	P=0.022	P<0.001
Cultivar	P<0.001	P=0.232	P<0.001	P=0.001	P=0.569	P<0.001
Region	P<0.001	P<0.001	P<0.001	P=0.002	P<0.001	P<0.001

In each column, different letters indicate significant difference (at 1 or 5% probability level, LSD).

The Malas cultivar of Sari showed the highest crown length (31.15 mm), which was significantly different from Malas and Yousefkhani in other regions. On the other hand, the lowest crown length was observed in Malas of Aliabad and Saveh regions, as well as in Yousefkhani of Aliabad (Table 2). These results are consistent with previous studies that reported crown lengths ranging from 11.7 to 30.2 mm for various cultivars (Tehranifar et al., 2010a; Khadivi & Arab, 2021). The highest length-to-diameter ratio of the crown was observed in Malas and Yousefkhani of Sari (1.51 and 1.66, respectively), while both cultivars grown in Aliabad and Malas of Saveh showed the lowest ratio.

According to the results of this study, Yousefkhani of Sari and Saveh regions had the highest percentages of fruit peel (35.39% and 36.11%, respectively). In contrast, the lowest percentage of fruit peel was observed in the Malas of Sari and Aliabad regions, as well as in Yousefkhani of Aliabad (Table 2). Fischer et al. (2013) previously reported that the leathery peel and fleshy mesocarp accounted for 9-12% and 22-38% of the entire fruit weight, respectively. Previous studies have also reported that the percentage of aril is inversely related to the percentage of peel (Tehranifar et al., 2010a; Tehranifar et al., 2010b). Furthermore, Chandra et al. (2013) found a significant difference among pomegranate cultivars in the percentage of fruit peel (22.53% to 41.19%), which confirms the results of this study. However, the percentage of peel obtained in this study was slightly lower than the findings of other studies (Tehranifar et al., 2010b).

Peel thickness is an important criterion for selection, as fruits with thin peel are preferred for processing, while those with thick peel that resist transport and storage are chosen for fresh consumption (Mansour et al., 2015). However, consumers generally prefer pomegranate fruit with thin peel because it reduces fruit waste and is easier to peel (Radunić et al., 2015). The study found that Malas and Yousefkhani of Saveh and Yousefkhani of Sari had the highest peel thickness (3.62, 3.77, and 3.75 mm, respectively). In contrast, the lowest peel thickness was observed in the Malas of Sari and Aliabad regions (Table 2). The peel thickness in this study is consistent with the results of some other studies (Tehranifar et al., 2010b; Al-Aslan et al., 2023). Gadže et al. (2012) observed significant differences in the peel thickness of the Glavaš cultivar grown at nine locations in western Herzegovina. They found that Buna4 had the highest value (9.89 mm), while Stolac3 had the lowest value (5.91 mm). Saei et al. (2014) reported that Malas, the sensitive cultivar to fruit cracking, had a thinner peel than Yousef-Khani, the resistant one. However, there was no significant relationship between peel thickness and resistance to fruit cracking in pomegranate. According to their theory, the role of fruit peel in pomegranate cracking depends on its mechanical properties. This study found that the peel thickness of Yousefkhani of Aliabad was similar to Malas of Aliabad, confirming that pomegranate peel thickness depends on the cultivar and growth region. Singh et al. (2020) reported that varietal differences in cracking were attributed to peel structure and epidermal cell size.

The research findings revealed that Yousefkhani of Sari had a higher number of membranous septa compared to the other cultivars and regions. On the other hand, Malas and Yousefkhani of Aliabad, as well as Yousefkhani of Saveh, had the lowest number of membranous septa (Table 2). Schwartz et al. (2009) reported that the number of white membranous walls inside each fruit was 1.14-1.25 times higher in fruits obtained from Neweyaar compared to those from southern Arava. They suggested that the environmental conditions at Neweyaar promote a higher formation of these membranous walls, which in turn affects the number of arils and peel thickness. This higher formation of membranous walls was also reported to be the main reason for the higher juice content of fruits from this habitat (Schwartz et al., 2009). The study found that Yousefkhani of Saveh had the highest percentage of fruit fleshy mesocarp (10.6%), while Malas of Sari had the lowest percentage

(6.85%) (Table 2). According to Saei et al. (2014), the fleshy mesocarp of the peel contains parenchyma cells with air bubbles between them, resulting in less extensibility. It can be hypothesized that since the fleshy mesocarp is responsible for water absorption, this part may act as a stress source in the fruit.

Fruit weight is an important factor in pomegranate fruit production and marketing (Holland et al., 2009). The study found no significant difference in fruit weight between the cultivars, but there was a significant difference among the growth regions. The highest fruit weight was observed in the Saveh region (478.53 g), while the lowest fruit weight was observed in the Aliabad region (340.50 g) (Table 3). These results are consistent with other studies that have reported fruit weights ranging from 133.8 to 509.82 g (Fawole & Opara, 2014). Schwartz et al. (2009) reported that in 2006, fruit weight was higher in fruits produced in the southern Arava (desert climate) compared to those from Neweyaar (Mediterranean temperate to subtropical climate). However, in 2007, the fruits obtained from Neweyaar were heavier than those harvested from the southern Arava. These findings align with the results of the present study, where fruits from the Saveh region, with a desert climate, had higher weight compared to the Aliabad and Sari regions with humid climates. Differences in fruit weight among various pomegranate cultivars have been attributed to ecological and genetic variations (Joshi et al., 2021). An increase in fruit size can be attributed to an increase in aril size and juice content as fruits reach maturity (Lyu et al., 2020).

The results of this study indicated that Yousefkhani had a significantly higher crown diameter compared to Malas (Table 3). Additionally, the Saveh region exhibited a higher fruit crown diameter (19.65 mm) compared to the other two regions. Previous studies have reported slightly lower (Nikdel et al., 2016), or a wider range of crown diameters (17.1 to 26.9 mm) (Radunić et al., 2015).

Table 3. The effects of cultivar and growing region on some physical properties of pomegranate fruit.

	Fruit weight (g)	Crown diameter (mm)	Membranous septum (%)	Membranous septum thickness (mm)	Peel moisture (%)	Fleshy mesocarp moisture (%)	Membranous septum moisture (%)
Cultivars							
Malas	417.29	17.61b	1.78	0.39	60.85	73.08	57.29
Yousefkhani	417.24	19.30a	1.91	0.35	61.37	74.29	57.63
Regions							
Sari	435.77b	18.02b	1.91b	0.32b	63.40a	75.52a	62.92a
Aliabad	340.50c	17.69b	1.43c	0.31b	62.21b	72.03b	49.22b
Saveh	475.53a	19.65a	2.18a	0.47a	57.73c	73.50b	60.24a
Cultivar × Region	*P*=0.213	*P*=0.418	*P*=0.074	*P*=0.973	*P*=0.512	*P*=0.118	*P*=0.187
Cultivar	*P*=0.998	*P*=0.004	*P*=0.316	*P*=0.213	*P*=0.209	*P*=0.081	*P*=0.844
Region	*P*<0.001	*P*=0.014	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001

In each column, different letters indicate significant difference (at 1 or 5% probability level, LSD).

Table 4. The analysis of variance of the effects of cultivar, growing region and sampling direction on pomegranate fruit peel deformation and peel extension force.

Sources of variation	df	Mean of squares	
		peel deformation	peel extension force
Cultivar	1	5.08 *	2370.704 ***
Region	2	30.913 ***	150.396 ns
Sampling direction	1	1.478 ns	158.292 ns
Cultivar × Region	2	0.427 ns	52.164 ns
Cultivar × sampling direction	1	0.311 ns	21.78 ns
Region × sampling direction	2	3.296 *	246.766 ns
Cultivar × Region × sampling direction	2	3.325 *	88.136 ns
Error	96	1.066	146.963

*** and *: Significant difference at 0.1% and 5% probability level, respectively. ns: no significant difference.

Membranous septa are papery tissues that compartmentalize groups of arils without attaching to them (da Silva et al., 2013). The results of this study showed no significant difference between the two cultivars in terms of the percentage and thickness of membranous septum (Table 3). However, there were significant differences in the percentage of membranous septum between the Saveh (2.18%) and Aliabad (1.43%) regions, with both regions differing significantly from Sari. Similarly, Saveh had the highest thickness of membranous septum (0.47 mm) among the growing regions. According to Kianmehr et al. (2011), 41.5% of pomegranate weight in the Alak cultivar consisted of epicarp and mesocarp. The present study found no significant difference in the percentages of moisture in the peel, fleshy mesocarp, and membranous septum between the two cultivars (Table 3). The results showed that Sari had the highest percentage of peel moisture (63.40%), while Saveh had the lowest (57.73%), with both regions differing significantly from Aliabad. The percentages of peel moisture found in Malas and Yousefkhani were slightly higher than those found in five Iranian pomegranate cultivars in South Khorasan Province (Nikdel et al., 2016). The highest percentage of fleshy mesocarp moisture was observed in Sari (75.52%), which was significantly different from the other regions. Additionally, the highest percentage of membranous septum moisture was observed in Sari and Saveh regions (62.92% and 60.24%, respectively). These results align with the findings of Kianmehr et al. (2011), who reported that the moisture content of pomegranate epicarp and mesocarp was 51.71% and 73.03%, respectively.

Extensional properties

The results of the analysis of variance (Table 4) showed that the triple interaction effect of cultivar, growth region, and sampling direction was significant at a 5% probability level for peel deformation, but not for peel extension force. The interaction effects of cultivar and sampling direction, cultivar and growth region, and growth region and sampling direction, as well as the main effects of growth region and sampling direction on peel extension force, were not significant. However, the main effect of cultivar on peel extension force was significant at a 0.1% probability level.

According to the findings of the current study, the highest peel deformation was observed in the transversal direction in Malas of Saveh (6.489 mm), while the lowest deformation was observed in the transversal direction in Malas and Yousefkhani of Sari, as well as in the longitudinal direction in Yousefkhani of Sari and in Malas and Yousefkhani of Aliabad (Fig. 3). Previous research by Sadrnia et al. (2009) found that Crimson Sweet watermelon had the highest failure deformation under load in the transversal direction, while Charleston Gray had the lowest failure deformation in the longitudinal direction for medium-sized fruits. They also noted that changes in the peel white layer affected the mechanical properties of these watermelon cultivars. Additionally, watermelon peel strength was higher in the transversal direction compared to the longitudinal direction. Environmental conditions such as temperature and relative humidity have a plasticizing effect on the mechanical properties of cuticles by reducing cuticle stiffness and strength (Dominguez et al., 2011).

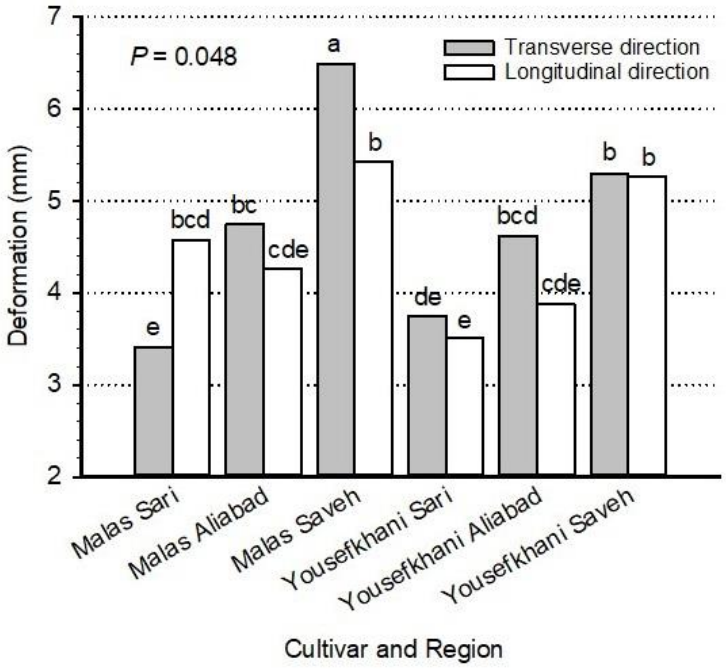

Fig. 3. The interaction effects of cultivar, growing region and sampling direction on pomegranate fruit peel deformation.

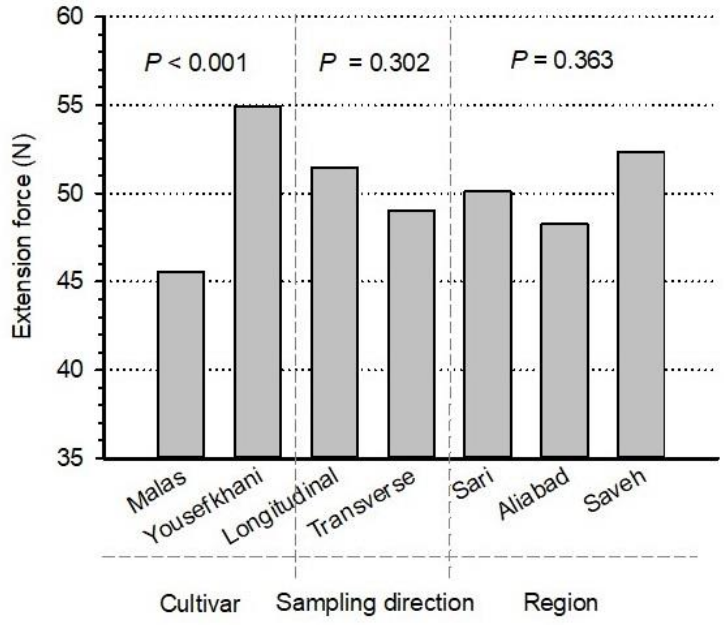

Fig. 4. The effects of cultivar, growing region and sampling direction on pomegranate fruit peel extension force.

The results of this study showed that the peel extension force varied significantly among the studied cultivars, with Yousefkhani (54.93 N) having a higher extension force than Malas (45.56 N) (Fig. 4). However, the differences among the sampling directions and growing regions were not significant. The fruit skin has a dual role as it protects the fruit from environmental stresses while also playing a critical role in resisting internal growth pressures, controlling fruit expansion, and maintaining fruit integrity (Joshi et al., 2021). Understanding the mechanical properties of pomegranate fruit peel is important for improving harvesting, transport, and postharvest handling of pomegranate fruits (Fawole & Opara, 2014). Some

pomegranate cultivars have relatively thinner and softer fruit peels, making them more susceptible to physical damage such as cracking and splitting (Holland et al., 2009).

According to Saeidirad et al. (2015), harvesting time affects the extension force of pomegranate fruit peel. The extension force of the fruit peel increased during prolonged harvesting periods, with a significant difference observed between the peel extension force in fruits harvested in late October and those harvested in mid-November. This increase in extension force can be attributed to fruit growth completion and a decrease in peel moisture content. The Shishe-Kab cultivar had the lowest extension force compared to Ardestani and Malas, despite having a thicker peel. The highest extension force was observed in Ardestani, indicating that an increase in peel thickness does not necessarily raise the peel extension force. These findings align with the present study, which found that peel extension force was consistent across different regions even when peel thickness varied. In other words, there is no direct relationship between peel thickness and extension force. Saei et al. (2014) reported that the correlation between peel thickness and resistance to fruit cracking in pomegranate was not significant. They also illustrated that the role of fruit peel in pomegranate fruit cracking depends on its mechanical properties. These results are consistent with the observations of this study, which found similar peel thickness in Aliabad for both Yousefkhani (resistant to cracking) and Malas (sensitive to cracking) cultivars. Therefore, it can be concluded that peel thickness depends on the cultivar and growing region, while resistance to cracking depends on the mechanical properties of the fruit peel.

CONCLUSION

Pomegranates are naturally adaptable to various climatic conditions, and they are cultivated in different regions worldwide. However, different climatic conditions can impact the quality of pomegranate fruit. According to the findings of this study, most pomegranate fruit characteristics were significantly influenced by cultivar and growing region. Yousefkhani, as a resistant cultivar to cracking, exhibited a thicker peel and a higher percentage of fleshy mesocarp compared to the sensitive cultivar, Malas. Yousefkhani, with its higher tolerance to extension force, is suitable for storage and transportation and may have a higher tolerance to cracking. Furthermore, this research indicated that fruits produced in arid and desert regions of Saveh had a higher weight. Given the increasing desire to grow pomegranates worldwide, it is crucial to consider the influence of climatic conditions on the qualitative properties of pomegranate fruit, particularly when introducing new superior cultivars to different planting regions. Further studies are necessary to explore the effects of climatic conditions on the qualitative characteristics of pomegranate flowers, fruit, and peel in other cultivars and regions.

Conflict of interest

The authors have no conflict of interest to report**.**

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Effect of gibberellin on *in vitro* bulblet induction of lily (*Lilium orientalis* L. cv. Santander)

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ABSTRACT

Purpose: Lily is one of the most economically ornamental plants and tissue culture plays a vital role in accelerating mass propagation of lily. In lily tissue culture, the production of bigger bulblets is highly desirable. The objective of the present investigation is to examine the impact of gibberellin on the *in vitro* growth of lily bulblets, administered at two distinct time intervals. **Research method:** In the present investigation, various concentrations of gibberellins (0, 0.1, and 1 µM) were employed at two distinct time points: the commencement of the culture period and the fifth week of culture period. After 11 weeks the fresh weight of bulblets, the number of bulblets, the fresh weight of leaves, the fresh weight of roots and the fresh weight of scale explant were scored and analyzed. **Findings:** The application of 1 µM gibberellin during bulblet induction yielded noteworthy outcomes, including a substantial 91% increase in the fresh weight of the bulblets, a significant 38% augmentation in the fresh weight of the leaves, as well as a 40% increase in the fresh weight of the roots. **Research limitations:** The quantification of endogenous phytohormones in lily scale explants was deemed unfeasible**. Originality/value:** The development of lily bulblets experienced a notable enhancement while the medium was supplemented with gibberellin in bulblet induction stage.

INTRODUCTION

Lilium is a genus of herbaceous flowering plants that originate from bulbs and belong to the lily family (*Liliaceae*) (Askari et al., 2018). The lily has gained significant popularity as a cut flower worldwide, accounting for approximately 25% of the total export value of flower bulbs (Ahmed et al., 2018). The breeding process for lilies is a time-consuming endeavor, with the introduction of a newly bred lily cultivar to the market historically taking up to 15 years. The initial phase of this process involves the selection of the most desirable clone, which requires several years. From the time of seed germination to the development of a flowering plant, approximately three years' elapse. Consequently, the evaluation of flower properties can only occur several years after the initial crosses have been made. Subsequently, additional years are necessary to assess quantitative traits such as yield and disease resistance. Once the best clone has been identified, the production of an adequate number of bulbs requires several more years due to the slow pace of available vegetative propagation methods, such as natural propagation and (artificial) "scaling" (Grassotti & Gimelli, 2011). However, the application of micropropagation has significantly reduced this propagation period and is now widely employed in most breeding programs (Askari et al., 2018). Through *in vitro* techniques, a large number of genetically identical bulblets can be produced from a single bulb within a short timeframe. As a result of the high propagation rates achieved through tissue culture, newly bred cultivars can be introduced to the market within a few years (typically 7-8 years) (Benschop et al., 2010). Consequently, tissue culture plays a crucial role in the rapid expansion of the lily assortment observed in contemporary times (Langens-Gerrits et al., 1997; Langens-Gerrits et al., 2003). The production of bulblets and other storage organs in *in vitro* condition has been observed to exhibit favorable characteristics as propagules. These propagules possess the advantages of being easily controllable, transportable, and storable, thereby eliminating the need for elaborate acclimatization procedures upon their transfer to soil (Askari et al., 2016; Thakur et al., 2006). In the micropropagation process of lilies, bulblets are generated as the final stage (Youssef et al., 2019). The size of the bulblets produced *in vitro* has a significant impact on their performance after planting. Studies have shown that small bulblets exhibit slower emergence, less uniformity, and a lower percentage of sprouting (Askari & Visser, 2022). Therefore, in tissue culture, it is recommended to produce heavy bulblets to ensure better performance after planting (Askari & De Klerk, 2020). Gibberellin is a category of plant hormones that promotes stem elongation, flowering, and germination (Li et al., 2020). These hormones are produced through the terpenoid pathway in plastids and undergo modifications in the endoplasmic reticulum and cytosol to attain their biologically active state (Castro-Camba et al., 2022). In tissue culture, gibberellins are employed to stimulate organogenesis, specifically the formation of adventitious roots (Ahmad et al., 2020; Willy John, 2022). Gibberellins have been discovered to hinder the process of tuberization in potatoes, instead inducing stolon to elongate rather than enlarge. Moreover, they impede the accumulation of starch and the synthesis of proteins that are specific to tubers in potatoes (Vreugdenhil & Sergeeva, 1999). The examination of the influence of inhibitors of gibberellin synthesis (Alar, Cycocel and Paclobutrazol) on the growth and development of oriental lily hybrids has been carried out, and it has been observed that the utilization of these growth retardants leads to enhance bulblet formation (Kumar et al., 2005). On the other hand, the *ex vitro* scaling of *Lilium davidii var. unicolor* was observed to have a beneficial impact due to the application of gibberellin. Scales treated with a concentration of 100 mg/L GA_3 resulted in a greater number of bulblets compared to the control. Additionally, the diameter of the bulblets was increased by GA_3 at both 100 and 150 mg/L (Tang et al., 2020). Additionally according to the findings of Ren et al. (2021), the

levels of endogenous gibberellic acid (GA_3) exhibited a notable rise during the competence stage (0–1 day) of culturing scale explants of Lycoris sprengeri in tissue culture. Subsequently, as the bulblet development progressed, the concentration of GA_3 gradually declined. Furthermore, a research revealed that during the *in vitro* rooting procedures of *Carpinus betulus*, the concentration of endogenous GA_3 reached its highest point in the middle of the rooting phase, whereas the IAA/ABA ratio declined in the middle of the adventitious rooting period (Zhu et al., 2017). In Bougainvillea, also the concentration of endogenous GA increased during the adventitious root induction (Huang et al., 2022). Due to the increasing and decreasing of endogenous gibberellin in different stages of adventitious organ formation (bulblet and adventitious root), as well as the lack of information about the impact of gibberellin on lily bulblet initiation and induction individually, this study was conducted to investigate the effects of gibberellin at two distinct time intervals (a) at the commencement of the experiment (bulblet initiation) and b) after the fifth week of the experiment (bulblet induction). This evaluation aims to determine whether gibberellin negatively affects bulblet initiation or it hampers bulblet induction in lily bulblet growth *in vitro.*

MATERIALS AND METHODS

Standard tissue culture conditions

The present study utilized field-grown bulbs of *Lilium orientalis* L. cv. Santander with a circumference of 18-20 cm. These bulbs were harvested, subjected to cold treatment to break dormancy, and stored at -1.0 °C until further use, as previously described by Askari et al. (2014). Prior to use, scales were surface-sterilized for 30 minutes in 1% (w/v) NaClO and rinsed for 1, 3, and 10 minutes with sterile water, as per the protocol established by Askari and De Klerk (2018).The sterilized scales were then stored in sterile water for an average of 1-2 hours until use. Explants were cut into standard (7×7 mm^2) sizes. The abaxial side of the explants (one explant per container) was placed on the medium in the container (red cap plastic container (2.5×5 cm), which was composed of macro- and microelements (Murashige & Skoog, 1962), 30 g l^{-1} sucrose, 0.4 mg l^{-1} thiamine, 100 mg l^{-1} myo-inositol, 7 g l^{-1} microagar (Fig. 1). The explants were maintained under standard growth conditions, which involved adjusting a temperature of 25°C and a light intensity of 30 µmol m^{-2} sec^{-1} (Philips TL 33) for 16 hours per day (Askari et al., 2022).

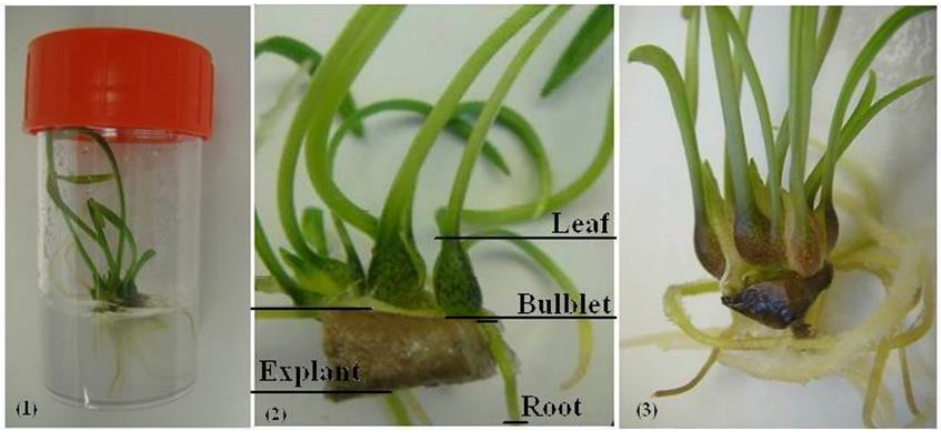

Fig. 1. The in vitro growth of Lily bulblets was observed through the following procedures. (1) Tissue culture containers including 15 ML MS medium, (2) Various Lily organs regenerated on the scale explant, and (3) The growth of Lily bulblets for duration of 11 weeks.

Gibberellins treatments

The experimental design encompassed two factors: 1) varying concentrations of gibberellins, specifically 0, 0.1, and 1 µM gibberellin (GA_3), and 2) two distinct application times, including a) at the commencement of the experiment (bulblet initiation) and b) after the fifth week of the experiment (bulblet induction). The experiment involved three replications and ten replicates for each treatment. To assess the impact of gibberellin on lily bulblet induction, a total of 60 explants were cultured on free hormone Murashige and Skoog medium (MS) (Murashige & Skoog, 1962): containing 30 g L^{-1} sucrose, 0.4 mg L^{-1} thiamine; 100 mg L^{-1} myo-inositol; 7 g L^{-1} microagar; and maintained at a temperature of 25±2 ºC and 30 µmol m^{-2} s^{-1} light (Philips TL 33) for 16 h per day for five weeks. After 5 weeks, the scale explants were subcultured on the same MS medium containing 0.1 and 1 µM gibberellin and incubated at the same condition for further growth. For evaluation of the effect of gibberellin on initiation of lily bulblets *in vitro* the scale explants were cultured on the MS medium (above mentioned) containing 0, 0.1, and 1 µM gibberellin from the beginning of the experiment. In order to eliminate the influence of subculturing on the results of the experiments, the explants that were subjected to gibberellin (0, 0.1, and 1 µM) at the beginning of the experiments were also subcultured simultaneously (at week five) onto the same medium.

Data collection

Following an 11-week growth period, measurements were taken of the fresh weights of bulblets, the number of bulblets per explant, the fresh weight of leaves and roots per explant, as well as the fresh weight of the explant itself. All the fresh weigh measured with analytical balance.

Statictis

The present investigation was executed as a factorial design in accordance with the principles of a completely randomized design (CRD), involving two factors: factors: 1) varying concentrations of gibberellins, specifically 0, 0.1, and 1 µM gibberellin (GA_3), and 2) two distinct application times, including a) at the commencement of the experiment (bulblet initiation) and b) after the fifth week of the experiment (bulblet induction). Data analysis was performed using SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). Specifically, a one-way ANOVA was employed to identify significant differences between individual treatments, with the Duncan test utilized to determine such differences. It should be noted that values sharing the same letter were found to not differ significantly at the 0.05 level. In the figures, the means are shown ± SE.

RESULTS

The impact of gibberellin on the growth of lily bulblets

The findings of this study indicate that the fresh weight of lily bulblets is influenced by varying concentrations of gibberellin, as depicted in Figure 2. Specifically, the addition of gibberellin to the culture medium at the initiation stage resulted in a 26% increase in the fresh weight of the bulblets. In addition, the introduction of gibberellin into the growth medium after the fifth week of the experiment (induction stage) resulted in a significant 91% increase in the fresh weight of the bulblets, particularly when exposed to a concentration of 1 µM gibberellin (Fig. 2). These results show that the timing of gibberellin application significantly impacts bulblet growth *in vitro.* Notably, adding gibberellin at the induction stage led to a 51% improvement in bulblet growth compared to adding gibberellin at the onset of the experiment (initiation stage). The application of gibberellin at the onset of the experiments did

not yield a remarkable impact on the growth of bulblets in 0.1 and 1 µM gibberellin concentration. However, the application of gibberellin after the fifth week alters the growth of lily bulblets, depending on the concentration used. Specifically, a higher concentration of gibberellin (1 µM) led to the production of larger bulblets (94 mg/bulblet) compared to the lower concentration of gibberellin (0.1 µM).

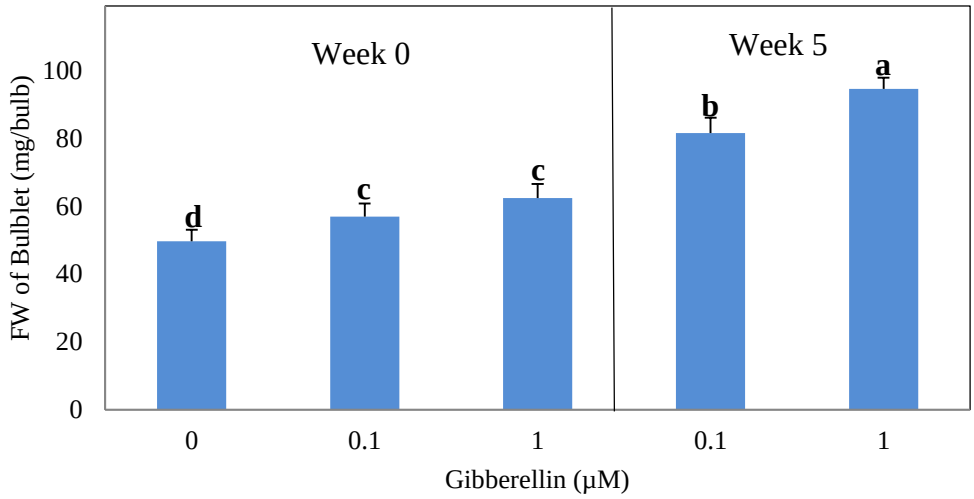

Fig. 2. Effect of different concentrations of gibberellin on FW (fresh weight) of bulblets. The error bar represents the standard error. The presence of letters above the error bars, as determined by Duncan's multiple range test, indicated the differences observed among the treatments (p=0.05).

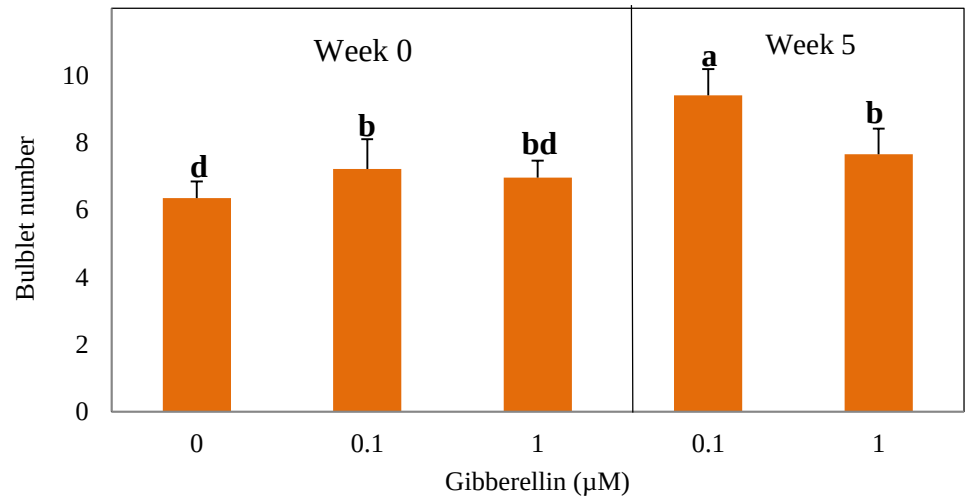

Fig. 3. Effect of different concentration of gibberellin on bulblets number. The error bar represents the standard error. The presence of letters above the error bars, as determined by Duncan's multiple range test, indicated the differences observed among the treatments (p=0.05).

The impact of gibberellin on the number of lily bulblets

Based on the findings presented in Figure 3, it is evident that the number of bulblets regenerated *in vitro* is subject to variation when exposed to different concentrations of gibberellins at two distinct application periods. The initial application of gibberellins at the commencement of the experiments did not yield any noteworthy influence on the number of bulblets regenerated when subjected to a concentration of 1 µM gibberellin. Conversely, the maximum number of bulblets was observed to increase by 50% when exposed to a concentration of 0.1 µM gibberellin, which was introduced into the medium after the fifth week (bulblet induction) (Fig. 3). The findings of this study indicate that a lower concentration of gibberellin is associated with a higher number of lily bulblets *in vitro*, while a higher concentration of gibberellin has a negative impact on lily bulblet number in both application times. Additionally, the application of gibberellin at week 5 demonstrated a positive response to the initiation of bulblets in terms of bulblet number. The findings indicated that the utilization of gibberellin during the initiation phase hindered the initiation of bulblets.

The impact of gibberellin on the growth of lily leaves

According to the data presented in Figure 4, the application of gibberellin at the initiation stage as well as at week 5 (induction stage) resulted in an increase in the fresh weight of lily leaves. However, it was observed that higher concentrations of gibberellin did not have any significant effect on the growth of lily leaves, regardless of the application time. Notably, the greatest increase in fresh weight of leaves was observed when 1 µM gibberellin was applied at week 5, exhibiting a 38% increase compared to the control (Fig. 4). Furthermore, the application of gibberellin at week 5 also led to a 20% increase in the growth of lily leaves compared to its application at the beginning of the culturing period. The findings of this study indicate that the application of gibberellin after the fifth week (induction stage) resulted in a more substantial increase in fresh leaf weight compared to applying the hormone at the beginning of the experiment. Furthermore, in the case of lily leaves, a higher concentration of gibberellin did not have a significant impact on their growth when compared to a lower concentration, regardless of the application time.

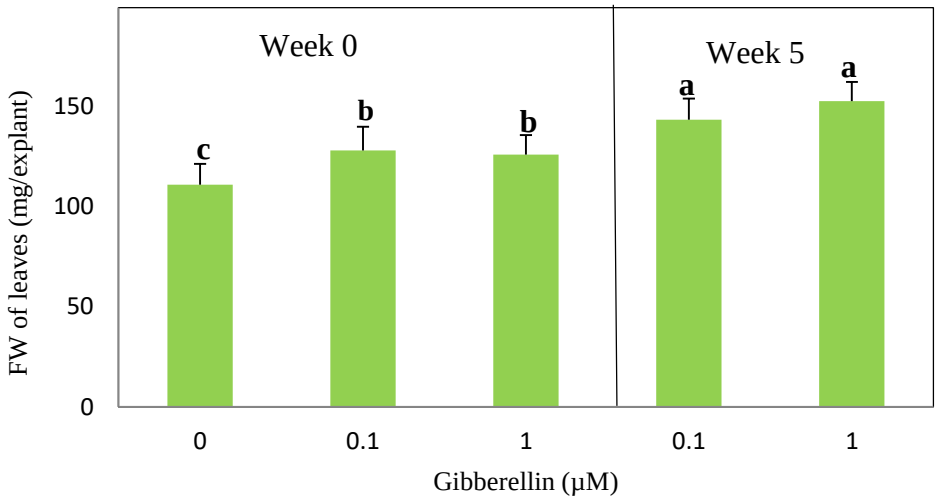

Fig. 4. Effect of different concentrations of gibberellin on FW (fresh weight) of leaves. The error bar represents the standard error. The presence of letters above the error bars, as determined by Duncan's multiple range test, indicated the differences observed among the treatments (p=0.05).

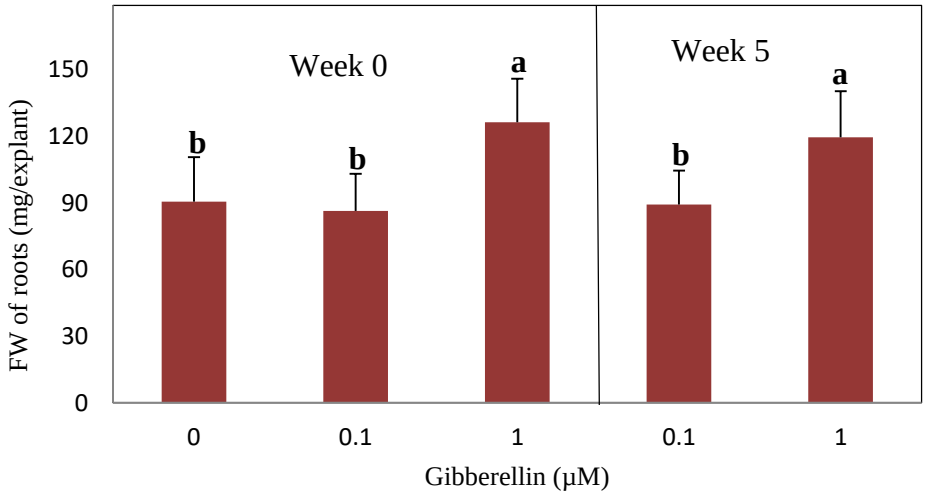

Fig. 5. Effect of different concentrations of gibberellin on FW (fresh weight) of roots. The error bar represents the standard error. The presence of letters above the error bars, as determined by Duncan's multiple range test, indicated the differences observed among the treatments (p=0.05).

Effect of gibberellin on lily root growth

The findings depicted in Figure 5 indicate that the fresh weight of the roots was solely influenced by elevated concentrations of gibberellin in both application periods. Conversely, lower concentrations of gibberellin did not enhance the growth of lily roots in comparison to the control in both application periods. Notably, the application of higher concentration of gibberellin resulted in a 40% and 32% increase in root growth when 1 µM of gibberellin was added at the initiation stage and at the fifth week, respectively (Fig. 5). The findings of these experiments indicate that the application of gibberellin at the onset of the experiment resulted in superior growth of lily roots. Specifically, the highest level of root growth (126.4 mg/explant) was observed when a concentration of 1 µM gibberellin was administered at the beginning of the experiment. It is important to note that there was no significant difference in root growth at a concentration of 1µM gibberellin, when it was applied initially or at week 5.

Effect of gibberellin on lily scale explants growth

The present study observed a reduction in the growth of scale explants under higher concentrations of gibberellin during both application times, as depicted in Figure 6. Conversely, at lower concentrations of gibberellin, no significant difference in the growth of explants was observed in comparison to the control at both application times. The findings of this study demonstrate the adverse impact of gibberellin on the growth of scale explants during lily bulblets regeneration *in vitro* at both application times.

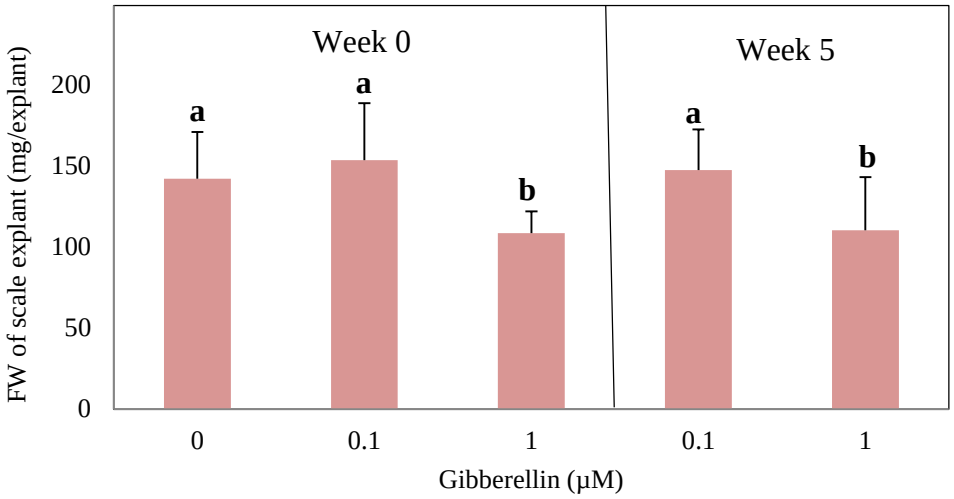

Fig. 6. Effect of different concentrations of gibberellin on FW (fresh weight) of scale explant. The error bar represents the standard error. The presence of letters above the error bars, as determined by Duncan's multiple range test, indicated the differences observed among the treatments (p=0.05).

DISCUSSION

Gibberellin is a phytohormone that plays a crucial role in regulating various developmental processes in plants, such as stem elongation, germination, dormancy, flowering, and enzyme induction (Hedden & Sponsel, 2015). Gibbrellin is a member of a major group of plant hormones that encompass around 136 distinct molecular structures., each with a skeleton of 19-20 carbon atoms (He et al., 2020). Gibberellins act as plant growth regulators by facilitating cell elongation, promoting plant height, and playing significant roles in germination, stem elongation, fruit ripening, and flowering (Shah et al., 2023). Gibberellic acids are primarily employed to stimulate the development of plantlets from adventitious embryos generated in culture (Cai et al., 2022). Nevertheless, their application is limited to certain tissue culture procedures, as they can exhibit inhibitory effects on certain plant species (Atif et al., 2021). Specifically, while gibberellins are crucial for promoting normal callus growth, they can impede organ development, such as root and shoot formation, as well as the process of somatic embryogenesis, in certain plants (Zdravković-Korać et al., 2023). In a study conducted by Le Guen-Le Saos et al. (2002), it was discovered that ancymidol, flurpirimidol, and paclobutrazol, which are known inhibitors of gibberellin biosynthesis, have the ability to stimulate bulb formation and enhance the percentage of bulbing in shallots. Meanwhile, in the context of *Hippeastrum* tissue culture, the presence of flurpirimidol not only influenced the rate of propagation but also had an impact on the size of the newly formed bulblets. More specifically, when explants were cultured in media containing flurprimidol, the bulblets were observed to be larger in size (Ilczuk et al., 2005). The findings of our study demonstrate that the addition of gibberellin to the medium significantly enhanced the growth of lily bulblets (91%), particularly when introduced after a period of 5 weeks (bulblet induction), at a concentration of 1 µM. Conversely, when gibberellin was added to the medium at the onset of the experiment (bulblet initiation), the growth of lily bulblets did not exhibit any noteworthy differences (26%) compared to the control. Furthermore, similar to the growth pattern observed in lily bulblets, both the number of lily bulblets and the fresh weight of leaves displayed comparable trends in response to the application of gibberellin. The

number of bulblets experienced a rise upon the introduction of gibberellin (0.1 µM) to the growth medium subsequent to the induction stage. However, it is noted that a higher concentration of gibberellin exhibited an adverse effect on the number of bulblets in comparison to a lower concentration. This suggests that gibberellin may have an inhibitory impact on the initiation of bulblets, but subsequently does not exert inhibitory effects on the *in vitro* bulblet induction of lily. In contrast, the response of root and scale explants to gibberellin exhibited divergent patterns of growth. Root growth was stimulated by higher concentrations of gibberellin, whereas the growth of scale explants was enhanced by lower concentrations of gibberellin, irrespective of the timing of gibberellin application. The findings of this study suggest that the application of gibberellin at various developmental stages (initiation and induction) can have a diverse impact on the growth of lily organs. Phytohormones appear to fulfill disparate functions when they are present during distinct stages of plant development. For instance, GA_3 is widely acknowledged as an inhibitor of adventitious root (AR) formation in cutting propagation (da Costa et al., 2013). However, a recent investigation on Bougainvillea uncovered that endogenous GA_3 levels experienced an initial decrease during AR development, followed by an increase during the induction and initiation stages, and ultimately a decrease during the expression stage. The researchers postulated that an elevated GA content could impede cell divisions in the early phase of rooting culture, consequently hindering the differentiation and formation of AR. Conversely, GA could enhance the elongation and growth of AR. (Huang et al., 2022). In the case of gladiolus, previous research conducted through *in vitro* studies has demonstrated that the presence of gibberellic acid can either hinder or have no impact on the formation of corms (Dantu & Bhojwani, 1995). Another study conducted on narcissus found that the application of gibberellic acid resulted in a reduction in both the quantity and weight of bulblets produced through twin-scaling (Tang et al., 2020). In the case of lycoris, the introduction of exogenous GA_3 (gibberellic acid) significantly impeded the propagation coefficient and weight of bulbs, with this inhibitory effect becoming more pronounced as the concentration of GA_3 increased (Xu et al., 2021). The inhibitory influence of gibberellin on bulblet formation of various crops has been reported while GA was administered simultaneously during the initiation and induction phases of bulblet regeneration *in vitro*, and there is no available evidence regarding the precise impact of gibberellin indivitually on either the initiation or induction stage. However, the current study presents the initial findings of utilizing gibberellin specifically on either the initiation or induction stage of lily bulblet development. Additional research is necessary to fully understand the effect of gibberellin on the different stages of lily bulblet regeneration *in vitro*.

CONCLUSION

The application of gibberellin to the growth medium in lily bulblet induction stage has been found to enhance the number of initiated bulblets and the growth of lily bulblets *in vitro*. It has been observed that various organs of the lily showed different response to gibberellin concentration. Specifically, higher concentrations of gibberellin have been found to promote the growth of lily bulblets and roots, while leaf growth does not respond positively to higher concentration of gibberellin. Conversely, the growth of scale explants is terminated by higher concentrations of gibberellin. Finally, the application of gibberellin at the initiation and induction stages individually exhibited diverse effects on the regeneration of lily bulblets *in vitro*.

Conflict of interest

The author has no conflict of interest to report**.**

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