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AHS
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Foliar application of potassium on antioxidant enzyme activities of tomato plants under drought stress

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Abstract: Water stress negatively affects productivity in crops, while the foliar application of potassium-containing compounds may be helpful in reducing the drought effects. This study evaluated the efficacy of foliar applied potassium chloride (control - distilled water spray -, 3 and 6 mM^{-1}) on tomato plants under drought stress. Three irrigation levels were maintained at 100, 75 and 50% according to evapotranspiration designated as well watered, moderate and severe drought stressed. Increasing drought stress significantly reduced plant growth and yield. The foliar applied KCl produced maximum leaf area, stem diameter and length, plant yield under each drought stress conditions compared to control. The minimum of growth factors were obtained by control under severe stress. Highest yield per plant was also recorded for foliar applied KCl under moderate condition than other treatments. Foliar applied KCl alone decreased the SOD, CAT and PPO in well-watered condition but KCl application on tomato plants under drought stress induced the antioxidant enzyme activities more than control well-watered treatment.

1. Introduction

The major limitation for plant growth and crop production in arid and semi-arid regions is soil water availability. Plants that are continuously exposed to drought stress can form ROS (Reactive oxygen species), which leads to leaf damage (Foyer *et al.*, 2002; Oerke and Dehne, 2004; Cakmak, 2005) and, ultimately, decreases crop yield. The decrease in soil water potential causes alteration in minerals uptake by plant roots and reduction in leaf expansion under drought stress conditions (Kaminek *et al.*, 1997; Pospisilova *et al.*, 2000).

Drought is becoming a serious threat to crop production worldwide resulting in 67 and 82% reduction in K uptake under mild and severe water stress conditions (Baque *et al.*, 2006). During drought stress, root growth and the rates of K^+ diffusion in the soil towards the roots were

both restricted, thus limiting K acquisition. The resulting lower K concentrations can further depress the plant resistance to drought stress, as well as K absorption, which ultimately leads to reduction in the fruit size, plant yield, lack in red color of tomato and fruit quality (Bidari and Hebsur, 2011; Afzal *et al.*, 2015), and deteriorated photosynthesis, enzyme activation in plants (Marschner, 1995; Garg *et al.*, 2004; Afzal *et al.*, 2015). Maintaining adequate plant K is, therefore, critical for plant drought resistance.

When plants were supplied with different K^+ concentrations and then subjected to drought stress, their stomatal conductance was more markedly reduced in normal K plants than in low K plants (Wang *et al.*, 2013). During drought stress, the stomata cannot function properly in K^+-deficient plants, resulting in greater water loss. Drought stress did not decrease water use efficiency (WUE), whereas it did increase WUE by rapid stomata closing during water deficit (Egilla *et al.*, 2005). Adequate levels of K nutrition enhanced plant drought resistance, water relations, WUE and plant growth under drought conditions (Wang *et al.*, 2013). Besides various adaptive mechanisms; potassium (K) sprayed under drought condition can improve the tolerance of crop plants to various types of abiotic stresses, and it also improved subsequent growth and yield. Mengel and Kirkby (2001) reported that K improves physiological processes by the regulation of turgor pressure and photosynthesis; translocation of cations and enzymes activation, while, Cakmak (2005) also observed that plant suffering from drought stress required more internal K. Yield limiting effect of water deficit could be overcome by increasing K supply (Damon and Rengel, 2007). In legumes, devastating effects of drought can be alleviated by rich K supply (Sangakkara *et al.*, 2000). A close relationship between K nutritional status and plant drought resistance has been demonstrated.

The bottom lines of the reviewed results in this section indicate that under drought stress conditions, yield losses can be minimized by the sufficient supply of K. However, its application effect at tomato growth stages is not well understood yet. The objective of present work was to study the possible role of K applied on tomato plant under drought, in mitigation of stress in terms of physiological components and antioxidant enzyme values.

2. Materials and Methods

This experiment was designed to observe the effect of KCl on tomato (*Solanum lycopersicum* L.) seedlings under drought stress. Seeds of tomato were sown in plastic trays and maintained in a greenhouse up to 4 real leafy stages, at Department of Horticulture, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran. The experimental design was a split plot design with three water stress plot as main plot and 3 KCl treatments (0, 3 and 6 mM^{-1}) as subsidiary plot with 3 replications. The subsidiary plot area was 1 m^2 (1×1 m) and consisted of four plants per plot. After 70 days from sowing, seedlings with uniform growth were transplanted to an experimental field with a 50 cm inter-seedling spacing. According to evapotranspiration (ET_{c} = crop evapotransipiration), the water stress was conducted on tomato plants at three levels well-watered (100% ET_c), moderate and severe drought stress (75 and 50% ET_c, respectively). According to Penman-Monteith equation, the crop evapotranspiration, ETc (Formula 1), is calculated by multiplying the reference crop evapotranspiration, ETo, by a crop coefficient, Kc and ETo (Formula 2) is calculated by Ep and Kp factors.

$$ET_c = K_c\, ET_o \qquad [1]$$

Where ETc= crop evapotranspiration [mm d^{-1}], Kc= crop coefficient [1.15 for tomato], ETo= crop evapotranspiration [mm d^{-1}].

$$ETo = Ep.\ Kp \qquad [2]$$

Where Kp= Pan coefficient (0.77), Ep0 pan evaporation.

The foliar spray was applied five times (during two month) to tomato plants during growth and fruit set with KCl at 0, 3 and 6 mM^{-1} dose.

Growth and plant analysis

Plant height and stem diameter were measured at the end of the harvesting season and presented as cm and mm respectively. The total yield of tomato fruits were measured by gram scales (g) in different harvesting times per plant. The leaf area (LA) measured by Windias (Delta-T Co, England) and presented as (mm^2).

The integrated water-use efficiency (formula 3) is typically defined as the ratio of biomass produced (D, kg h^{-1}) to the rate of total water irrigation (W_i, m^3ha^{-1}) and rainfall (W_p, mm) during the drought stress treat-

ment.

$$WUE = D/(Wp + Wi) \quad [3]$$

Assays of enzymatic and non-enzymatic antioxidants

Fruits were randomly selected from each treatment, at the end of the experiment. Total soluble proteins were quantified by following the protocol devised by Bradford (1976). For determination of enzymatic antioxidants, fruit samples were extracted in 50 mM phosphate buffer (pH 7.8). The extract was centrifuged at 15,000 rpm 4°C and the supernatant was used for further assay of catalase (CAT) Chance and Maehly, 1955 and poly phenol oxidase (PPO) (Siriphanich and Kader, 1985) and super oxide dismutase (SOD) activities (Giannopolitis and Ries, 1977). Tomato juice was squeezed from the fresh tomatoes onto a digital refractometer (PR-100, Atago Co. Ltd., Tokyo, Japan) to measure total soluble solids (TSS) and the results were expressed in Brix according to AOAC method 932.12 (2005). For measured the proline content, leaves were randomly selected and experimented according to Bates *et al.* (1973) method.

Statistical analysis

Effects of water stress treatments; KCl and corresponding interactions were determined by analysis of variance according to the general linear model procedure of SAS (version 8.2; SAS Institute, Cary, N.C.). Means were compared using Least Significant Difference (*LSD, p≤0.05*) according to method described by Tukey HSD. Analysis showed a significant interaction between KCl and watering treatment for the some measured parameters. The graphs draw by using excels 2010 software.

3. Results

Increasing drought stress (DS) levels significantly reduced plant growth and yield, but foliar applications of KCl improved the harmful effects of drought stress.

Comparison of means indicate that leaf area (LA) was gradually decreased with increasing drought stress (Table 1). The minimum leaf area was obtained under extreme drought conditions (50% ETc). Water stress decreased LA but exogenous application of KCl ameliorated the negative effects of water stress significantly than water spray only (Fig. 1a). However, highest LA in each treatment was recorded for foliar applied KCl than control under well-watered, moderate and severe drought stress. A minimum LA was recorded under severe drought stress, especially control × 50% ETc treatment (Fig. 1a).

The increasing water stress decreased stem length (Fig. 1b). A maximum stem length was observed under well-watered conditions by exogenous application of 6 mM^{-1}KCl while in moderate and severe drought, foliar applied KCl had no significant effects. Minimum stem length was noted under extreme water stress without foliar KCl application. Stem diameter decrease in response to DS (Table 1). Foliar KCl application helps to plant tolerance with increasing stem diameter under DS condition. Data showed (Fig. 1c) the increasing in KCl doses (0 up to 6 mM^{-1}) lead to increasing in stem diameter in all of treatments.

The tomato yield displayed a significant reduction in response to the increasing levels of water deficit treatments. For example, under effect of 50% ETc condition, the yield decreased by 21% (Table 1) compared to control. In the other hand, plants had more vegetative growth and less yield in well-watered condition (100% ETc) than moderate DS (75% ETc). In non-DS condition, foliar spray of KCl showed similar fruit yield under well-watered, moderate and severe drought conditions. But foliar KCl application had effective enhancement on plant yield under drought stress (Fig. 1d). Tomatoes irrigated with 50% ETc without KCl foliar application also produced fruits with significantly higher juice brix value than other

Table 1 - Effects of different drought stress levels on tomato plants

Drought	LA (mm^2)	St. Length (cm)	St. Diameter (mm)	WUE	Yield plant $^{-1}$	Brix ^{0}	Proline (µg/g.fw)	Protein (mg g^{-1} fw)	SOD (IU mg^{-1} min^{-1} protein)	CAT (IU mg^{-1} min^{-1} protein)	PPO (IU mg^{-1} min^{-1} protein)
Control	7555.156 a	52.66 a	14.69 a	4.360 b	1225.861 a	7.00 b	6734.877 c	1.405 a	967.28 a	178.35 c	80.777 b
75% ETc	5464.41 b	47.44 b	14.41 a	6.187 a	1404.72 a	7.11 b	9468.355 a	1.540 a	1240.31 a	196.63 b	142.66 b
50% ETc	3294.32 c	42.66 c	11.82 b	2.462 c	963.62 b	7.722 a	8161.222 b	1.382 a	1133.79 a	207.83 ab	258.11 a

Means in each column followed by similar letters are not significantly different at 0.05 probability level, using LSD (Least Significant Difference) test.

treatments (Fig. 1e).

The results in figure 1 (f) revealed that DS on tomato had higher significantly record in water use efficiency than those normally irrigated. Because of fewer yield in control treatment than 75% ETc, the results affirmed that the treatment 75% ETc and then control showed the highest water use efficiency than 50% ETc treatments. Foliar application of KCl had no significant effects of WUE under non-DS condition. Finally, the foliar application of KCl × 75% ETc showed the highest significant record in WUE in plants subjected to DS, respectively. Meanwhile, the treatment 6 mM^{-1} KCl × 75% ETc recorded the highest significant WUE compared to other studied treatments under well-watered and sever DS.

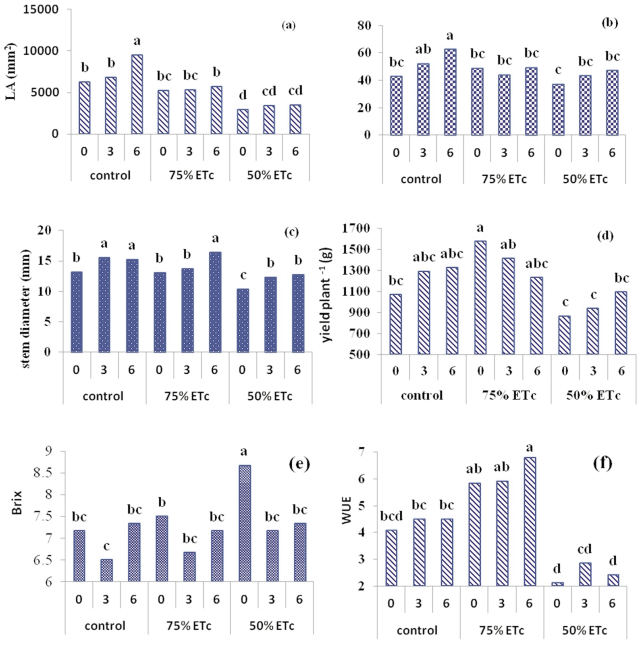

Fig. 1 - Effect of exogenous application of different KCL level on leaf area (a), stem length (b), stem diameter (c), yield (d), Brix (e), and WUE (f) of tomato under drought conditions. (LSD, p≤0.05).

Leaf proline concentration responded differently to drought and K supply. Drought stress (Table 1) increased proline concentration in leaves but exogenous KCl decreased the proline content under none DS conditions (Table 2). The tomato leaves were sprayed with KCl had more proline content under drought stress than control conditions (Fig. 2 a). At 0 mM^{-1}KCl level with no DS, the proline content in leaves was 1168.4 µM/g F.wt. which increased to 13368.5 and 12111.5 µM/g F.wt. with increasing DS stress in 50% ETc with 3 and 6 mM^{-1} KCl foliar application, respectively.

Increase in KCl doses enhanced the total soluble proteins in tomato fruits (Table 2). Maximum fruit protein content was evident at moderate stress with 3 mM^{-1}KCl followed by 0 mM^{-1}KCl with minimum value under well-watered conditions (Fig. 2b). Foliar spray with KCl had impressive effects on protein contents under well-watered and drought stress. However, the exogenous application of KCl performed better than control for fruit total soluble protein.

A rise in drought stress also amplified the antioxidants status of tomato fruits (Fig. 2c, d, e). Results showed that the enzymatic antioxidant contents in tomato fruits were sprayed by KCl, were decreased. So, the maximum SOD with no significant differences were recorded for foliar applied 0 and 3 mM^{-1}KCl under severe drought and 0 mM^{-1}KCl under moderate conditions (Fig. 2c). Increased CAT activities with no significant differences were found under medium and severe drought stress (Fig. 2d). Decreasing water level increased PPO contents for all treatments (Fig. 2e) and the highest value of PPO was obtained under sever DS but increasing in foliar application of KCl doses from 0 to 6 ($m\mu^{-1}$) caused to decrease the PPO fruit content (Fig. 2e).

Table 2 - Foliar application effects of different KCl levels on tomato plants

KCl	LA (mm^2)	St. Length (cm)	St. Diameter (mm)	WUE	Yield plant $^{-1}$	Brix ^{0}	Proline (µg/g fw)	Protein (mg g^{-1} fw)	SOD (IU mg^{-1} min^{-1} protein)	CAT (IU mg^{-1} min^{-1} protein)	PPO (IU mg^{-1} min^{-1} protein)
Control	4836.40 b	43.11 b	12.23 c	4.014 a	1168.46 a	7.777 a	8953.16 a	1.286 b	1539.26 a	230.56 a	167.44 ab
3	5197.64 b	46.55 b	13.88 b	4.426 a	1211.41 a	6.777 ab	7590.68 c	1.562 a	761.61 b	196.29 ab	204.66 a
6	6279.84 a	53.11 a	14.81 a	4.014 a	1214.32 a	7.277 b	7820.60 b	1.479 ab	1040.52 ab	155.96 b	109.44 b

Means in each column followed by similar letters are not significantly different at 0.05 probability level, using LSD (Least Significant Difference) test.

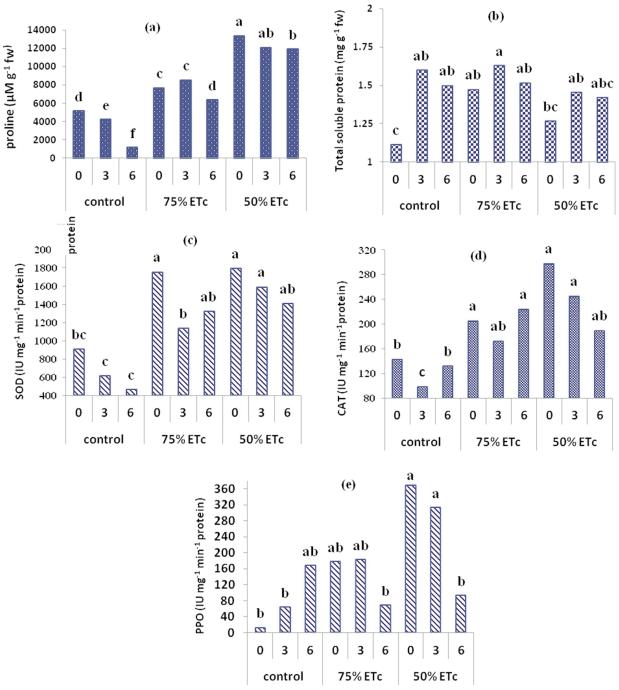

Fig. 2 - Effect of exogenous application of different KCl levels on enzymatic and non-enzymatic antioxidants; proline (a), total soluble protein (b), superoxide dismutase (c), catalase (d) and polyphenol oxidase (e) of tomato fruits under drought conditions. (LSD, $p \leq 0.05$).

4. Discussion and Conclusions

Increasing water stress has direct impact on crop growth and yield reduction and similar observations for reduction in growth and fruit yield of tomato were found in present study. Reduction in tomato yield under restricted water availability might have been due to reduction in photosynthetic area such as leaf area and leaf number (Chaves *et al.*, 2011; Khan *et al.*, 2015). In the present study, yield, leaf area and stem length were drastically reduced due to drought effect, whilst KCl foliar application improved these characters in tomato plants.

It has been reported that the foliar application of KCl improved the growth factors in agronomy crops (Ahmad and Jabeen, 2005; Yasmeen *et al.*, 2013). Besides adaptation role, positive effect of KCl on protein contents was also pronounced under drought stress. Proline is a well-known amino acid that generally accumulates when plants are exposed to environmental stresses (Kavi-Kishor *et al.*, 2005). Enhanced proline synthesis is a common response of tomato plants to drought and may determine the stress tolerance (Doan and Maurel, 2005; Khan *et al.*, 2015). Proline is believed to acts as a signaling molecule that initiates adaptation to the stress (Maggio *et al.*, 2002), acts as osmolyte for osmotic adjustment (Hayat *et al.*, 2012), helps in stabilizing membranes/proteins and scavenges free radicals (Ashraf and Foolad, 2007). Thus, it decreases the adverse effects of cytoplasmic acidosis and maintains proper $NADP^+$ /NADPH ratios (Liang *et al.*, 2013). In plants grown under drought conditions, proline induces the expression of drought stress responsive genes and, thus, decreases the damage due to excessive Na^+ ions accumulation (Chinnusamy *et al.*, 2005). Proline act as a compatible solute in the plants (Mansour, 2000) and, generally, increases with increase in both the salinity stress and drought stress duration (Kishor and Sreenivasulu, 2014). Thus, it is likely to observe enhanced proline synthesis with increasing drought stress duration. Antioxidant enzymes activities were considered as indicators of scavenging ROS and reducing oxidative stress (Dionisio-Sese and Tobita, 1998; Lin and Kao, 2002). For example, SOD may convert superoxide radicals into H_2O_2 and H_2O_2 was further decomposed by CAT and POD (Redman *et al.*, 2011). In this study, drought stress significantly increased the antioxidant enzymes activities. The results of many researchers' studies proved that adequate external K supply significantly decreased antioxidant enzymes activities and proline in drought stressed plants might be caused by enhancing plant physiological metabolism and reducing ROS production and MDA content (Wei *et al.*, 2013; Yasmeen *et al.*, 2013; Bahrami-Rad and Hajiboland, 2017). In the other hand, the combination of drought stress and exogenous KCl application improved the antioxidant activities and proline content than well-watered conditions. So, rise in drought stress with exogenous application of KCl induce tolerance in crops (Yasmeen *et al.*, 2013). A similar trend was followed by all the treatments in case of antioxidant enzyme activities under each water stress treatment.

The results of this study clearly demonstrated that water deficit at any critical crop growth stage severely affected the physiological and antioxidant and non-antioxidant parameters of tomato. Foliar application of K on a drought stressed plants at all growth stages improved the physiological performance and plant tolerance but reduced antioxidant enzyme activities. All these findings lead us to recommend that for tomato crop under drought farmers should spray the crop with 6 ($m\mu^{-1}$) KCl to minimize the negative effect of drought. This can have a dual benefit:

improving the physiological performance of tomato and supplying K nutrient to plants. For the foliar spray on a small scale, a common hand-boom sprayer can easily be used, whereas on large scale use of a mechanical boom-sprayer is advised.

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AHS
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Improving 'Piyarom' date palm fruit quality with fruit thinning and bunch covering treatments

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Abstract: 'Piyarom' dates are one of the most important commercial date cultivars grown in Iran but fruit quality and postharvest losses can be a problem. Thinning and the use of bunch covers were examined to improve the date fruit quality. Three different levels of fruit thinning were applied (without thinning, removal of one third of total strands from terminal tips during pollination and removal of one third of terminal tips of central strands in early kimri). In addition, the effects of bunch covers were assessed with the use of polyethylene bunch covers that were applied in the early stages of Khalal. The results showed that bunch covering reduced the lightness of the fruit but increased bunch weight, fruit length, total antioxidant activity and total phenolic compounds compared to the control. Thinning during pollination increased the weight of the fruit compared to the control. The thinning during Kimri stage increased the percentage of Tamar and ascorbic acid content. These results show that the use of covers play a positive role in increasing quality and bunch weight and thinning at both pollination and Kimri ripening stage is recommended to produce high quality 'Piyarom' dates.

1. Introduction

Date palms (*Phoenix dactylifera* L.) are an ancient horticultural crop cultivated for its sweet fruit which are also rich in nutrients, antioxidants and phenolic compounds (Hussain *et al.,* 2016). Iran is one of the largest producing countries of dates growing 171,647 hectares of date palms with an annual production of 1,204,158 tons (FAO, 2018).

Date fruit has five stages of growth and ripening; (1) Hababouk (cell division and elongation), (2) Kimri (firm full colored), (3) Khalal (physiologically mature with a hard and crisp texture and a moisture content between 50-85%), (4) Rutab (partially browned, reduced moisture content 30-45%, fibres softened, perishable), and (5) Tamar (fruit colour from amber to dark brown with a moisture content further reduced - below 25% down to 10% and less, and the texture is soft pliable to firm to hard)

(Awad and Al-Qurashi, 2012). Date palm (*Phoenix dactylifera* L.) cv. Piyarom is one of the most important commercial semi-dry dates in Iran which is consumed at tamar ripening stage and fruit reaches full maturity. It is widely grown in Hormozgan province of Iran with increasing export production. In commercial production, larger fruit with higher quality are more marketable and therefore fruit thinning is one of the main agronomic methods to improve fruit size and quality. Fruit thinning is widely used in horticultural production which increases fruit quality, reduces bi-annual bearing and ensures a physiological balance between vegetative and reproductive parts due to reduced competition for water and food absorption (Slatnar *et al.,* 2020). There are different times and methods for date palm thinning. For example, thinning 1/3 of the bunch at pollination period or 1/3 terminal tips of central strands in early kimri stage of fruit development, as has been described by El-Badawy *et al.* (2018) and Moustafa *et al.* (2019). However some date palm cultivars can react contrarily to the different treatments of thinning for example, Awad and Al-Qurashi (2015) reported that thinning 5-10 cm bunch four weeks after pollination in 'Barhee' date did not affect the qualitative characters of fruit. However, Ahmed *et al.* (2019) showed that thinning 1/3 terminal tips of central strands in early Kimri stage of fruit development of 'Zaghlool' decreased titrable acidity and increased soluble solid content. However there has been no research on the effects of different thinning options on the quality of 'Piyarom' date where most farmers in southern Iran currently use bunch thinning only at pollination period.

Pre-harvest covering of fruit is practiced in many crops and its usage is increasing in many countries to improve fruit quality. However, the effects of fruit covering on final fruit quality is variable and reflect differences in the covering type, fruit age at covering and cultivar response (Sharma *et al.,* 2014). For example Kassem *et al.* (2010) stated that 'Zaghlool' dates covering with dark polyethylene at kimri stage did not affect total acidity (TA) and total soluble solid content (TSS). However, Awad and Al-Qurashi (2012) concluded that bunch craft bagging after pollination increased bunch weight, TA and TSS, ascorbic acid and decreased total phenols and Rutab percentage of 'Barhee' date. Harhash and Al-Obeed (2010) showed that bunch bagging increased fruit and bunch weight and quality of 'Succary' and 'Khalas' dates. Harhash *et al.* (2020) further showed that bunch weight was greatly increased by covering bunches with white paper and white and brown cloth as compared to control. Fruit weight was increased remarkably by using white or kraft paper and white or brown cloth in covering bunches of 'Barhee' date palm while TSS was increased in the fruit which were produced from bunch covering with white or kraft paper and white or brown cloth over control.

Due to its high consumer appeal, 'Piyarom' date is a promising cultivar for export but a more consistent high fruit quality is required. Therefore, this study assessed the effects of both date fruit thinning and the use of bunch covers on the bunch weight and quality of 'Piyarom' dates over two seasons.

2. Materials and Methods

Experimental procedure

Fruits selected for this trial were from 'Piyarom' date palms of the same age (18 years old) and size which were grown on sandy loam soil and drip irrigated at the Hajiabad Agricultural Research Station in Hormozgan province in Iran for two years during 2018-2019. All regular cultural practices were conducted according to the normal commercial schedules. Pollination was conducted using a local 'Green' male cultivar to manually pollinate the female flowers in April each year with a total of eight 8 bunches were left on each experimental tree.

This trial was a factorial trial with fruit thinning and bunch covers as the two factors. Within the fruit thinning treatment, three levels of thinning were examined; (1) removal of one third of the end of total strands from terminal tips of bunch at the time of pollination, (2) removal of one third of terminal tips of central strands in early Kimri (performed in June), and (3) untreated flowers (control). The second factor of experiment was bunch covering at two levels (uncovered bunches and bunch covering with polyethylene mesh which consisted on a 100 cm long and 80 cm wide bag with mesh holes (diameter of each hole is about 2 mm) which were applied in early stage of Khalal. The thinning treatments and bunch coverings were performed on four bunches in each palm tree, and fruit sampling of these bunches was performed at harvest. In November on each tree, all bunches were harvested and bunch weight per palm was recorded. The Tamar percentage was also calculated. Fruit samples were collected at the Tamar stage for physicochemical characteristics measure-

ments.

Physico-chemical characters

At harvest time (Tamar stage), 100 fruit subsamples from each replicate were sampled from palm trees and the factors related to the colour of the fruit measured by Minolta (CR-300, Minolta Corp, Osaka, Japan) colorimeter and expressed as L*, C* and h°.

Fruit weight, length and diameter of each fruit and seed were measured on 25 fruit per replicate. Fruit and seed length and diameter were measured by a digital caliper and the weight of the fruit and seed were measured by a digital scale. Fruit total soluble solid content (TSS), ascorbic acid, moisture content, total phenolic compounds and total antioxidant activity were measured on a composite sample of 20 fruit per replicate. TSS was measured as Brix % with a digital refractometer (DBR95, Taiwan). The spectrophotometric method was used for measuring ascorbic acid content (Etemadipoor *et al.,* 2019). Ten ml of distilled water used for homogenizing one gram of fruit tissue. Then, the solution filtered and 0.1 ml of it was added to 1% metaphosphoric acid. Then, solution combined with 9 ml of 2, 6-dichloro indophenol (0.0025%). After that, the mixture absorbance was evaluated by spectrophotometer (Cecil, CE2501, England) at 517 nm. The results were reported accordingly (mg/100 g fresh fruit tissue). For moisture content 20 g of fruit sample from each replicate was cut into small pieces by a sterilized knife. Then, the fresh weight of each sample was measured. Then, dried to a constant weight in oven at 70°C. Dry weight was measured and fruit moisture expressed as percentage (Marzouk and Kassem, 2011).

Total antioxidant activity (TAA), total phenolic content (TPC)

Measuring TAA involved adding 1 ml 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) (0.1 mM) to 0.1 ml methanolic extract. This solution was subsequently mixed with 1 ml of Tris-HCl (pH 7.5). The absorption value of each sample was determined at 517 nm wavelength via spectrophotometer (Cecil, CE2501, England) (Etemadipoor *et al.,* 2020). The antioxidant activity was determined as follows:

$$\text{Antioxidant activity (\%)} = [1 - (\text{Abs sample} / \text{Abs control}) \times 100]$$

The Folin-Ciocalteu reagent was used for measuring TPC (Ehteshami *et al.,* 2019). Briefly, fruit tissue (0.5 g) was squashed and mixed into methanol (3 ml, 85%). The resultant solution was centrifuged at 12,000 × *g* at 4°C for 20 min. The extract was considered as supernatant. Then, 300 µl of extract was combined with sodium carbonate (1.2 ml, 7%). This mixture was stored at ambient temperature for 5 min. After that Folin-Ciocalteu (10%, 1.5 ml) was added. The absorbance was determined at 760 nm and TPC was presented as mg GAE 100 g^{-1} FW.

Statistical analysis of data

The experimental was as factorial in a randomized complete block design with three replicates (block) and each replicate consisted of two palm tree. Two factors included bunch thinning and bunch covering. Bunch thinning consisted of 3 levels and bunch covering consisted of 2 levels. The treatment unit consisted of four bunches on each of the two tree treatment blocks. Data were analyzed by Statistical Analysis System version 9.1 (SAS Institute Inc., Cary, NC, USA). The means were compared using LSD at a significance level of p= 0.05.

3. Results and Discussion

Bunch weight and Tamar percentage

The results of this experiment showed that the coverage did not have a significant effect on the percentage of Tamar, but date palm fruit thinning in the Kimri and pollination stages significantly increased Tamar percentage of both years (Table 1). The Tamar percentage was approximately 94.25-113.75% higher than the control group and occurred as a result of thinning in the Kimri stage during both years of experiment. Also, the bunch weight was significantly higher in the control as compared to bunch weights that occurred after date palm fruit thinning at the pollination and Kimri stages, for both years (Table 1). In this research, the Tamar percentage was not affected by covering. In the available literature, there are inconsistent results on the use of covering for fruit ripening. For example, Awad and Al-Qurashi (2012) stated that bunch bagging of 'Barhee' date can eventually reduce the percentage of Rutab in comparison with the control. However, Kassem *et al.* (2011) stated that spathe-bagging increased fruit ripening in 'Zaghlool' date, as compared to the control. Tamar is the last stage of 'Piyarom' date fruit maturity. However, date fruit do not ripen evenly, and even in a bunch Rutab and Khalal can be observed at harvest. This causes economic loss of fruit. Therefore, increasing Tamar percentage in the bunch can rise the commercial value of product. The

Table 1 - Main effects of bunch date palm fruit thinning and covering on bunch weight and Tamar percentage of 'Piyarom' date

Treatment	Bunch weight (kg)	Tamar (%)
2018		
Thinning		
Control	15.49 a[z]	29.16 c
Removal of one third of total strands from terminal tips during pollination	13.44 b	49.33 b
Removal of one third of terminal tips of central strands in early Kimri	11.37 c	62.33 a
LSD	4.89	3.82
Bunch covering		
Control	13.09 b	47.66 a
Bunch covering	13.77 a	46.22 a
LSD	3.99	3.12
Thinning x bunch covering	NS[y]	NS
2019		
Thinning		
Control	15.51 a	32.00 c
Removal of one third of total strands from terminal tips during pollination	14.05 b	45.66 b
Removal of one third of terminal tips of central strands in early Kimri	11.00 c	62.16 a
LSD	3.23	5.10
Bunch covering		
Control	13.29 b	46.33 a
Bunch covering	13.75 a	46.88 a
LSD	2.64	4.16
Thinning x bunch covering	NS	NS

[z] Means within each column for each treatment followed by the same letter are not significantly different at P=0.05.

[y] NS= non significant.

increase in the percentage of Tamar, as a result of the thinning treatment, can be due to a lowered level of competition between fruit for the absorption of water and nutrients, thereby accelerating fruit maturity and increasing the percentage of Tamar (Radwan, 2017). The results of this study on increasing bunch weight of the control, as compared to the date palm fruit thinning treatment, are consistent with previous results (Moustafa *et al.,* 2019) on the Khadravi cultivar. However, the primary goal is to determine an optimum method of date palm fruit thinning so as to improve fruit quality, obtain a reasonable bunch weight and thus enhance marketability.

TAA, TPC

The antioxidant properties of date fruit differ depending on the amount of phenolic compounds in the fruit (Hussain *et al.,* 2016). Phenolic compounds account for most of the antioxidant properties of dates. They exhibit a range of biological effects such as the prevention of nucleic acid damage. There has been growing interest in the topic of antioxidants, regarding the ability of antioxidants to scavenge free radicals associated with various diseases (Aleid, 2014). Thus, dates can be used as antioxidative functional food ingredients (Aleid, 2014). Fruit of different date palm cultivars have different total phenolic contents and antioxidant activities (Al-Turki *et al.,* 2010).

In the current study, comparison of the main effects of date palm fruit thinning on total antioxidant activity (TAA) showed that date palm fruit thinning during the Kimri phase increased the TAA as compared to the control (Table 2). Also, date palm fruit thinning during the Kimri phase increased the TPC as compared to the control for both years. Moreover, bunch covering increased TAA and TPC

Table 2 - Effect of fresh organic amendments on nematode population, gall number and galling index

Treatments	TAA (%)	TPC (mg GAE 100 g fw)
2018		
Thinning		
Control	49.66 c[z]	130.16 b
Removal of one third of total strands from terminal tips during pollination	67.26 b	135.45 ab
Removal of one third of terminal tips of central strands in early Kimri	70.43 a	138.21 a
LSD	2.89	5.73
Bunch covering		
Control	58.73 b	131.23 b
Bunch covering	66.17 a	137.98 a
LSD	2.36	4.68
Thinning x bunch covering	NS[y]	NS
2019		
Thinning		
Control	48.18 c	126.51 b
Removal of one third of total strands from terminal tips during pollination	67.83 b	141.16 a
Removal of one third of terminal tips of central strands in early Kimri	71.10 a	140.06 a
LSD	2.21	4.15
Bunch covering		
Control	57.31 b	134.28 b
Bunch covering	67.43 a	138.21 a
LSD	1.80	3.39
Thinning x bunch covering	NS	NS

[z] Means within each column for each treatment followed by the same letter are not significantly different at P=0.05.

[y] NS= non significant.

compared to the control for both years (Table 2). Little research exists on the effects of bunch thinning and covering on TAA and TPC of date. Hussain *et al.* (2016) stated that thinning treatments of 'Hillawi' and 'Khadrawi' increased TPC in comparison with the control, probably because of a greater light exposure to the fruit in response to less fruit density in each bunch. Indeed, light plays an important role in processes that are responsible for the accumulation of phenolic compounds. On the other hand, in fruit of the control group, there were lower amounts of light and air circulation available to the fruit, thereby affecting the rate of photosynthetic carbon assimilation rate and TPC (Hussain *et al.*, 2016). The contradictory effects of covering on phenolic-compound might be due to differences in the covering material, exact time and period of covering, cultivars and climatic conditions (Sharma and Sanikommu, 2018). Chen *et al.* (2012) stated that fruit bagging reduced the concentration of phenolic compounds in 'Golden Delicious' apple, whereas Griñán *et al.* (2019) reported that pomegranate fruit bagging increased the antioxidant content.

Physiochemical characteristics

Comparison of main effects of coverage on color lightness, chroma and hue showed that coverage significantly reduced the lightness and chroma, while at the same time increased the hue in comparison with the control for both years (Table 3). Also, the comparison of main effects of thinning on lightness, chroma and hue showed that thinning in pollination and the early Kimri stage significantly reduced the lightness and chroma, compared to the control. In addition, this treatment increased the hue in fruit, compared to the control for both years (Table 3). The colour of date fruit is one of the most important factors that can largely influence customers and can determine prices. So far, the available literature does not include measurements of lightness, chroma and hue when bunch covering and thinning are used as treatments for date palm. The positive correlation

Table 3 - Main effects of bunch date palm fruit thinning and covering on colour, TSS, ascorbic acid and moisture of 'Piyarom' date

Treatment	L (*)	c (*)	H (◦)	TSS Brix (%)	Ascorbic acid (mg 100 g^{-1})	Moisture (%)
2018						
Thinning						
Control	30.31 a [z]	11.99 a	40.83 c	61.11 b	6.06 c	12.13 a
Removal of one third of total strands from terminal tips during pollination	25.66 b	9.45 b	46.98 b	63.11 b	11.25 b	11.25 a
Removal of one third of terminal tips of central strands in early Kimri	22.58 c	7.12 c	51.80 a	66.05 a	13.46 a	11.86 a
LSD	2.19	1.49	2.60	2.41	1.11	1.19
Bunch covering						
Control	27.50 a	10.19 a	45.21 b	63.01 a	10.68 a	11.95 a
Bunch covering	24.84 b	8.85 b	47.86 a	63.84 a	9.83 a	11.54 a
LSD	1.79	1.22	2.13	1.97	0.90	0.97
Thinning x bunch covering	NS [y]	NS	NS	NS	NS	NS
2019						
Thinning						
Control	28.75 a	11.14 a	44.71 c	62.01 c	6.81 c	10.76 a
Removal of one third of total strands from terminal tips during pollination	24.06 b	8.05 b	50.30 b	64.88 b	12.13 b	11.60 a
Removal of one third of terminal tips of central strands in early Kimri	21.90 b	6.36 c	55.73 a	67.45 a	13.66 a	10.41 a
LSD	2.41	1.25	3.34	2.26	1.28	1.61
Bunch covering						
Control	25.90 a	9.17 a	47.87 b	64.96 a	11.34 a	11.09 a
Bunch covering	23.91 b	7.86 b	52.62 a	64.60 a	10.40 a	10.75 a
LSD	1.96	1.02	2.72	1.85	1.04	1.31
Thinning x bunch covering	NS	NS	NS	NS	NS	NS

[z] Means within each column for each treatment followed by the same letter are not significantly different at P=0.05.

[y] NS= non significant.

between TAA and hue has been shown in table 4. Based on our results, bunch covering increased TAA which can be effective in improving the colour of the fruit (Siddiq *et al.,* 2013). The mechanism involves the formation of dark pigments on fruit, thereby reducing the brightness of the fruit and increasing hue in fruit (Siddiq *et al.,* 2013). On similar accounts, covering has reportedly improved the colour of apple (Sharma and Pal, 2012). A more pronounced fruit colouring is one of the most important goals of thinning. The increase in fruit colour, as a result of the date palm fruit thinning treatment, is due to the absorption of more nutrients in the remaining fruit and also due to the release of more sugars that lead to antioxidant compounds and dark pigments in fruit (Hussain *et al.,* 2016). These results are comparable with previous results reported by Ahmed *et al.* (2019) where date palm fruit thinning increased the colour index in 'Zaghlool' date fruit. The results of statistical compound analysis showed that bunch covering did not affect significantly on soluble solid content (Table 3). Comparison of mean values of the main effects of thinning on TSS showed that thinning in the early Kimri caused a significant increase in TSS compared to thinning at the pollination stage and in the control group (Table 3). With the softening of the fruit, astringency is lost and TSS increases, thereby sweetening the fruit (Serrano *et al.,* 2001). The increase in TSS due to thinning usually occurs because of the fact that fruit use more leaf area for the production of photosynthetic materials, soluble carbohydrates and soluble solids as the fruit ripens (Moustafa *et al.,* 2019). The main effects of bunch covering on ascorbic acid content showed that bunch covering did not affect this variable compared to the control. Moreover, comparison of the mean values of the main effects of date palm fruit thinning on ascorbic acid content showed that date palm fruit thinning in the early Kimri caused a significant increase in ascorbic acid compared to the control and also compared to thinning at the pollination stage (Table 3). Moreover, Ahmed *et al.* (2019) reported that date palm fruit thinning increased ascorbic acid in "Zaghlool" dates. Also, Awad and Al-Qurashi (2012) reported that bagging 'Barhee' dates increased ascorbic acid content. However, the mechanism by which bunch covering affects ascorbic acid is not clear. The main effects of bunch date palm fruit thinning and covering on the percentage of fruit moisture were not significant (Table 3). The percentage of fruit moisture is usually affected by many environmental factors and tree management. In some cultivars, thinning of dates reduces the percentage of moisture in the 'Saidy' date palms fruit (Samouni *et al.,* 2016). However Moustafa *et al.* (2019) showed that date palm fruit thinning does not affect the moisture content of 'Khadrawi' date fruit.

The results of statistical compound analysis showed significant effects of bunch date palm fruit thinning on fruit length, fruit weight and seed length. Comparison of mean values on the main effects of covering bunches revealed that fruit length increased as compared to the control for both years (Table 5). Mean comparison of the main effects showed that date palm fruit thinning caused a significant increase in the length and weight of fruit in comparison with the control for both years. Also, date palm fruit thinning at the time of pollination significantly increased the diameter of fruit and the length of the seeds compared to the control and thinning in the early Kimri stage for both years. Thinning did not affect the weight and diameter of the seeds for both years (Table 5). The effects of coverage on different fruit have been investigated. However, there are contradictory reports on the effects of covering on fruit size. For example, in mango, covering increased the size of the fruit (Chonhenchob *et al.,* 2011). In another study, bagging reduced fruit size in bananas (Hasan *et al.,* 2001). Fruit weight and size usually affect the marketing of dates. Larger sizes have better marketability (Al-Qurashi and Awad, 2011). Thinning can be an important treatment in the orchard which improves the quantitative and qualitative properties of fruit for export. In dates, date palm fruit thinning can be performed until the middle of the Kimri phase. Growth occurs during the cell division and enlargement stages. Cell division occurs after fertilization and continues until the end of the

Table 4 - Pearson correlation between TAA, lightness, chroma and hue

Correlation	TAA	Lightness	Chroma	hue
TAA	1	-0.88**	-0.84**	0.82**

**= Correlation is significant at the 0.01 level.

Kimri phase. Then, cellular growth ensues. A proper distribution of nutrients during cell division and enlargement increases the size of the fruit and causes better marketability. In the process of fruit growth and development, there is competition for water and nutrients. Thinning reduces competition between fruit for their absorption of water and nutrients, while providing adequate conditions for growth. This reduces the number of fruit and increases the length of the fruit. These results agree with the results of Ahmed *et al.* (2019) that thinning in 'Zaghlool' dates at an early stage of fruit development can have a greater effect on fruit size due to a more comprehensive allocation of water and nutrients.

4. Conclusions

The results of this study showed that covering the date bunch reduced the brightness of the fruit, but increased the bunch weight, fruit length, total antioxidant activity and total phenolic compounds. Thinning at the pollination stage increased the weight of the fruit compared to the control. Thinning at the Kimri stage increased the percentage of Tamar, ascorbic acid content, and total antioxidant activity. We conclude that bunch covering at the pre-harvest stage is a simple, grower-friendly method. It is safe to use and has several beneficial effects on the physiochemical traits of fruit. This approach can be an integral part of fruit production in orchards. Moreover, fruit thinning and covering increased total antioxidant activity and total phenolic compounds of fruit which are important quality factors. Also, thinning is recommended for 'Piyarom' dates at both pollination and Kimri stages.

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Table 5 - Main effects of bunch date palm fruit thinning and covering on fruit and seed length, diameter and weight of 'Piyarom' date

Treatments	Fruits length (cm)	Fruits diameter (cm)	Fruits weight (g)	Seed length (cm)	Seed diameter (cm)	Seed weight (g)
2018						
Thinning						
Control	3.42 c [z]	1.78 b	6.75 c	2.30 b	1.06 a	0.99 a
Removal of one third of total strands from terminal tips	4.95 a	2.76 a	10.46 a	3.10 a	1.08 a	1.05 a
Removal of one third of terminal tips of central strands in	4.30 b	1.95 b	8.77 b	2.10 b	1.00 a	1.11 a
LSD	0.42	0.52	1.45	0.32	0.21	0.18
Bunch covering						
Control	3.97 b	2.33 a	8.34 a	2.50 a	1.11 a	1.06 a
Bunch covering	4.48 a	2.00 a	8.98 a	2.50 a	0.98 a	1.04 a
LSD	0.34	0.42	1.18	0.26	0.17	0.15
Thinning x bunch covering	NS [y]	NS	NS	NS	NS	NS
2019						
Thinning						
Control	3.75 b	1.96 b	7.08 c	2.23 b	0.75 a	0.98 a
Removal of one third of total strands from terminal tips	4.75 a	3.31 a	11.50 a	3.25 a	0.81 a	0.99 a
Removal of one third of terminal tips of central strands in	4.66 a	2.26 b	8.73 b	2.06 b	0.78 a	1.05 a
LSD	0.31	0.45	1.04	0.36	0.20	0.18
Bunch covering						
Control	4.05 b	2.41 a	8.90 a	2.62 a	0.82 a	0.95 a
Bunch covering	4.72 a	2.62 a	9.31 a	2.41 a	0.74 a	1.06 a
LSD	0.25	0.37	8.85	0.29	0.16	0.14
Thinning x bunch covering	NS	NS	NS	NS	NS	NS

[z] Means within each column for each treatment followed by the same letter are not significantly different at P=0.05.

[y] NS= non significant.

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Postharvest quality of pepino melon (*Solanum muricatum* Aiton) as influenced by NPK fertilizer rates, growing environment and storage temperature

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Abstract: The present study evaluated the effect of NPK fertilizer (17:17:17) rates (0, 100, 200, 300 and 400 kg ha^{-1}) on the postharvest quality of field and greenhouse grown pepino melons (*Solanum muricatum* Ait.) stored at room temperature (15-22°C) and at low temperature (7°C). The study was carried out in randomized complete block design with fruits from the field and greenhouse, five NPK fertilizer rates as treatments and the two storage temperatures replicated three times. Data were collected on percentage fruit weight loss (PWL), total soluble solids (TSS), firmness and shelf life. Results indicated that greenhouse and field grown fruits from the control and plants supplied with 100 kg NPK ha^{-1} had low PWL at both storage temperatures. Field grown fruits from the control stored at room temperature had the highest TSS and were firmer after 28 days of storage. Field grown fruits not supplied with fertilizer and stored at low temperature had a shelf life of 27 and 26 days in trial one and two respectively. Application of 100 kg NPK ha^{-1} and storage of pepino melon fruits at low temperature can be used to enhance quality and shelf life.

1. Introduction

Pepino melon (*Solanum muricatum* Aiton) is a little-known vegetable crop which belongs to the family solanaceae. It originated from the tropical and subtropical region of Andes and is grown for its edible fruits (Heiser, 1964). Pepino melon fruits are aromatic, juicy, scented, mild sweet, and vary in size, shape and colour depending on the cultivar (Martinez-Romero *et al*., 2003). The fruits mature 30 to 80 days after pollination and the skin is usually golden yellow with purple stripes (Nuez and Ruiz, 1996). Several studies have reported significant losses in horticultural produce after harvest (Toktam *et al*., 2019). Such losses are caused by dehydration, decay, and physiological disorders during postharvest handling. Fresh fruits and vegetables also undergo rapid transformation in nutritional and sensory quality after harvest, some of which contribute to loss of market value (Ahmad and Siddiqui, 2015). The losses can

be reduced through good management of pre- and postharvest factors (Toktam *et al*., 2019).

Postharvest quality is also affected by climatic factors such as temperature and light intensity, and other pre-harvest factors like soil type, fertilization, irrigation, mulching, and other cultural practices (Toktam *et al*., 2019). Temperature affects growth and development of fruits and vegetables as well as cellular compounds, their structure and this in turn affects produce firmness (Toktam *et al*., 2019). Fertilizers have also been shown to influence postharvest quality of most fruits and vegetables. The type of fertilizer used and the amount applied will dictate the quality of the resulting vegetables (Arah *et al*., 2015). Application of potassium fertilizers on tomato has been shown to improve fruit colour, reduce the occurrence of yellow shoulder and enhance titratable acidity (Passam *et al*., 2007). On the other hand, application of high doses of nitrogenous fertilizers to greenhouse grown tomatoes reduces fruit quality by reducing total soluble solids (Passam *et al*., 2007).

Temperature management between the time of harvesting and consumption has been shown to be effective in maintaining the quality of harvested vegetables. High temperatures increase metabolic activities and ethylene production but this is dependent on other factors like oxygen or carbon dioxide levels, time of exposure and the ripening stage (De Wild *et al*., 2003). Storage of vegetables at low temperature slows down metabolic processes and hence extends the shelf life of horticultural produce (Arah *et al*., 2015). The present study sought to investigate the effect of NPK fertilizer rates, growing environment and storage temperature on the postharvest quality of pepino melon.

2. Materials and Methods

Experimental site description

The experiment was conducted at the Horticulture Research and Teaching Field, Egerton University, Njoro. The field lies at a latitude of 0° 23' South, longitudes 35° 35' East in the Lower Highland III Agro Ecological Zone (LH3) at an altitude of approximately 2,238 m above sea level. Average maximum and minimum temperatures range from 19°C to 22°C and 5°C to 8°C, respectively, with a total annual rainfall ranging from 1200 to 1400 mm. The soils are predominantly mollic andosols (Jaetzold and Schimdt, 2006). The greenhouse used was 8 m by 60 m and the covering material was polythene with a thickness of 12×150 microns purchased from Amiran Kenya Ltd. The mean monthly temperatures in the greenhouse and field during the experiment are presented in Table 1.

Plant material and experimental design

Pepino seedlings (Ecuadorian Gold variety) were obtained from Garlic and Pepino Farm, Nakuru and planted in the field and greenhouse. The experimental design was randomized complete block design (RCBD) with fruits from the five NPK fertilizer treatments, the two growing environments and two storage temperatures replicated three times. The five NPK fertilizer treatments were [0, 100, 200, 300 and 400 NPK (17:17:17) kg ha^{-1}], two growing environments (field and greenhouse) and two storage temperatures (room and low temperature). Mature green pepino fruits were harvested from the field and greenhouse experiments and stored at low temperature (7°C) in a refrigerator and at room temperature (15-22°C) in the biotechnology laboratory of

Table 1 - Average monthly field and greenhouse temperature (°C) in trial one and two

Experimental conditions	Temperature						
	2018		2019				
Trial one	Nov	Dec	Jan	Feb	Mar	Apr	May
Field	20.9	19.7	20.9	21.7	22.8	22.6	21.2
Greenhouse	30.3	21.0	33.4	30.2	29.4	34.0	35.8
			2019				2020
Trial two	July	Aug	Sept	Oct	Nov	Dec	Jan
Field	19.1	19.2	20.5	19.3	19.3	18.9	19.1
Greenhouse	18.5	29.4	30.0	28.0	32.0	28.0	35.3

Egerton University. The experimental design was RCBD consisting of fruits from the five treatments replicated three times. The experiment therefore comprised of five treatments similar to those of the field and greenhouse experiments each replicated three times giving a total of thirty experimental units each represented by a plastic tray. Each experimental unit comprised of twenty pepino fruits randomly selected from the harvest of the individual respective treatments in the field and greenhouse experiments.

Data collection and analysis

Data were collected and recorded on percentage fruit weight loss (PWL), firmness, total soluble solids (TSS) and shelf life. To determine PWL, five fruits in each replication for each treatment were marked before storage and weighed using a digital balance (HANGPING JA 12002, Japan). The same fruits were weighed at the beginning of the experiment and weighing continued at an interval of 7 days for 28 days. The results were expressed as the percentage loss of initial weight using the formula:

$$\text{Percentage weight loss} = \frac{\text{Initial weight-final weight}}{\text{Initial weight}} \times 100$$

Fruit firmness was determined using hand held penetrometer (model 62/DR, UK) from the beginning of the experiment and continued at an interval of 7 days for 28 days. The results were reported in kg Force. TSS was determined on the same fruits used for determination of firmness using a hand held refractometer (0-30°Brix) (RHW refractometer, Optoelectronic Technology Company Ltd, UK) was used as per the procedure described by Tigchelaar (1986). Results were expressed as °Brix. This was done at the beginning of the experiment and continued at an interval of 7 days for 28 days.

The shelf life of pepino fruits was determined by counting the number of days at which at least 50% of the fruits had reached senescence and were not marketable (too soft, wrinkled or with fungal rots). Quality evaluation was done using a rating scale of 1-5 (Miguel and Marita, 1996).

Data collected were subjected to Analysis of Variance (ANOVA) and significant means separated using Tukey's honestly significant difference (Tukey's HSD) test at $p \leq 0.05$. The SAS statistical package (SAS Institute, 2005) was used for data analysis.

3. Results

Percentage weight loss (PWL)

NPK fertilizer rates, growing environment and storage temperature had a significant effect at $p \leq 0.05$ on PWL of Pepino fruits after 28 days of storage in trial one. During this trial, highest PWL of 10.863% and 15.77% were recorded in greenhouse grown fruits from plants supplied with 300 and 400 kg NPK ha^{-1} during production as well as in field grown fruits from plants supplied with 400 kg NPK ha^{-1} regardless of the storage temperature (Table 2). The lowest PWL was, on the other hand, recorded in greenhouse and field grown fruits without NPK fertilizer application (control) stored at low temperature although the difference in weight loss for this treatment was not significantly different from that of other treatment combinations. In trial two, NPK fertilizer rates, growing environment and storage temperature had a significant effect on PWL from day 7 to day 28 of the study. On day 28, greenhouse and field grown fruits from plants supplied with 400 kg NPK ha^{-1}, stored at room temperature had the highest PWL of 19.38% and 15.54% respectively (Table 2).

It was noted that as the fertilizer rates increased the PWL also increased in both growing environments and storage temperatures in both trials. Generally, fruits stored at low temperature had lower PWL compared to those stored at room temperature in both trials. Greenhouse grown fruits also had a higher PWL compared to field grown fruits in both trials.

Total soluble solids (TSS)

NPK fertilizer rates, growing environment and storage temperature had a significant effect at $p \geq 0.05$ on TSS of pepino fruits from day 7 to day 28 in both trials. In trial one, field grown fruits from plants which were not supplied with fertilizer (control) and stored at room temperature had the highest TSS from day 7 to day 28. The highest TSS was recorded after 28 days of storage where field grown fruits from plants not supplied with NPK fertilizer and stored at room temperature had a TSS of 8.67 °Brix which was significantly higher than that recorded from fruits from all other treatment combinations. Greenhouse grown fruits from plants supplied with the highest fertilizer rate of 400 kg NPK ha^{-1} and stored at low temperature had the lowest TSS of 4.40°Brix after 28 days (Table 3). In trial two, field grown fruits from plants not supplied with NPK fertilizer (control) and stored at room tem-

Table 2 - Effect of NPK fertilizer rates, growing environment and storage temperature on percentage weight loss of pepino fruits in trial one and two

Storage temperature	Environment	Fertilizer (kg ha^{-1})	Weight loss			
			Day 7	Day 14	Day 21	Day 28
Trial one						
Room temperature	Field	0	1.067	2.050	2.837	3.987def
		100	1.423	2.800	3.620	5.303 def
		200	1.980	3.580	4.713	6.383 cdef
		300	2.500	5.147	5.717	9.120 bcd
		400	4.417	7.280	11.353	12.457 ab
	Greenhouse	0	1.213	1.840	2.820	3.413 ef
		100	2.037	4.317	5.590	7.047 cde
		200	2.673	5.540	7.160	8.913 bcd
		300	3.167	6.400	8.720	10.863 abc
		400	5.810	9.327	11.410	15.770 a
Low temperature	Field	0	0.523	1.043	1.283	1.557 f
		100	0.917	1.720	1.997	4.300 def
		200	1.057	2.170	2.763	2.310 ef
		300	1.740	3.883	4.040	7.120 cde
		400	2.737	5.497	8.020	15.123 a
	Greenhouse	0	0.543	1.087	1.630	2.173 ef
		100	1.063	1.353	2.070	2.633 ef
		200	1.543	2.417	2.867	4.012 def
		300	2.240	2.977	5.080	3.183 ef
		400	3.950	6.107	9.067	8.630 bcd
Trial two						
Room temperature	Field	0	0.940 ij	1.173 kl	1.443 kl	4.187 fghi
		100	1.893 fghi	2.633 fghijk	3.123 fghij	7.100 defg
		200	2.507 efgh	3.130 defghi	4.590 cdefg	8.723 cde
		300	3.667 bcd	4.773 cde	5.653 cd	11.690 bc
		400	4.550 b	7.363 b	10.883 b	15.543 ab
	Greenhouse	0	1.156 hij	1.670 ijkl	1.943 ijkl	3.360 fghi
		100	3.067 cdef	3.077 efghi	4.230 defgh	7.293 def
		200	3.703 bcd	4.333 cdef	5.327 cd	9.290 cd
		300	4.113 bc	4.813 cd	6.190 c	11.600 bc
		400	6.617 a	10.477 a	14.703 a	19.397 a
Low temperature	Field	0	0.663 j	0.917 l	1.283 l	1.653 i
		100	1.047 ij	1.420 ijkl	1.997 ijkl	2.243 hi
		200	1.970 efghi	2.173 hijkl	2.783 hijkl	2.487 hi
		300	2.720 defg	2.887 fghij	3.533 efghi	3.017 ghi
		400	4.127 bc	3.997 cdefg	4.740 cdef	5.967 defgh
	Greenhouse	0	1.607 ghij	1.337 jkl	1.720 jkl	2.190 hi
		100	2.313 efgh	2.473 ghijkl	2.993 ghijk	4.593 efghi
		200	3.117 cde	3.520 defgh	4.223 defgh	5.330 defghi
		300	3.693 bcd	4.163 cdefg	4.823 cde	4.880 efghi
		400	4.473 b	5.487 c	6.20 c	5.930 defgh

* Means followed by the same letters in a given day and trial are not significantly different according to Tukey's Honestly Significant Difference Test at $p \leq 0.05$. Room storage temperature varied between 15 and 22°C. Low temperature was 7°C.

Table 3 - Effect of NPK fertilizer rates, growing environment and storage temperature on TSS (°Brix) of pepino melon fruits in trial one and two

Storage temperature	Environment	Fertilizer (kg ha^{-1})	TSS (°Brix)				
			Day 0	Day 7	Day 14	Day 21	Day 28
Trial one							
Room temperature	Field	0	4.00	6.00 a	6.93 a	7.83 a	8.67 a
		100	4.00	4.37 cde	5.73 b	6.13 c	6.40 bcde
		200	4.00	4.53 cde	4.97 c	5.73 cd	6.13 cdef
		300	4.00	4.53 cde	4.90 c	5.47 de	5.80 defg
		400	4.00	4.17 de	4.67 cde	4.80 ghi	5.20 ghi
	Greenhouse	0	4.00	5.00 b	6.00 b	6.77 b	7.07 b
		100	4.00	4.40 cde	4.90 c	5.47 de	5.80 defg
		200	4.00	4.47 cde	4.90 c	5.23 ef	5.67 fgh
		300	4.00	4.17 de	4.63 cde	4.86 fghi	5.10 ghij
		400	4.00	4.10 e	4.30 ef	4.53 hijk	4.80 ij
Low temperature	Field	0	4.00	5.00 b	5.63 b	6.00 c	6.83 bc
		100	4.00	4.60 bcd	4.83 c	5.13 efg	5.77 efg
		200	4.00	4.43 cde	4.63 cde	4.97 fg	5.27 ghi
		300	4.00	4.30 de	4.40 def	4.73 ghij	4.97 hij
		400	4.00	4.10 e	4.13 f	4.33 jk	4.56 ij
	Greenhouse	0	4.00	4.77 bc	5.00 c	5.90 c	6.50 bcd
		100	4.00	4.60 bcd	4.77 cd	4.93 fghi	5.23 ghi
		200	4.00	4.47 cde	4.63 cde	4.80 ghi	4.97 hij
		300	4.00	4.30 de	4.33 ef	4.50 ijk	4.73 ij
		400	4.00	4.17 de	4.10 f	4.20 k	4.40 j
Trial two							
Room temperature	Field	0	4.00	5.60 a	6.70 a	7.60 a	8.133 a
		100	4.00	4.50 cde	5.36 c	5.67 d	6.10 cd
		200	4.00	4.33 defg	4.97 cde	5.26 ef	5.77 def
		300	4.00	4.17 efg	4.53 fghi	4.80 ghi	5.00 hij
		400	4.00	4.60 g	4.13 ijk	4.27 j	4.50 jkl
	Greenhouse	0	4.00	4.70 bc	5.23 c	6.07 c	6.50 bc
		100	4.00	4.50 cde	4.80 def	5.10 efg	5.50 efgh
		200	4.00	4.33 defg	4.97 cde	4.87 ghi	5.13 ghi
		300	4.00	4.13 fg	4.53 fghi	4.60 ij	4.90 ijk
		400	4.00	4.03 g	4.07 k	4.27 j	4.47 kl
Low temperature	Field	0	4.00	5.27 a	5.83 b	6.47 b	6.93 b
		100	4.00	4.60 bcd	5.00 cde	5.37 de	5.83 def
		200	4.00	4.30 defg	4.70 efg	4.93 fghi	5.43 fgh
		300	4.00	4.13 fg	4.50 fghij	4.70 hi	5.00 hij
		400	4.00	4.07 fg	4.10 jk	4.27 j	4.60 jkl
	Greenhouse	0	4.00	4.87 b	5.17 cd	5.67 d	6.00 cde
		100	4.00	4.50 cde	4.77 defg	5.00 efgh	5.53 efg
		200	4.00	4.30 defg	4.53 fghi	4.73 ghi	5.00 hij
		300	4.00	4.13 fg	4.37 ghijk	4.60 ij	4.83 ijk
		400	4.00	4.03 g	4.13 ijk	4.23 j	4.30 l

* Means followed by the same letters in a given day and trial are not significantly different according to Tukey's Honestly Significant Difference Test at $p \leq 0.05$. Room storage temperature varied between 15 and 22°C. Low temperature was 7°C.

perature had the highest TSS from day 14 to day 28. On day 7, field grown fruits from plants not supplied with NPK fertilizer and stored at room or under low temperature had the highest TSS of 5.6 and 5.27 °Brix, respectively (Table 3). The highest TSS was recorded after 28 days of storage where field grown fruits from plants not supplied with NPK fertilizer and stored at room temperature had a TSS of 8.13 °Brix which was significantly higher than for greenhouse grown fruits from plants supplied with 400 kg NPK ha^{-1} and stored

at low temperature with a TSS of 4.3°Brix. It was observed that TSS increased as the storage time progressed and decreased as the fertilizer rates increased regardless of the environment under which the fruits were produced and the temperature during storage. Generally, field grown fruits had higher TSS compared to greenhouse grown fruits regardless of the storage temperatures in both trials. On the other hand, fruits stored at low temperature had lower TSS values compared to those stored at room temperature in both trials.

Firmness

NPK fertilizer rates, growing environment and storage temperature had a significant effect on firmness of pepino melon fruits on day 7 and day 28 in trial one, and day 21 and 28 in trial two. In day 7 of trial one, field grown fruits from plants which were not supplied with NPK fertilizer and stored at either room or low temperature had the highest firmness of 4.67 kg F and 4.83 kg F, respectively. However, this was not significantly different from the firmness of 4.57 kg F recorded for greenhouse grown fruits harvested from plants not supplied with NPK fertilizer and maintained under low temperature during storage. On day 28, the highest firmness was recorded in field grown fruits from plants not supplied with NPK fertilizer and stored at low temperature with a firmness of 3.83 kg F. The lowest firmness of 0.52 kg F was recorded in greenhouse grown fruits from plants supplied with 400 kg NPK ha^{-1} and stored at room temperature after 28 days of storage (Table 4).

In trial two, NPK fertilizer rates, growing environment and storage temperature had a significant effect on firmness of pepino melon fruits at day 21 and day 28 of storage. In day 21 of storage, field grown fruits from plants not supplied with NPK fertilizer and stored at low temperature had the highest firmness of 4.13 kg F but this was not significantly different from the firmness of 3.93 kg F recorded from greenhouse fruits from plants not supplied with NPK fertilizer and stored at low temperature, field grown fruits from plants not supplied with NPK fertilizer stored at room temperature with a firmness of 3.70 kg F and field grown fruits from plants supplied with 100 kg NPK ha^{-1} maintained under low temperature with a firmness of 3.67 kg F (Table 4). The lowest firmness of 1.70 kg F was recorded in greenhouse grown fruits from plants supplied with 400 kg NPK ha^{-1} stored at low temperature but this was not significantly different from the other treatment combinations.

Generally, it was observed that field grown fruits were firmer compared to greenhouse grown fruits and fruits stored at low temperature were firmer compared to those stored at room temperature. Firmness also decreased as the fertilizer rates and storage days increased.

Shelf life

NPK fertilizer rates, growing environment and storage temperature had a significant effect ($p \leq 0.05$) on the shelf life of pepino melon fruits in both trials. In trial one, field grown pepino fruits from plants which were not supplied with NPK fertilizer (control) and stored at low temperature (7°C) had the longest shelf life of 27 days. Field grown fruits from plants supplied with 100 kg NPK ha^{-1} stored at low temperature had shelf life of 22 days but this was not significantly different from the shelf life of greenhouse grown fruits from plants not supplied with NPK fertilizer and those supplied with 100 kg NPK ha^{-1} and maintained at low temperature with a shelf life of 21 and 19 days respectively (Table 5). The lowest shelf life of 11 days was recorded in greenhouse grown fruits from plants supplied with 400 kg NPK ha^{-1} and stored at room temperature although this was not significantly different from that of field grown fruits from plants supplied with 300 and 400 kg NPK ha^{-1} and stored at room temperature, greenhouse grown fruits from plants supplied with 200 and 300 kg NPK ha^{-1} and stored at room temperature and field grown fruits from plants supplied with 400 kg NPK ha^{-1} and stored at low temperature.

In trial two, field grown pepino fruits from plants not supplied with NPK fertilizer and stored at low temperature had the longest shelf life of 26 days, followed by field grown fruits from plants supplied with 100 kg NPK ha^{-1} stored at low temperature with a shelf life of 21 days. The shelf life recorded for fruits from this treatments was, however, not significantly different from that of greenhouse grown fruits from plants not supplied with fertilizer, those supplied with 100 kg NPK ha^{-1} and stored at low temperature and field grown fruits from the control and stored at room temperature (Table 5). The lowest shelf life was recorded in greenhouse grown fruits from plants supplied with 400 kg NPK ha^{-1} stored at room temperature with a shelf life of 10 days but this was not significantly different from the shelf life recorded for greenhouse grown fruits from plants supplied with 200 and 300 kg NPK ha^{-1} stored at room temperature and that of field grown fruits from plants supplied with 400 kg NPK ha^{-1} and stored at room temperature.

Generally, it was observed that fruits stored at

Table 4 - Effect of NPK fertilizer rates, growing environment and storage temperature on firmness (kg) of pepino melon fruits in trial one and two

Storage temperature	Environment	Fertilize (kg ha^{-1})	Firmness (Kg)				
			Day 0	Day 7	Day 14	Day 21	Day 28
Trial one							
Room temperature	Field	0	5.00	4.67 a	4.03	3.60	3.03 b
		100	5.00	3.90 c	3.27	3.03	2.77 bc
		200	5.00	3.57 cde	3.20	2.87	2.37 cdef
		300	5.00	3.03 ef	2.57	2.00	1.57 hijk
		400	5.00	2.83 f	2.33	1.50	1.02 kj
	Greenhouse	0	5.00	3.97 bc	3.57	2.90	2.47 cdef
		100	5.00	3.73 cd	3.13	2.23	2.07 efgh
		200	5.00	3.43 cdef	2.83	2.13	1.80 ghij
		300	5.00	2.93 ef	2.20	1.83	1.50 ijk
		400	5.00	2.17 g	1.87	1.27	0.52 l
Low temperature	Field	0	5.00	4.83 a	4.77	4.33	3.83 a
		100	5.00	3.97 bc	3.67	3.37	2.70 bcd
		200	5.00	3.83 cd	3.40	3.07	2.50 bcde
		300	5.00	3.57 cde	3.20	2.83	2.17 defg
		400	5.00	3.33 cdef	2.97	2.17	1.50 ijk
	Greenhouse	0	5.00	4.57 ab	4.40	3.30	3.03 b
		100	5.00	3.53 cde	3.30	2.73	2.27 cdefg
		200	5.00	3.20 def	2.93	2.40	1.93 fghi
		300	5.00	3.00 ef	2.57	2.13	1.57 hijk
		400	5.00	2.87 f	2.20	1.80	1.27 jk
Trial two							
Room temperature	Field	0	5.00	4.63	4.07	3.70 abc	2.73 bc
		100	5.00	3.90	3.67	3.10 def	2.60 bc
		200	5.00	3.80	3.17	2.87 efgh	2.13 def
		300	5.00	3.43	2.87	2.17 ijk	1.87 fg
		400	5.00	3.00	2.43	1.90 jk	1.20 ij
	Greenhouse	0	5.00	4.27	3.80	3.07 def	2.47 cde
		100	5.00	3.93	3.27	2.80 fgh	2.03 efg
		200	5.00	3.60	3.00	2.40 hij	1.70 fgh
		300	5.00	3.13	2.63	2.00 jk	1.30 hi
		400	5.00	2.90	2.20	1.70 k	0.85 j
Low temperature	Field	0	5.00	4.70	4.37	4.13 a	3.83 a
		100	5.00	4.40	3.93	3.67 abc	2.83 bc
		200	5.00	4.13	3.60	3.33 cde	2.93 b
		300	5.00	3.93	3.37	3.07 def	2.63 bc
		400	5.00	3.63	3.07	2.77 fgh	2.07 defg
	Greenhouse	0	5.00	4.27	4.07	3.93 ab	3.40 a
		100	5.00	4.03	3.87	3.50 bcd	2.83 bc
		200	5.00	3.83	3.20	2.97 efg	2.50 bcd
		300	5.00	3.43	2.87	2.53 ghi	2.10 def
		400	5.00	3.20	2.53	2.17 ijk	1.63 ghi

* Means followed by the same letters in a given day and trial are not significantly different according to Tukey's Honestly Significant Difference Test at $p \leq 0.05$. Room storage temperature varied between 15 and 22°C. Low temperature was 7°C.

low temperature had a longer shelf life than those stored at room temperature. Field grown fruits had a longer shelf life compared to greenhouse grown fruits. The shelf life decreased as the NPK fertilizer rates increased.

4. Discussion and Conclusions

There was a progressive increase in percentage fruit weight loss as the storage days advanced. Field and greenhouse grown fruits from plants which

Table 5 - Effect of NPK fertilizer rates, growing environment and storage temperature on shelf life (days) of pepino melon fruits in trial one and two

Storage temperature	Environment	Fertilizer (kg ha^{-1})	Shelf life (Days)	
			Trial 1	Trial 2
Room temperature	Field	0	18 cd *	20 bcd
(15-22°C)		100	17 cde	18 cdef
		200	15 defg	16 fghij
		300	14 fghi	14 ijk
		400	12 hi	11 lm
	Greenhouse	0	18 cd	17 efgh
		100	16 defg	14 hijk
		200	13 ghi	12 klm
		300	12 hi	10 lm
		400	11 i	10 m
Low temperature (7°C)	Field	0	27 a	26 a
		100	22 b	21 b
		200	18 cd	17 defg
		300	16 def	15 ghij
		400	14 fghi	13 jkl
	Greenhouse	0	21 b	21 bc
		100	19 bc	19 bcde
		200	17 cdef	16 efghi
		300	16 defg	15 ghij
		400	15 efgh	14 ijk

* Means followed by the same letters are not significantly different according to Tukey's Honestly Significant Difference Test at p≤0.05. Room storage temperature varied between 15 and 22°C. Low temperature was 7°C.

received 400 kg NPK ha^{-1} and stored at room temperature had the highest PWL. Similar results were reported in sweet potato in which excessive application of nitrogen led to an increase in percentage weight loss during storage (Mark *et al*., 2003). Nitrogen fertilizer rates affect the rate of water loss in fruits and vegetables (Warner *et al.*, 2004). Transpiration is the main cause of deterioration because it results in direct loss of weight. Weight loss is the major cause of softening and shriveling of fruits and vegetables damaging the appearance of fruits and loss of market value (Wilson *et al*., 1999). The quality of most fruits and vegetables is affected by weight loss but this depends on the temperature and humidity during storage (Perez *et al*., 2003). Storage of pepino fruits at room temperatures (15-22°C) could also have resulted in production of high levels of ethylene, increased respiration and subsequent weight reduction. High temperatures during storage leads to increased water loss resulting to shriveling and loss of fresh appearance of the fruits (Wills *et al*., 1989). Fruits lose weight when metabolic activities increase and this is accelerated by an increase in temperature around the produce resulting in loss of water. Weight loss is mainly as a result of water loss and as temperature increases the rate of water loss also increases. In this study, pepino melon fruits were harvested when green mature and as ripening progressed there was an increase in ethylene production which led to senescence and shriveling of the fruits during storage (Wills *et al*., 1989). Greenhouse fruits had a higher percentage weight loss probably because of the high preharvest temperature (Table 1). At room temperature the temperatures were higher than at low temperature (7°C) and this could have resulted to faster ripening, increased respiration rates and hence high PWL. Fruits stored at low temperature had lower PWL compared to those stored at room temperature. Vanitha and Mehalai (2016) also reported that pepino fruits stored at room temperature had a higher weight loss compared to those stored at low temperature. Temperatures above 20°C can lead to abnormal physiological processes in fresh produce, respiration occurs and water is lost to the surrounding environment and hence reduction in weight. Although there was an increase in PWL as the storage days increased, the rate was much lower in pepino fruits stored at low temperature. Loss in weight could also be due to activity of polygalacturonase which increases cell wall permeability and hence increase in transpiration. Low temperature reduces respiration and metabolic processes thereby slowing down the rate of fruit weight loss during storage. Low temperature also reduces the sensitivity of fruits to ethylene and senescence is reduced (Wills *et al*.,1989). In both trials, field and greenhouse grown pepino fruits supplied with the highest NPK fertilizer rate had the highest PWL. This is in agreement with the findings of Hailu *et al*. (2008) and Mark *et al*. (2003) where application of highest nitrogen fertilizer rates had the highest physiological weight loss of carrots and sweet potatoes during storage. The increased PWL due to increased level of nitrogen supply may be attributed to the higher moisture content in the fruits which may lead to decreased shelf-life due to rapid metabolic activity, moisture loss and shrinkage in storage (El-Tantawy and El-Beik, 2009). On the other hand, fruits from the control (no NPK fertilizer) had the lowest PWL which could be attributed to low moisture content in the fruits, slowed metabolic activities and hence reduced moisture loss. The PWL decreased as the phosphorous and potassium rates

in the NPK fertilizer increased. This could be due to the fact that potassium plays a role in maintaining fruit firmness but high rates do not result to further increase in firmness. The firmer the fruit the less the PWL and reduction in firmness results to more PWL. On the other hand, high nitrogen levels coupled with high phosphorous levels reduce fruit quality because most of the carbohydrates are translocated to the shoots rather than to the developing fruits resulting to dense vegetative growth. Fruits produced by plants which have dense vegetative growth tend to be less firm resulting to high PWL while fruits from plants with less vegetative growth are firmer and hence low PWL. In summary, PWL progressively increased with increase in storage time in both room and low temperatures. As the fruit continues to ripen the rate of respiration also increases and this also leads to increase in weight loss. However, low temperature leads to delayed ripening and hence reduced respiration resulting to low PWL compared to ambient room temperatures.

TSS increased as the storage days increased. Our results are in agreement with Harman *et al.* (1986) and Hailu (2016) who reported that as pepino melon and mango fruits mature TSS increases significantly during maturation and ripening. The increase in TSS might be due to alteration of cell wall structure and the breakdown of complex carbohydrates into simple sugars. At room temperature, the temperatures were high and this led to an increase in metabolic processes, respiration and ripening resulting to high TSS. Increase in TSS could also be due to excessive moisture loss of fruits which led to increased concentration of pepino fruits stored at room temperature (Nath *et al*., 2011). At high temperatures the rate of ripening is higher than at low temperatures and this increases TSS. Field grown pepino fruits had a higher TSS compared to greenhouse grown pepino fruits in both storage temperatures. The high TSS recorded in field grown pepino fruits could be due to high light intensity and thus high photosynthesis leading to more accumulation of sugars in the fruit compared to greenhouse grown fruits where the light intensity was low leading to reduced photosynthesis and hence low accumulation of sugars in the fruits (Beckmann *et al*., 2006). Any factor that interferes with photosynthesis will affect glucose and sucrose accumulation in the fruit and thus alter TSS (Rana *et al.*, 2014). High relative humidity in the greenhouse may also have led to reduced transpiration and this enhances flow of water in the xylem vessels and this is good for the fruits because fruits act as drains for high concentrations of organic molecules leading to low water potential (Bertin *et al*., 2000). The low water potential in the fruits promotes absorption of water by the fruits leading to "dilution effect" making the fruits to have low TSS compared to those grown in the field (Rana *et al*., 2014). The low TSS recorded in greenhouse grown fruits could also be due to the fact that high temperatures during ripening of pepino melon reduce sugar content of the fruits (Pluda *et al*., 1993). Fruits stored at low temperature had a lower TSS compared to that of fruits stored at room temperature. This could be due to delayed fruit ripening and slow conversion of carbohydrates into simple sugars. During ripening there is breakdown of complex carbohydrates into simple sugars and this increase TSS. At high temperatures the conversion of carbohydrates into simple sugars is accelerated and this results to high TSS whereas at low temperature ripening is delayed and the hydrolysis of carbohydrates to sugars is slower, resulting to low TSS. In the present study, TSS ranged from 4.00-7.07 and 4.00-8.13 °Brix in trial one and two respectively. Other studies have reported lower TSS of pepino melon in the range of 4.91-5.40 °Brix (Kola, 2010) and 5.04-5.46 °Brix (Maruapey and Yuwono, 2016). The low TSS could be attributed to high water content in pepino fruits in the range of 90-92% (Gonzalez *et al*., 2000) and the fact that the quality of pepino melon fruits is greatly influenced by the environment in which these studies were conducted which is quite different from the environment in this study. TSS decreased as the fertilizer rates increased in both growing environments and storage temperatures in both trials. Field grown fruits from control plants had the highest TSS and this could be due restriction of vegetative growth because no NPK fertilizer was applied and thus the fruits became the only sink for sugars and hence increase in TSS (Pluda *et al*., 1993). Greenhouse grown fruits from plants supplied with the highest fertilizer rate of 400 kg NPK ha^{-1} had the lowest TSS and this might be due to excessive vegetative growth of both the main and side shoots therefore most of the photosynthates were directed to the young developing shoots rather than to the fruits leading to low sugar concentration in the fruits (Pluda *et al.*, 1993). Excess nitrogen fertilizers make plants be more succulent, thus fruits from plants supplied with 400 kg NPK ha^{-1} had a high water content and this might have led to dilution of sugars in the fruit resulting to low TSS.

Fruit firmness decreased as the storage days increased. Decrease in firmness is strongly related to increased weight loss because as the fruits lose weight they become soft hence decreased firmness. In this study, firmness decreased as the NPK fertilizer rates increased. Fruits from plants which were supplied with 400 kg NPK ha^{-1} had the lowest firmness and this could be due to the fact that plants with dense vegetative growth are less firm than those with low or moderate vegetative growth (Toktam *et al.*, 2019). Fruits which were not supplied with NPK fertilizer (control) were firmer due to decreased vegetative growth. Loss of moisture and enzymatic changes results to change in firmness (Ball, 1997). Hemicelluloses and pectins become more soluble and this causes changes and loosening of the cell wall (Paul *et al*., 1999). In the present study, both trials field grown pepino fruits were firmer than greenhouse grown fruits. This could be due to the fact that lower temperature during the growing season increases firmness (Anagnostou and Vasilakakis, 1995). In the greenhouse the temperatures (Table 1) were high and it has been reported that high preharvest temperatures tend to decrease firmness (Paul *et al*., 1999). Previous studies reported that loss of firmness in pepino melon is due to softening which is caused by breakdown of structural cell wall carbohydrates and an increase in soluble pectic substances during storage (Heyes *et al*., 1994). Increase in pectic substances leads to weakening of cell walls and reduction of cohesive forces binding cells together resulting to loss of firmness (Heyes *et al*., 1994). In summary, fruit softening is caused by structural as well as compositional changes in various components of the cell wall carbohydrates partly as a result of fruit softening enzymes (Abbasi *et al*., 2011). Other studies have reported that fruit softening is as a result of cell wall digestion by pectinesterase, polygalacturonase and other enzymes and this is increased by an increase in storage temperature (Ahmed *et al*., 2009). Low temperature storage maintained firmness of pepino melon fruits.

Pepino fruits stored at low temperature had a longer shelf life compared to those stored at room temperature. This could be attributed to reduced ethylene production, respiration, ripening, weight loss, senescence, retention of firmness and reduction of other metabolic activities and this enhances shelf life and quality of produce (Lei Yi *et al*., 2019). On the other hand, pepino fruits stored at room temperature had a shorter shelf life because high temperature results to increased ethylene production, respiration, ripening, weight loss, senescence, loss of firmness and other metabolic processes and this reduced shelf life (Mutari and Debbie, 2011). Field grown fruits had a longer shelf life compared to greenhouse grown fruits. This could be attributed to lower temperature in the field during the growing season (Table 1) as low temperatures have been reported to increase firmness (Anagnostou and Vasilakakis, 1995). In the greenhouse the temperatures were high and it has been reported that high temperatures tend to decrease firmness (Paul *et al*., 1999). Therefore, field grown fruits remained firmer than greenhouse grown fruits and hence the former had a longer shelf life. Fruits from the control had the longest shelf life and this could be attributed to low nitrogen levels and low water content in this fruits hence they remained firmer. On the other hand, fruits from plants supplied with high NPK fertilizer rates had a short shelf life and this might be due to high water content in the fruits due to excess nitrogen which also leads to postharvest decay especially fruits which were stored at room temperature.

Based on the foregoing results and discussion, we conclude that application of high amounts of NPK fertilizer leads to increased weight loss, less firm fruits and low TSS of pepino fruits stored at room temperature. Storage of pepino melon fruits at 7°C maintains quality through reduced weight loss and maintaining firmer fruits. We therefore recommend application of 100 kg NPK ha^{-1} for both field and greenhouse grown pepino melon and storage at low temperature (7°C) for enhanced quality and shelf life of the fruits.

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The impact of cumin essential oil on cold stored-radish tubers

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Kew words: Antioxidant capacity, Malondialdehyde, protease, protein, total phenol content

Abstract: Notable biological compounds in radish, made it as one of the most popular crops in the raw vegetables global market. However, storing it under low temperature conditions is associated with browning and taste changing. The present research aimed to evaluate the effects of different concentrations of cumin essential oil (0, 1.56, 3.13, 6.25, 12.5 and 25 ppm) and the storage period (0, 3, 6 and 9 days) on antioxidant parameters of radish tubers under low temperature conditions. The results indicated declining trends in the L* and a* values, beside ascending trend in b* value after nine days of storage. However, over the storage period of tubers, these parameters increased in cumin essential oil treated tubers. According to our findings, the application of cumin essential oil increased protein content, but reduced the malondialdehyde content, polyphenol oxidase and peroxidase activities. The cold-stored radishes received the most effective treatment of cumin essential oil at the concentration of 12.5 and 25 ppm.

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1. Introduction

Radish (*Raphanus sativus* L.), belongs to the Cruciferae family, is a part of the human diet worldwide. This root-product is usually used as a crunchy vegetable, mainly in salads (Banihani, 2017). Radish contains carbohydrates, sugars, dietary fibers, proteins, vitamins and minerals (Khattak, 2011). In addition, it has been found to be rich in some bioactive compounds, including isothiocyanates (e.g. indole-3-carbinol and sulforaphane) and glucosinolates (e.g. glucoraphanin, glucoerucin, glucobrassicin, glucoraphasatin, neoglucobrassicin, 4-methioxyglucobrassicin and 4-hydroxyglucobrassicin) (Malik *et al*., 2010; Ishida *et al*., 2015; Baenas *et al*., 2016), which prevent the proliferation of the colon (Nakamura *et al*., 2008), prostate (Steinbrecher *et al*., 2009), lung (Wang *et al*., 2014) and breast (Pawlik *et al*., 2017) cancer cell lines.

The global marketing of radish has improved due to be included in easy-to-prepare foods especially in the northern European countries like Holland and Germany (Salerno *et al*., 2005). However, the storage of radish under ambient conditions leads to shrinking and hence failing its

marketing (Luegno and Calbo, 2001).

Low temperature conditions are commonly used in the food industry to maintain the quality of horticultural products in storage, reduce the respiration rate and delay metabolic processes (Patel *et al*., 2016). But lipid peroxidation, the most important deteriorating factor during low temperature storage, affects the nutritional value and sensory evaluation of food products (Liang *et al*., 2020), i.e radish lose up to 5% of its weight and 43% of its nutritional content after exposure to low temperature (10°C) for 10 days (Del Aguila *et al*., 2006).

The antioxidants are essential compounds that prevent or delay the lipid peroxidation (Khalid *et al*., 2016). Food industries use artificial antioxidants such as BHA (Butylated hydroxylanisole), BHT (Butylated hydroxyltoluene), TBHQ (Tertiary-butyl hydroquinone), and PG (Propyl gallate) to extend the shelf life of cold-stored products. However, the side effects of these synthetic components have caused consumers to be concerned (Andre´ *et al*., 2010; Liang *et al*., 2020) and drawn the attention of researchers to safe alternatives such as natural derived products. Plants essential oils (EOs) are a rich source of antioxidants which reduce the production of reactive oxygen species (ROS) or scavenge the formed ROS (Khalid *et al*., 2016).

Browning and taste changing are the main disorders which influence the quality of radishes under low temperatures (Del Aguila *et al*., 2008; Ramachandran *et al*., 2013). Using antioxidant compounds, such as citric acid and ascorbic acid (Del Aguila *et al*., 2006; Lee *et al*., 2007) or covering the tuber with chitosan (Ramachandran *et al*., 2013) have reduced the browning intensity under cold storage conditions. Packed radishes can also be stored for six days under low temperatures with no browning symptoms (Nicola *et al.,* 2004; Ayub *et al*., 2013).

Plant EOs can serve as efficient strategies in the quality preservation in cold-stored horticultural products (Misharina and Samusenki, 2008). The Cumin (*Cuminum cyminum* L.), an herbaceous annual member of the Apiaceae family, is frequently used as flavor (Thippeswamy and Naidu, 2005). The main components of cumin EO are cuminal, cuminic alcohol, terpinene, safranal, p-cymene and pinene (Thippeswamy and Naidu, 2005; Ravi *et al*., 2013).

Despite several reports on the antimicrobial characteristics of cumin EO (Thippeswamy and Naidu, 2005; Gachkar *et al*., 2007; Milan *et al*., 2008; Dua *et al*., 2012), there is no research on its potential to serve as an antioxidant with ROS scavenging capacity, specifically in applying on cold-stored products. Therefore, the present research aimed to evaluate the effects of different concentrations of cumin EO, as an organic antioxidant compound, on the biochemical and antioxidant parameters of stored radishes under low temperature conditions.

2. Materials and Methods

Plant material preparation

Radish (*Raphanus sativus* L. var 'Cherry Belle') seeds were planted (October 1st 2019) in the research greenhouse (RH=50%, temperature: 26 ± 1°C day /20± 0.5°C night, and 50% shade) at the University of Hormozgan (53° 33′ E 28° 30′ N, 10 m), Iran. The plastic pots (25×18 cm^2) were filled by a media mixture (soil-sand-silt in 1:1:1 ratio). Daily drip irrigation system was used for all experimental units. The tubers were harvested 40 days later. The experiment was repeated next months (November 1st 2019-December 10th 2019). The radish tubers were then transferred to the laboratory. After wards, well-formed and uniform tubers (1.5×1.5 cm^2) which were healthy, smooth, firm and free from decay, damage or cracks were selected, washed and dried.

The experimental design

The factorial experiment was a completely randomized design with six replications (10 samples per each experimental unit). The factors were cumin EO concentrations (0, 1.56, 3.13, 6.25, 12.5 and 25 ppm) and the storage period (0, 3, 6 and 9 days). The control (day 0) measurement was done before applying the treatments. The cumin EO was prepared from Zardband Pharmaceuticals industry, Tehran, Iran. The phytochemical and microbiological properties of the EO were described by Zarband Company (Table 1). The Cumin oil was obtained by steam distillation of seed. Ground seed were sieved and then subjected to water distillation using a Clevenger apparatus (3 hours) (Beis *et al*., 2000).

The cumin EO was diluted with distilled water (0, 1.56, 3.13, 6.25, 12.5 and 25 ppm) and the radishes were immersed at 20°C for 10 min. Then all radishes were air-dried under room temperature for 1 h, put in polyethylene plastic containers (10 tubers per each container) and placed in the cold storage (5°C, 95% RH). The tubers were selected for the following measurements at 0, 3, 6 and 9 days of storage.

Table 1 - Physicochemical and microbiology of analysis of Cumin EO

Test	Results
Physicochemical test	
Appearance	Clear liquid
Color	Pale Yellow
Odor	Confirmed characteristics
Density	0.89 Kg m^{-3}
Refractive index	1.46
Assay (Cumin aldehyde)	36.33%
Microbiology limit test	
Total bacterial count	< 10 Colony Forming Units per mL
Total mold and yeasts	< 10 Colony Forming Units per mL
Escherichia coli	Absence
Salmonellae	Absence

Plant used parts: fruits, state: liquid.

Color parameters

Tuber skin color was measured using a colorimeter (Konica-CR-400 Minolta, Japan) under reflected light in CIE L*a*b* system, where L* expressed color lightness from 0 (black) to 100 (white), a*defined the proportion of red (+a) to green (-a), and b* represented the proportion of yellow (+b) to blue (-b). The average of six records was considered for every color parameter.

Malondialdehyde content

According to Heath and Packer (1968), 0.5 g of the tuber was homogenized in 5 ml of 1% Trichloroacetic acid (TCA), centrifuged (10000 rpm, 5 min) and the supernatant (250 µl) was mixed with 1 ml of Malondialdehyde (MDA) solution containing 20% TCA and 5% TBA (Thiobarbituric acid). It was then incubated in a hot water bath (95°C) for 30 min, immediately cooled and re-centrifuged (10000 rpm, 10 min). The absorbance of the sample was measured at 532 and 600 nm (using a Cecil CE2501 spectrophotometer). The MDA content was calculated using the equation (1).

$$\text{MDA (mg g}^{-1}\text{ FW)} = [(A_{532} - A_{600}) \times W \times 1000]/116 \quad (1)$$

Where A_{532} and A_{600} are the sample absorptions at 530 and 600 nm, respectively, W is the sample weight (mg) and 116 is dilution factor

Total phenol content

The total phenol content was determined by the Folin-Ciocalteu procedure (Spanos and Wrolstad, 1990). Tuber tissue (0.5 g) was homogenized with 10 ml of 80% methanol. The mixture was centrifuged (10000 rpm, for 10 min). Then 10 µl of the supernatant, 490 µl of distilled water and 500 µl of Folin-Ciocalteu reagent were mixed and incubated under dark ambient conditions (24±1°C) for 3 min. Then 500 µl of sodium carbonate (1%) was added and the mixtures were re-incubated under the same conditions for 30 min. The absorbance was measured at 750 nm using a Cecil CE2501 spectrophotometer and the phenol content was expressed in µg gallic acid g^{-1}FW, using a gallic acid (0-0.1 mg ml^{-1}) standard curve.

Antioxidant activity (DPPH)

The DPPH (2,2-Diphenyl-1-Picrylhydrazyl) assay was done according to Singleton *et al*. (1999) procedure. Briefly, 0.1 g of tuber tissue was powdered in liquid nitrogen and then 100 ml of 96% ethanol was added. After centrifuging (at 3500 rpm, 5 min), 950 µl of 0.1 N DPPH was added to 50 µl of each sample and stirred immediately. Each sample was then kept in ambient dark conditions for 30 min. Finally, the absorption of the extract was measured at 517 nm. The antioxidant activity was evaluated using equation (2).

$$\text{Antioxidant activity (\%)} = [(\text{Acont} - \text{Asamp})/\text{Acont}] \times 100 \quad (2)$$

Where Acont and Asamp are absorptions of the standard and the sample, respectively.

Protein content

Tuber tissue (0.1 g) was homogenized in 1 ml of 50mM sodium phosphate buffer (containing 129.18 mM NAH_2PO_4, 383.96 mM NA_2HPO_4, 12.66 mM EDTA, pH=7). The homogenates were then centrifuged (10000 rpm, 4°C for 10 min). The Bradford solution (1 ml) was added to the supernatant (50 µl) and the absorbance was measured at 595 nm. The protein content was evaluated according to the standards curve of Bovine serum albumin (BSA) and expressed in mg g^{-1} fresh weight (Bradford, 1976).

The assay of protease, catalase, peroxidase and polyphenol oxidase activities

The radish tuber (0.5 g) was powdered with liquid nitrogen and mixed with 1ml of the extraction solution (containing 100 ml of 50 mM phosphate buffer, 1.27 mM of EDTA and 4Mm of PVP). The mixture was then centrifuged (1000 rpm, 15 min). Afterwards the supernatant, as an enzyme extract, was used for determining the activity of the following enzymes (Dhindsa *et al*., 1981).

The protease activity was determined using a pro-

cedure defined by Homaei and Samari (2017). Briefly, 50 µl of the enzyme extract was mixed with 350 µl of 50mM sodium phosphate buffer (pH=7.5) and then 800 µl of 1% casein was added. The mixture was incubated for 10 min at ambient temperature. Then 400 µl of 10% TCA was added and the mixture was again re-incubated at ambient temperature for 20 min. Finally, the samples were centrifuged (10000 rpm, 10 min) and the absorption at 280 nm was measured. The coefficient of excitation was 26.40 mM^{-1} cm^{-1}.

To assay the catalase activity, 50 µl of the enzyme extract was mixed with 1 ml of the catalase reaction solution (containing 50 mM phosphate buffer with pH=7 and 15 mM H_2O_2). Then the absorption was measured at 240 nm and the coefficient of excitation was 39.4 $mM^{-1}cm^{-1}$ (Dhindsa *et al*., 1981).

To determine the peroxidase activity, 33 ml of the enzyme extract was mixed with 1 ml of peroxidase reaction solution (containing 13 mM guaiacol, 5 mM H_2O_2 and 50 mM phosphate-potassium buffer with pH= 7). The sample absorption was then measured at 470 nm and the coefficient of excitation was 26.6 mM^{-1} cm^{-1} (Chance and Maehly, 1995).

In order to assay the polyphenol oxidase (PPO) activity, 100 µl of the enzyme extract was mixed with 1 ml of pyrogallol reaction solution (containing 2.5 ml of 50 mM potassium phosphate buffer and 200 µl of 0.2 M pyrogallol). The sample absorbance was then measured at 280 nm and the coefficient of excitation was 26.4 $mM^{-1}cm^{-1}$ (Kar and Mishra, 1976).

Data analysis

The statistical analysis was done using SAS (version 9.1.3) (SAS Institute Inc. Cary, NC, USA, 1990). The Shapiro-Wilks test confirmed the data normality (procedure: PROC UNIVARIATE, SAS). The Multivariate Analysis of Variance was performed related to the observation period and cumin levels, both of which were considered as independent variables (procedure: PROC GLM, SAS). Pillai's trace test confirmed the variance homogeneity (procedure: PROC GLM, SAS). Tukey's test was used in order to compare the mean values (procedure: Files, Sedit, Factor, Range, P<0.01, MSTATC). Excel 2013 was used to draw the figures. The presented mean values are the average of two growing seasons.

3. Results

Color parameters

The results indicated that all color parameters were influenced during the observation period. A declining trend was observed in the L* value after nine days of storage. The highest L* value (35.1±0.56) was observed on day 0 (in control plants) and the lowest value (23.19±0.97) on 9 days (in control plant). However, over the storage period of tubers, this factor increased in cumin EO treated tubers. Increase of 17.43 % and 17.32% in L* value in the 9 days of treatment was the results of 12.5 and 25 ppm cumin EO application, respectively (Fig. 1A).

The results indicated that over the storage period, the a* trend was declining. The highest (30.32±0.75) and the lowest (19.35±0.88) values were observed on the 0 and 9 days, respectively, in the control after being under cold storage. But, increasing the concentration of cumin EO in each measurement period (3, 6 and 9 days), led to an increase in a* value. Based on the results, the highest value (29.42% increment) occurred at 25 ppm cumin EO on 3 days (Fig. 1B).

According to our results the b* value showed an ascending trend and rose from 10.5±0.65 on day 0 to 16.87±0.59 on 9 days of 25 ppm EO treatment. Moreover, increase in the concentration of cumin EO made significant increments in b* value (8.37%,

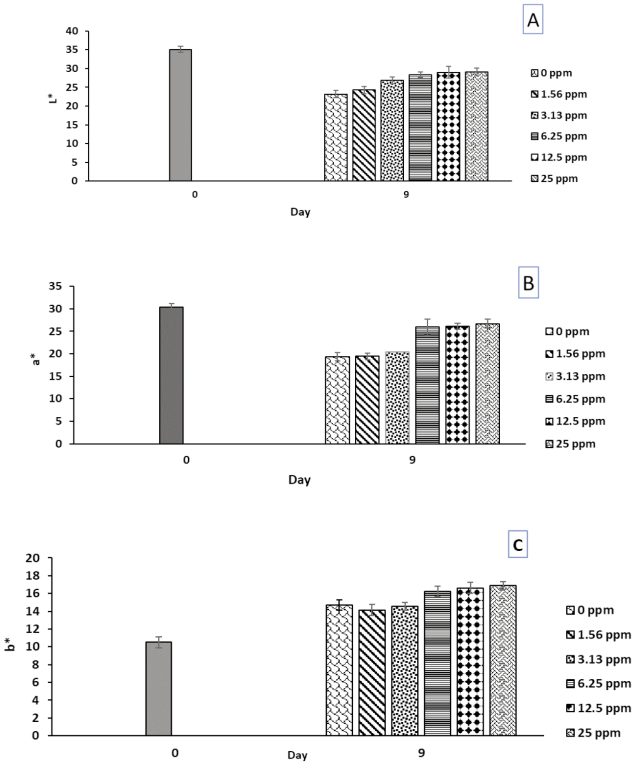

Fig. 1 - The influence of cumin EO concentrations on the L* (A), a* (B), and b* (C) values under different observation periods in cold stored-radishes. Means ± SD of six replicates of two seasons are given (Tukey, p<0.01).

3.43% and 12.64%, respectively on 3, 6 and 9 days) (Fig. 1C).

MDA, phenol contents and antioxidant activity

The MDA and phenol contents, along with the antioxidant activity, were significantly affected by the EO levels and storage duration. During the nine days of radish storage, the MDA, total phenol content and the antioxidant activity showed ascending trend. Furthermore, the cumin EO treatment resulted in a significant reduction in MDA and phenol contents (Figs. 2A, B).

According to our findings, malondialdehyde generally increased as a result of storage period extent. The MDA content of the first assessment, day 0, (0.75±0.16 mg g^{-1} fresh weight) rose to 3.29±0.15 mg g^{-1} fresh weight in the last assessment (9 days of storage). The cumin EO-treated tubers had 0.76±0.14 mg g^{-1} fresh weight MDA content at 25 ppm on 9 days. However, in each assessment period, a declining trend in malondialdehyde content was a result of increment in the concentration of cumin EO. The highest amount of MDA was related to the control treatment value on the 9 days (3.29±0.15 mg g^{-1} fresh weight) and the lowest value (0.55±0.14 mg g^{-1} fresh weight) was recorded at the 25 ppm cumin EO on the 3 days (Fig. 2A).

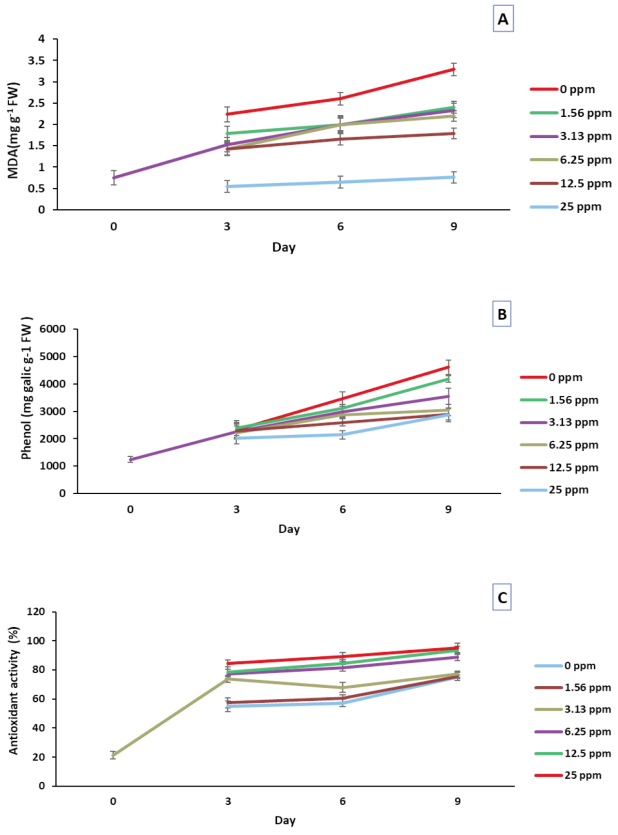

Fig. 2 - The influence of cumin EO concentrations on the MDA (A), phenol content (B), and antioxidant activity (C) under different observation periods in cold-stored radishes.

The results indicated that the total phenol content showed an increasing trend over the storage period. The phenol content rose from 1243±116.19 µg of gallic acid g^{-1} fresh weight, in the first observation (day 0) to 4613.05±258.90 µg of gallic acid g^{-1} fresh weight on the last day of storage. Although, the total phenol content showed a declining trend, with increasing the concentration of cumin EO. The lowest content of total phenol was observed at 25 ppm cumin EO on the 3 days (2024.03±212.84 µg of gallic acid g^{-1} fresh weight). (Fig. 2B).

Regarding the antioxidant activity, over the duration of storage period and also with increasing the concentration of cumin EO, an ascending trend was observed compared to the control. The value of antioxidant activity on day 0 (21.2±2.49%) for control plants, improved significantly on days 3 (54.98±3.62%), 6 (57.19±3.33%) and 9 (75.24±1.41%). The 25 ppm cumin treated-tubers showed a same trend, which was reached from 84.37±2.41% on 3 days to 95.18±3.07% on 9 days (Fig. 2C).

Protein content and enzyme activities

The effect of low temperature storage on the radishes caused in the reduction in the protein content along with an increase in the activity of protease. Different levels of cumin EO in each observation period had a significant effect on both traits (Figs. 3A, B). The results indicated that the protein content decreased over the storage period of radish. The protein content in the control treatment on the day 0 measurement was 27.1±0.33 mg g^{-1} fresh weight and reached to 15.48±0.94 mg g^{-1} on the 9 days. The highest content of protein was related to 1.56 ppm cumin EO (26.78±0.33 mg g^{-1} fresh weight) on the 3 days and the lowest content was related to 3.13 ppm cumin EO (26.4±0.32 mg g^{-1} fresh weight). On the 6 and 9 days of storage, the highest value was occurred in 25 ppm cumin EO (25.29±1.22 and 19.40±1.32 mg g^{-1} fresh weight increment) (Fig. 3A).

The protease activity on day 0 (1.1±0.16 µmol $min^{-1}g^{-1}$ fresh weight) had an ascending trend during 9 days of cold storage. Also increasing the concentrations of cumin EO (6.25±0.11 ppm and more), increased the protease activity. This trait was varied from 1.35±0.15 to 2.53±0.20 µmol $min^{-1}g^{-1}$ fresh weight on the 3 days and from 4.93±0.11 to 6.93±0.14 µmol $min^{-1}g^{-1}$ fresh weight on the 6 days, as a result of different cumin EO levels. However, on

the 9 days of storage, the protease activity varied from 9.55±0.11 to 11.9±0.15 µmol $min^{-1}g^{-1}$ fresh weight as a result of different EO concentrations (Fig. 3B).

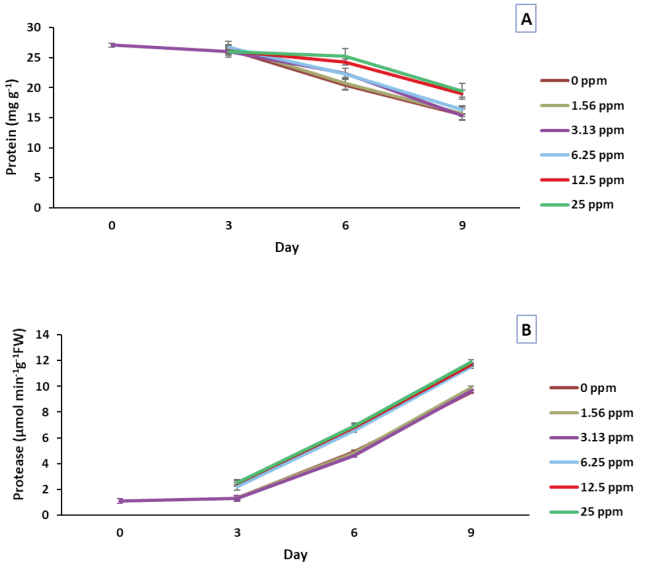

Fig. 3 - The influence of cumin EO concentrations on the protein content (A) and the activity of protease (B) under different observation periods in cold stored-radishes. Means ± SD of six replicates of two seasons are given (Tukey, p<0.01).

There were enhancements in the activities of radish antioxidant enzymes (Figs. 4A, B, C). According to figure 4A, a weak increment was observed in the catalase activity, by increasing the storage days until 6 days. Nonetheless, the first measurement did not differ much than the second measurement. However, on 9 days, the highest activity was observed in all concentrations compared to the control. Different concentrations of cumin EO showed various patterns on different days. On 3 days, the lowest catalase activity was observed in the control (106.34±12.39 µmol $min^{-1}g^{-1}$ fresh weight) and the highest was at 1.56 ppm cumin EO (132.51±22.27 µmol $min^{-1}g^{-1}$ fresh weight). The highest enzyme activity on the 6 days was at 3.13 ppm (209.40±18.38 µmol $min^{-1}g^{-1}$ fresh weight) and the lowest was at 6.25 ppm (202.27±17.46 µmol $min^{-1}g^{-1}$ fresh weight). On 9 days, the trend was completely declining, as the highest enzyme activity was in the control (1013.45±19.26 µmol $min^{-1}g^{-1}$ fresh weight) and the lowest was observed at 25 ppm cumin EO (602.72±19.07 µmol $min^{-1}g^{-1}$ fresh weight) (Fig. 4A).

Peroxidase activity increased over the storage period and changed from 78.23±2.4 µmol $min^{-1}g^{-1}$ fresh weight on day 0 to 178.94±4.39 µmol $min^{-1}g^{-1}$ fresh weight in the last observation (9 days). In addition, increasing in the EO concentration reduced the activity of this enzyme. Accordingly, the lowest values (29.81±4.73, 45.90±4.42 and 61.41±3.76 µmol $min^{-1}g^{-1}$ fresh weight) of the peroxidase activity were observed at 25 ppm cumin EO on the 3, 6 and 9 days, respectively and the highest activity was in the control (113.80±4.60, 149.79±3.72 and 178.94±4.39 µmol $min^{-1}g^{-1}$ fresh weight, respectively) on 3, 6 and 9 days, respectively (Fig. 4B).

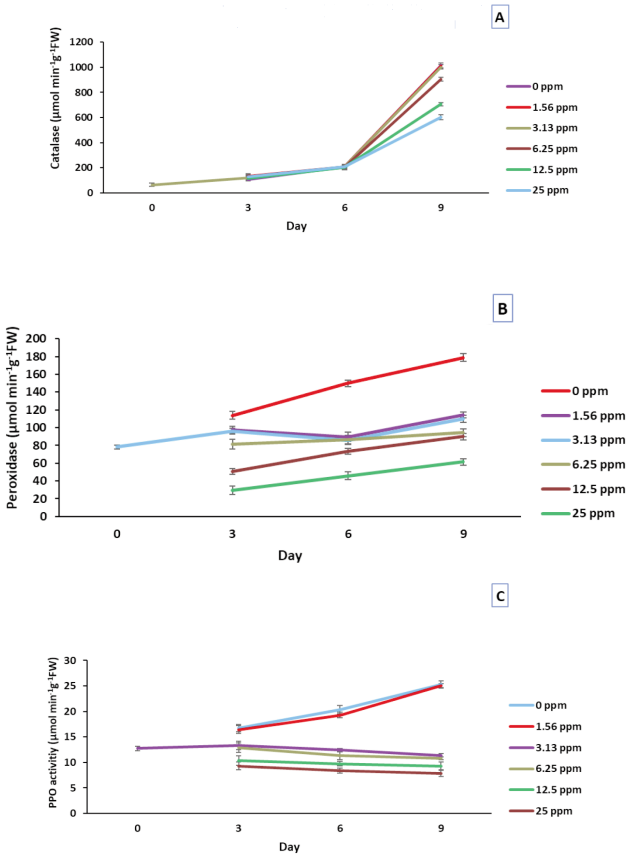

Fig. 4 - The influence of cumin EO concentrations on the activity of catalase (A), peroxidase (B), and PPO (C) under different observation periods in cold-stored radishes. Means ± SD of six replicates of two seasons are given (Tukey, p<0.01).

The activity of polyphenol oxidase enzyme increased over the storage days (from day 0 to 9 days) at zero and 1.56 ppm; as the highest activity (25.31±0.73 µmol $min^{-1}g^{-1}$ fresh weight) was observed in cumin EO-free tubers on 9 days. Changes in the activity of polyphenol oxidase on 3 and 6 days showed similar trends. Its activity on day 0 (12.76±0.45 µmol $min^{-1}g^{-1}$ fresh weight) rose to 25.31±0.73 µmol $min^{-1}g^{-1}$ fresh weight on the 9 days. In the cumin EO treated tubers, the PPO activity indicated a declining trend, over time and the lowest activity (8.44±0.49 µmol $min^{-1}g^{-1}$ fresh weight) was observed at 25 ppm, on 6 days (Fig. 4C).

4. Discussion and Conclusions

Excess production of ROS and oxidative burst, under cold conditions, elicit some plant physiological reactions. The formed ROS interact with cellular components, trigger cascade of oxidative responses, peroxidase lipids, degrade proteins, inactivate enzymes and damage the DNA (Mittler, 2002). Exposure to cold conditions causes the discoloration of radish and production of cracks on its edible parts (Abdel, 2016). In addition, low temperatures influence carbohydrate accumulation in radish roots, accelerate plant growth rates and increase the shoot/root biomass ratio (Sirtautas *et al*., 2011).

Color is an important factor in the consumer's acceptance of foods. The L*, a* and b* values describe color parameters of food products (Walkowiak-Tomczak *et al*., 2008). The color of stored-fruits and vegetables mostly changes and turns darker over time. Hernandez-Munoz *etal*. (2008) reported a decrease in the values of color indices of cold stored-strawberry fruits. In the present study, there was a significant reduction in L* and a* values, despite an increase in b*of radish tubers through storage period, which was related to a reduction in water content and product browning (Hassani *etal*., 2012).

The impact of organic compounds on the color characteristics of horticultural products has been reported previously (Raybaudi-Massilia *etal*., 2008; Asghari Marjanlo *et al*., 2009). Clove EO prevented browning of grapes by preventing water loss (Martinez-Romero *et al*., 2007). According to our findings, treatment with different cumin EO concentrations caused different color values in the radishes.

Cold disturbs the electron transfer chain in mitochondria and chloroplasts. This happens by an excess production of ROS and causes oxidative damage to the membrane, thereby accelerates the lipid peroxidation and MDA over production (Larkindale and Huang, 2004). The results of this study showed a significant increase in the malondialdehyde content during the nine days of cold storage. Antioxidant compounds inhibit free radical's generation, interrupt its functions or lead to ROS destruction (Melo *et al*., 2005; Srivsatava *et al*., 2011). The cinnamon EO has reportedly prevented the membrane lipids peroxidation in peaches (Montero-Prado *et al*., 2011). Our findings regarding the cold-stored radishes treated with cumin EO, confirm this finding.

In this work, the antioxidant capacity of cold-stored radishes was assayed using the scavenging ability of DPPH radicals, which is used widely for evaluating the radical scavenging effects of chemical and organic materials. DPPH functions by absorbing electrons or hydrogens, thereby becomes a stable molecule (Sagar and Sing, 2011). The results indicated that the scavenging capacity of cumin-treated tubers was significantly higher than those of the control, proving that cumin improved the scavenging ability in radish tubers. According to our results, cumin EO displayed a dose-dependent manner in the scavenging of free radicals, as previously reported by Dua *et al*. (2012). Similarly, the antioxidant properties of peppermint and clove oils can be attributed to the ability of free radical scavenging (Tripathi and Dubey, 2004).

Polyphenol compounds naturally show an ability to scavenge ROS. They are considered to be the effective non-enzymatic antioxidants (Ma *et al*., 2011; Zrig *et al*., 2011).

The application of organic compounds was reportedly capable in improving the phenol content of cold-stored mangoes (Wang and Lin, 2000; Razzaq *et al*., 2015). This can occur through changes in both polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activities (Sun *et al*., 2010; Deng *et al*., 2015). In our work, keeping the radish tubers in cold storage conditions made an increase in their phenol content. The application of cumin EO improved the antioxidant capacity and gradually decreased the phenol content.

The lethal impact of oxidative stress on biological organisms is correlated to the destruction of proteins and the inactivation of enzymes (Srivsatava *et al*., 2011). In the present study, the cold stored radishes displayed a visible decrease in protein content and an increase in antioxidant enzyme activities. The antioxidant enzymes act as agents that trigger detoxification at low temperatures (Mittler, 2002) and convert free radicals to the compounds that eventually release water and oxygen (Zheng and Tian, 2006). Superoxide dismutase detoxifies ROS by generating H_2O_2. Then, ascorbate peroxidase and catalase breakdown the H_2O_2 (Wang, 1995). Catalase converts free radicals to oxygen and water (Zheng and Tian, 2006). Our results of increases in catalase, peroxidase and PPO activities in cold stored radishes, confirmed the previous researches.

Essential oils can be considered as agents that aid the antioxidant defense system, reduce the release of radicals, prevent the destruction of cells and thus increase stress tolerance of plants (Holley and Patel, 2005). The usage of carvacrol and anethole in raspberry causes a reduction in antioxidant enzyme activ-

ity (Jin *et al*., 2012). Lipoxygenase activity is also reported to be reduced in cinnamon-treated peaches (Montero-Prado *et al*., 2011).

Microbiology limit test confirmed that bacteria, mold and yeasts in our essential oil sample can be ignored. Also it has no *Salmonellae* neither *Escherichia.coli*. The density of cumin essential oil is 0.90 (Lewis, 1999). The pale yellow color and strong odor of our essential oil sample was similar to standard reference. Cumin seeds contain flavonoids (β-pinene, p-cymene, cumin aldehyde and cuminyl alcohol) are recognized to have antioxidant activity and scavenging capacity of the superoxide anion (Sowbhagya 2013). The refractive index of an essential oil is a unique number that designates how the oil responds to and bends light. Essentially, it is a measurement that tests how the speed of light is altered when passing through the oil. The refractive index of our essential oil sample (1.48) and cumin aldehyde content (36.33%) were close to which reported earlier (1.49 and 45%, respectively) (Fahlbusch *et al*., 2005). Cumin aldehyde is a constituent of the essential oils of eucalyptus, myrrh and cumin (Morshedi *et al*., 2015). β-pinene is a monoterpene, an organic compound found in cumin essential oil (Li and Jiang, 2004). The presence of polyphenolic compounds in cumin EO inhibits protein oxidation and enzyme inactivation (Melo *et al*., 2005). According to our findings, cumin EO improved the antioxidant system of tubers so caused in decreased catalase, peroxidase and PPO activities.

The market for chilled-fresh products has a dramatic rise in the recent decade, encouraged mostly by the consumer request for nutritious, fresh and additive-free products. Hence, the food industry has to respond with novel preservation, storage and handling strategies. In our study, the storage of greenhouse-harvested radishes at low temperatures declined the L* and a*values, and protein content during nine-day of storage, but increased the b* value, phenol content, antioxidant capacity, MDA level, PPO, peroxidase, catalase and protease activities. The application of cumin EO reduced the MDA content, polyphenol oxidase and peroxidase activities. Finally, cold-stored radishes received the most effective treatment of cumin EO at the concentration of 12.5 and 25 ppm.

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A new pneumatic harvester for improvement and facilitation the harvesting of the olive fruits

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Abstract: The objective of this study was to measure and evaluate the performance of a New Pneumatic Harvester (NPH) for harvesting the olives fruit and compare the results with those of similar cases. The study involves two sections, namely, the NPH and the collector system. Two oily types of olive ('Mari' and 'Yellow') were selected to evaluate the NPH and the collector system. The randomized complete block design and Duncan's multi-scope test were used for variance analysis and means comparison purposes, respectively. The dependent parameters used to evaluate the NPH were harvesting productivity, harvesting efficiency, and leave downfall percent. Also, the fruit damage was selected for the collector system. Results showed that the NPH can harvest 92% of olive fruits. The highest amount of harvesting productivity belongs to NPH of 29.47 kg/h. The percent of leaves downfall in the harvesting process was found to be 2.55%. Using the collector system can reduce the level of damaged fruit from 60.8% to 25.12% and from 60.54% to 24.54% in the Mari and Yellow variety.

1. Introduction

The olive tree is one of the Mediterranean plants that its fruits used as a cannery and oil extraction (Barbera *et al*., 2013). Olive has traditionally been known among people, which is also important for health and used as a strategic food in some countries (Fiorino *et al.,* 2010).

One of the major problems with the olive plant is harvesting which is mostly done in different ways: (a) picking up the fruit on the ground (b) harvesting manually (c) harvesting via branch shaker (d) harvesting mechanically (Lupi *et al.,* 2012). Harvesting is the final step in field production of olive fruits, but if done at the wrong time or in the wrong way it can markedly affect the grower's net return. The economic success of super-intensive olive plantations is mainly achieved due to the full mechanization of harvesting (Ottanelli *et al*., 2019). The olive harvest is expen-

sive in terms of wages and adequate labor supply has brought about many problems (Zipori *et al*., 2014). Fruit damage, damages related to the olive tree, quality reduction, human injuries, time consumption, and costly process in the traditional ways are problems existing in the harvesting process. Mechanized harvesting is done in many different types, such as the trunk and limb shakers. Because of device feature like power supplement implementation of this shakers are difficult (Çakmak *et al.*, 2011). Although, the mechanized harvesting methods are very efficient but they have an high usage costs. Therefore, the best harvest system should be inexpensive and highly efficient.

Designing of shakers, harvesting efficiency analyzing and tree damage for olive fruits are the attractive topics in the mechanization field, so in this regards researchers studied the effect of shaking force and the amplitude of the shaking on the mechanized harvesting of olive. They showed that an increase in olive tree diameters trunks led to an increase in shaking force and a decrease in amplitude of the shaking. So, for mechanized harvesting in the big olive trees with big trunk diameters more power must be applied (Babanatsas *et al.*, 2019). A comparative study was done to investigate the various ways of olive harvesting. Results show that harvesting through shaking the stem is the best way to remove the olive fruit from a tree (Yousefi *et al*., 2010). Five different olive harvesters were investigated. The flat type olive harvester has more vibration value index (Çakmak *et al.*, 2011). The effect of two types of olive tree harvesting (Trunk Shaker and Mechanical Harvest Aid) on harvesting time were studied. Results show that harvesting the olive trees with mechanical harvest aid can save more time (Nayeri and Torkashvand, 2016). Results concerning the examination of harvesting type on harvesting efficiency show that with increasing the fruit removal force the harvesting efficiency was decreased (Zipori *et al*., 2014). A study on four types of olive harvester; plastic combs, small shakers, rotating combs, and vibrating combs were done. Researchers indicated that all types of harvesters except the small shaker improve the worker's productivity (Bentaher and Ben Rouina, 2002). Results of the study on a pneumatic harvester showed that manual harvesting and mechanical harvesting methods had a significant effect. The pneumatic comb machine had higher bruised fruit than the manual harvesting (Ahmad, 2018). With an increase in the operating velocity, the productivity harvesting and damage percentage increased and the 1250 rpm operating velocity value could be a good alternative for harvesting (Mansour *et al*., 2018). Two methods of harvesting olive fruit, namely, mechanized harvesting with Pneumatic Harvester (PH) and Manual Harvesting (MH) were investigated, which indicated that due to the presence of the collector system, using mechanized harvesting can reduce the fruit damages (Plasquy *et al*., 2019). Mechanical canopy and trunk shaking were investigated in line with the harvesting mechanization of table olive orchards. Accumulating the trunk shaker and shaker combs can increase the bruise index of olive fruits. The lowest bruise index of olive fruits belongs to the manually-type harvest with a 0.5% value (Sola-Guirado *et al*., 2020).

The designing and fabrication of olive fruit harvester have long since been the subject of many types of research. Applying some of these systems may exert large costs on farmers, and the efficiency of these machines requires significant economic costs that many producers cannot afford. For this large group of farmers, new models have recently been presented. Nevertheless, the use and utility of some of these machines still entail difficulties, such as the large weight, difficult access to the trunks, or handling the fruit boxes. In this study, results concerning the evaluation of a new design of portable pneumatic olive harvester with a fruit collector system are presented to fix some of the harvesting problems. The evaluation parameters of harvesting machine (such as: the harvesting efficiency, harvesting productivity, and olive tree damage) were compared both with the manual method and similar harvesting methods. Finally, the effect of fruit collector on fruit damage was investigated.

2. Materials and Methods

Vegetal material

Two different varieties ('Mari' and 'Yellow') of olive fruits were selected to evaluation that both of them are Iranian variety and commonly grown for oil and canned production. The first step in designing a new harvesting machine is cognition about crop characteristics such as detachment force from tree branches. For measuring fruit detachment force from tree branches, three tree of each verity were selected and ten olive fruits of each tree accidentally were selected. The force required to detach them was

measured by a mechanical force gauge. The mechanical gauge of FG-5020 model was used and calibrated in the Precision Process Calibration Laboratory with the approval of No. 681 of the Standard Institute of Iran.

The fruit detachment force employed to determine the applied force on the pneumatic jack. So to determine this amount assuming that all of the jaw surface covered by the olive fruit and the detachment force in this situation was calculated.

Tests were conducted in the Ashrafieh gardens in Qazvin province in Iran and the fruit ripening index was 4.52%. The age of olive trees was 20 years and the height of trees was up to 3.5 m.

Harvester machine design

The pipe diameter is a very important factor for designing pneumatic systems. When the low pipe diameter was applied, the pressure dropped and the airflow velocity increased accordingly. In the case of water penetration into the pipe, the clogged pipes situation was expected to happen. Two conditions, namely, 5-8 m/s and 0.5 bar were found to be appropriate for the airflow velocity and the pressure dropped.

The first step for jack design is to define the required pressure and velocity of air to detach the olives from the tree. Equation (1) shows the jack piston, which was used to calculate the air force.

$$F_m = P \times a \quad (1)$$

After selecting jaws frequency and active volume of the piston, the cam length and Air Mass Flow (AMF) were calculated. The amount of AMF for jaw movement to open the jaw was calculated by equation (2), and in the closed cycle, equation (3) was used for the AMF. Finally, the total AMF to open and close the jaws was calculated by equation (4).

$$Q_{ext} = \pi \times (D^2/4) \times \chi \times n \times (P_1 - P_0)/P_0 \quad (2)$$

$$Q_{ret} = \pi \times [(D^2-d^2)/4] \times \chi \times n \times (P_1 - P_0)/P_0 \quad (3)$$

$$Q_{tot} = \pi \times [(2D^2-d^2)/4] \times \chi \times n \times (P_1 - P_0)/P_0 \quad (4)$$

Assuming that the total pneumatic energy was converted to the kinematic energy, the maximum velocity could be determined using Equation (5).

$$E = \frac{1}{2} m (v_{max})^2 \quad (5)$$

The total energy required was calculated by equation (6).

$$E = [\frac{1}{2} P \times \pi D^2 \times m (0.5)^2] / (2g \times 4) \quad (6)$$

The total energy for opening the jaw was calculated by equation (7):

$$E = 5 \times 10^{-4} \times P (D^2 - d^2) \quad (7)$$

The determinations show that the detachment force of olive fruit from the tree and the pressure required for the pneumatic jack were 10 N and 7 bar, respectively. The machine had one handle and it was gripped by one operator's hand and a telescoping rod up to a maximum length of 3 m. The harvesting mechanism had two jaws and each jaw contained 7 fingers that allowed for easy and deep access to all types of foliage without getting caught in the branches. 90 degrees was selected for the angle between two jaws based on the previous researches. The jaws had 18 cm length and the distance between them must be equal to the olive fruit width, so 1 cm distance was considered. We assume that in every impact of jaws, 23 olive fruits were detached from tree branches. So, the total force required for each impact was determined. The force related to the pneumatic cylinder was determined by equation (8).

$$2f_d Cos (\Theta/2) = F \quad (8)$$

The amount of force of each jaw for the olive fruit detached from the tree was 325.3 N, and for calculating the piston diameters equation (9) was used.

$$F = A \times P \quad (9)$$

After determining the piston diameters, the length of the cam was calculated and the standard jack with these characters was selected.

The cylinder provides a closed system which assists the movement of the piston. For moving the piston across the cylinder in high frequency, valves must be used or the manual control applied. Because of the operation conditions, the pneumatic valve was selected to change the airflow direction. When the pneumatic valve was in position A, high air pressure pushed the piston to the right side (Fig. 1). By moving the piston to right, the airflow was transferred to the C input of the pneumatic valve which changed the direction of airflow, resulting in the activation of the B position of the pneumatic valve. When high-pres-

sure air moves to the B output of the pneumatic valve, the piston moves to the left side, and air moves toward the D input of the pneumatic valve, and such a cycle is replicated. After assembling the parts, the frequencies of these cycles were measured by an electronic eyes sensor. The frequency of the piston movement is 5.21 Hz in 7 bar air pressure. So, after defining design parameters, the mechanical value of No. G1/8'' was selected. Other parts of the harvester such as the chain, cam, and pneumatic value along with the parts were designed.

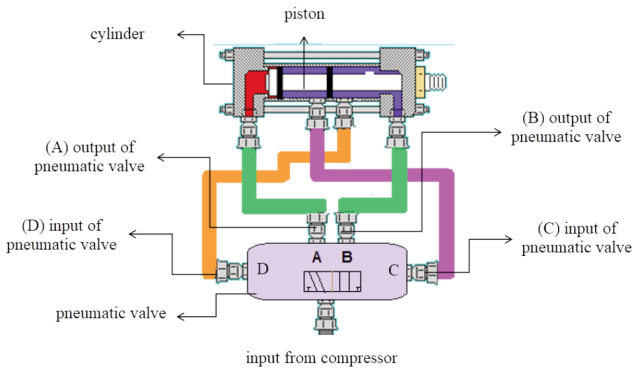

Fig. 1 - Schematic of the piston, cylinder, valve, and connector pipes

Figure 2 shows the details of NPH, including harvester jaws, cylinder and pneumatic valve, power transmission, and telescoping rod.

The common properties of the collector system for olive fruit are shown in Table 1. By applying this system, the olive fruits falling from the tree were collected into a box. The driving power of the collector system are provided by labor.

It is possible to conclude from figure 3 that the collector system is ready to collect the fruits in the box. The collector system includes the wheels, steering wheel, bendable bars, wire for hold cover, polyester cover, trunk holder, position for boxes, and outlet, all of which are assembled on chassis.

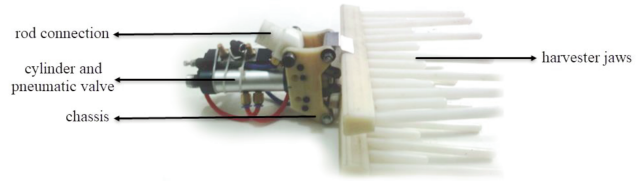

Fig. 2 - The different parts of the new pneumatic harvester.

Table 1 - Collector system properties

Parameters	Value
Chasses length	140 cm
Chasses width	95 cm
Trunk holder height	60 cm
Trunk diameter	up to 40 cm
Cover diameter	300 cm

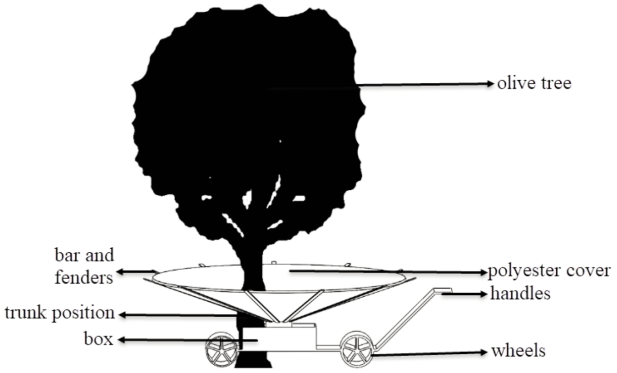

Fig. 3 - Collector system parts.

Statistical design

The variance analysis was done in randomized complete block design with three replicate and the statistical computations as well as analyzing the data were performed by the MSTAT-C software. Duncan's multi-scope test was used for comparison of the means.

The performance of the developed harvest machine was measured by the Harvesting Productivity (HP), harvesting efficiency (HE), and leaves downfall percent (LD). The performance of the developed collector system was measured by the olive fruit damage (FD). For measuring the harvesting productivity of olive fruit harvester, the total mass of detachment fruits was recorded using the machine. The total harvesting time includes selecting, detaching fruit, and the time required for moving machine between olive trees inside the field. The productivity of the operated harvester was calculated using equation (10) (Polat *et al.*, 2007):

$$HP = k_1/T \quad (10)$$

Harvesting efficiency was calculated by equation (11) (Srivastava *et al.*, 2006).

$$HE = [k_1/(K_2 + K_1)] \times 100 \quad (11)$$

The percent of olive leaves downfall is defined as the percent of branches and leaves fall down by the harvester and was calculated by equation (12):

$$TD = M_{bl} / M_{tbl} \times 100 \quad (12)$$

The total weight of branches and leaves of the olive trees previously measured. Some trees that had to be removed were collected and weighed separately, finally the means weight of the branches and leaves of three trees were used in the equation (12).

Fruit damage includes the visual inspection and the fruit appearing broken to the naked eye. Fruit damage was calculated by equation (13).

$$FD = M_d / M_t \times 100 \quad (13)$$

The main criteria for designing the New Pneumatic Harvester (NPH) were as follows: easy design, simple to use, low weight, high productivity, less fruit damage, and low operating costs. The main novelty of this type of harvester is low operating costs and use in gardens far from the energy networks. A double-sided jack was used to provide jaw movement so, the required airflow rate, pressure drop, airflow control valve, and jack size must be defined.

To show the advantages and disadvantages of the present device with existing devices, the results of the evolution of the NPH compared with a Pneumatic Harvester (PH) that the features of the PH are shown in Table 2.

Table 2 - PH technical features

Number	Feature	Amount
1	model	Campagnola srl
2	weight	1 kg
3	length	1.8 - 3 m
4	Max pressure	7 bar

3. Results and Discussion

Results of the harvesting tests

Harvesting productivity was calculated among 3 types of different harvesters' system (MH, PH and NPH), using the equation number "10" (Table 3). Results showed that the three harvesters have a significant difference at 5% levels of probability. Due to different treatments, the means were compared and the NPH was found to have more HP placed in a group (a) with an amount of 29.47 kg/h. The PH was placed in a group (b) with an amount of 21.33 kg/h and the MH was placed in a group (c) with an amount of 9.37 kg/h.

Table 3 - Result of variance analysis velocity (m s^{-1}) for harvesting productivity

Parameters	df	Sum of squares	Means of squares	F
Block	2	34.925	17.462	1.6126 *
Harvest method	2	283.554	141.777	13.093 *
total	4	43.314	10.828	
CV	25%			

* = significant at 5% of probability.
** = significant at 1% of probability.
NS = not significant.

Figure 4 shows the HP means for three harvest methods. Due to the special properties of NPH such as the power of vibration, frequency, and shape of a bar, the HP of this machine was more when compared to two other methods of harvesting. Kermani (2016) indicated that the mounted branches shaker machine has 130.72 kg/h of HP, but it fails to be used in traditional gardens. This type of harvester needs a tractor to perform, which is not feasible for small gardens (Kermani, 2016).

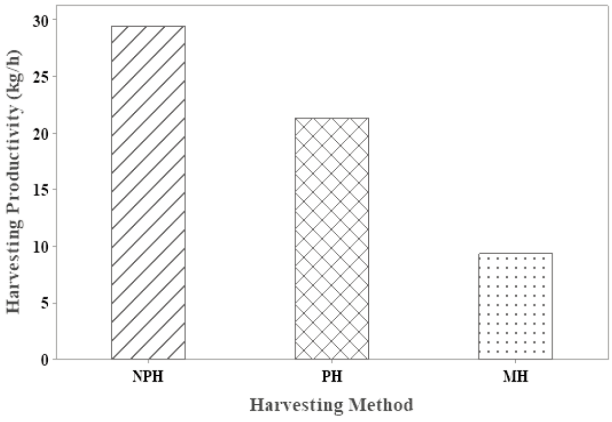

Fig. 4 - Harvesting productivy means for three harvesting methods. NPH= New pneumatic harvester; PH= pneumatic harvester; MH= manual harvesting.

Table 4 shows the results of variance analysis related to the difference between two types of harvesting machine concerning the Harvesting Efficiency (HE). The analysis shows that the effect of machine type on HE is significant at 1% probability. The pneumatic harvester can detach 86% of olives from the tree, but NPH shares 92% of HE. Because of the availability of all olives on the tree, the HE of this method

is considered as 100% for labor in the case of manual harvest (Fig. 5).

It is very important to understand how to place the harvesting head on the telescoping rod. The angle between the harvester head and telescoping rod in the NPH is fixed and the jaws move between 123 to 213 degrees, and such a property differs from the PH. The other important difference resulting in increased HE is the jaws speed, where they move quicker in NPH compared to the PH. Some places of olive fruits on the tree are not available for NPH and PH. The harvester rod plays the important role in the HE. Whatever the harvesters rod has high maneuverability the more olive fruits can detected and the HE increased. Kermani (2016) showed that the olive tree branches shaker machine has 84.51% HE, but compared to other machines, the leaves downfall percent in this harvesting method is high (Kermani, 2016).

Equation 12 was used to determine Leaves downfall percent (LD). After harvesting, all the branches and leaves were collected and weighted. By increasing in branches and leaves that fall from the tree the LD was increased. Table 5 shows the variance analysis among the three harvesting methods and olives types. The effect of variety (type) on LD was not significant, but harvesting methods have a significant effect on LD. Figure 6 shows the LD for three harvesting methods; NPH has more LD than the two other harvesting methods. The speed of jaws in the PH is 1080-1150 rpm, while in the NPH the minimum speed of jaws is 1290 rpm. This difference in jaws speed led to an increase in the leaves' downfall percent.

Table 5 - Result of variance analysis for leaf downfall

Parameters	df	Sum of squares	Means of squares	F
Block	2	0.286	0.143	42503 NS
Harvest method	2	1.006	0.503	14.934 *
total	4	0.135	0.034	
CV	8.93%			

* = significant at 5% of probability.
** = significant at 1% of probability.
NS = not significant.

Table 4 - Result of variance analysis of for harvesting efficiency

Parameters	df	Sum of squares	Means of squares	F
Between data	1	717.883	717.838	118.644 **
Inside data	4	24.203	6.05	
CV	3.25%			

* = significant at 5% of probability.
** = significant at 1% of probability.
NS = not significant.

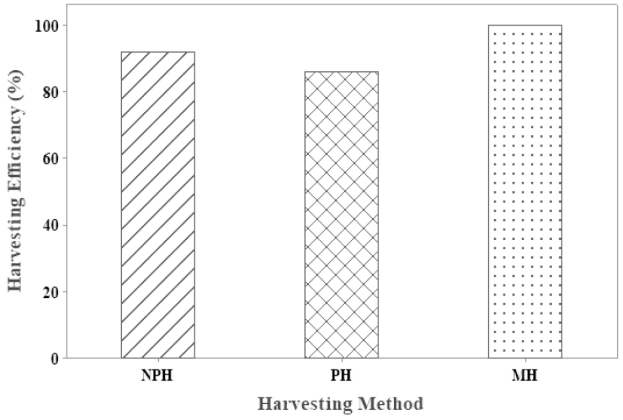

Fig. 5 - Harvesting efficiency means for three harvesting methods.NPH= New pneumatic harvester; PH= pneumatic harvester; MH= manual harvesting.

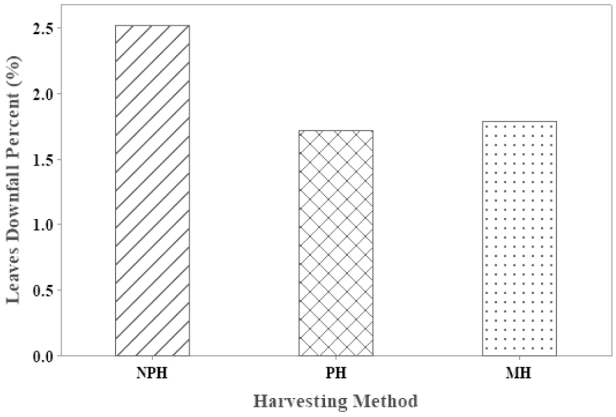

Fig. 6 - Leaf downfall (%) means for three harvesting methods. NPH= New pneumatic harvester; PH= pneumatic harvester; MH= manual harvesting.

Comparison of the means of harvesting method shows that the NPH with an amount of 2.52% LD has the highest detachment of leaves from the tree. The PH and MH were not significantly different, and they were placed in a group (b). The electrical rotation bar in Kermani's (2016) research has the highest LD with a 2.20% damage (Kermani, 2016). Due to the problems with the head location on the handle in the NPH, the amount of LD was increased, however, such a problem can be surmounted.

Product quality results with the use of the collector system

Olive fruits fall on the ground after harvesting and get damaged as a result of this matter. To prevent the olive fruits from damage, a collector system gathering the fruits into the box was designed and manufactured. For evaluating the collector system, two types of olive trees were harvested with the new pneumatic harvester, and the percent of damaged and undamaged olive fruits were investigated. Table 6 shows the damaged and undamaged olive fruit in the NPH with the collector systems and manual harvest without the collector system.

Results show that the use of a collector system can decrease the fruit damage (FD) from 60.80% to 25.12% in the 'Mari' variety. Similar results were achieved for the 'Yellow' variety. Table 6 shows the damaged and undamaged olive fruit for the harvesting of 'Yellow' olive fruit variety in the two harvesting methods. Figure 7 shows the injured, bruised, and uninjured olive fruit percent in fourteen measured samples for the manual harvest. Due to the difference in the weight of olive fruits, the percent of each group shown was based on the number and weight of olive fruits. In the manual harvest, a large portion of the fruits of the olive falls on the ground, then picked up and collected into the box. This process led to the bruising of more than half of the olive fruits. Table 7 shows the means, standard deviation, average deviation, variance, minimum and maximum of weight along with the number of injured, bruised, and uninjured olive fruits in MH and NPH. Results show that after harvesting the fruits of the olive in the manually harvest method, 45.29% of olives fruits were bruised. Bruising occurred on the skin of the olive fruits as a result of fruit hitting on the soil. Also, the injuring situation occurred when fruits were hit by sharp branches or stones.

Table 6 - Damaged and undamaged fruits in two types of harvesting method in Mari and Yellow variety

Cultivar	Manual harvesting		New pneumatic harvester	
	Undamaged fruit (%)	Damaged fruit (%)	Undamaged fruit (%)	Damaged fruit (%)
Mari	39.2	60.8	74.88	25.12
Yellow	39.45	60.54	75.45	24.54

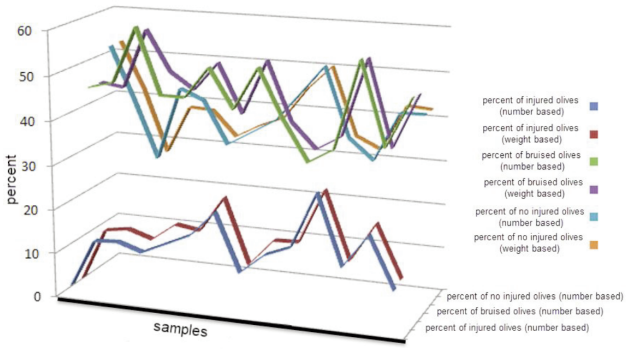

Fig. 7 - Illustration of the injured, bruised, and no injured olive fruit in the manually harvest.

The percent of injured, bruised, and uninjured olive fruits for NPH based on the number and weight is shown in figure 8. The results improved significantly. Most parts of the olives fruit had no injury in the harvesting process. Olive fruits that were detached from the tree fall in the collector systems and avoid contact with the stones or the soil/ground. Table 7 also shows the results of measuring related to the injured olive fruit parameters. The means of unin-

Table 7 - Results of measuring the injured parameters for the olive fruits in manual harvesting and new pneumatic harvester

Harvesting type	Number of injured olives	Weight of injured olives	Number of bruised olives	Weight of bruised olives	Number of no injured olives	Weight of no injured olives
Manual harvesting	14.87±1.70 *	15.51±1.71	45.42±2.04	45.29±1.98	39.71±1.95	39.20±1.87
New pneumatic harvester	7.98±1.14	8.33±1.36	15.98±2.75	16.79±2.79	76.02±2.96	74.88±2.98

* Means ± standard deviation.

jured olive fruits in the NPH increased from 39.71% to 76.02%. This means that the NPH was able to save almost half of the olive fruits. The jaws hitting olive fruits and detached fruits from brunches after that fruits falling from the tree and collide with the branch and trunk of the tree. This process caused almost 24% damage to the olive fruits. 16% of the olive fruits in the harvesting process were bruised and the rest of the damaged fruits got the ruptured skin, which was then classified as the injured fruits.

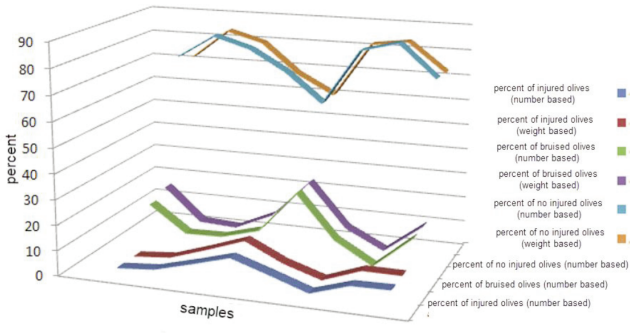

Fig. 8 - Schema of the injured, bruised, and no injured olive fruit in harvesting with new pneumatic harvester.

4. Conclusions

Harvesting is one of the important steps in the olive farming and the device that used also strongly effected on the quality of the harvesting. In this study, tried to investigate the New Pneumatic Harvester performance to show its advantage in compared with other pneumatic harvesters that exist in the market. When considering the fruit ripening index of 4.52%, one can say only 8% of olive fruits remained on the trees. On the other hand, the amount of HP was 29.47 kg/h. This result shows that NPH can detach 92% of olives on the tree in a short time. In the case of the traditional and small gardens, this machine is very practical because it can work effectively in the garden in different situations. The collector system saves more time and prevents olive fruit from damage and can be easily used for every harvesting method.

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AHS
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Endogenous hormone causes flower and fruit drop of wax apple (*Syzygium samarangense* cv. Citra)

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Key words: ACC, cytokinins, fruit drop, IAA, GA_3, wax apple.

Abstract: The aim of this study was to obtain information about the content of endogenous hormones that causes flowers and fruit drop of wax apple. The variables observed in the six stages of flower and fruit development that drop easily and retention of indole-3-acetic acid (IAA), cytokinin (zeatin and kinetin), gibberellins (GA_3), 1-amino cyclopropane-1-carboxylic acid (ACC), total sugar, and starch. Six stages of development of wax apple fruit: (1) Bud Development (initial flowering) 0-3 days before anthesis. (2) Anthesis (perfect blooming flowers), 0-7 days after anthesis. (3) Fruit set, 7-14 days after anthesis. (4) Fruit development, 14-28 days after anthesis. (5) Fruit Maturation, 28-35 days after anthesis. (6) Fruit ripening, 35-50 days after anthesis. The results showed that the content of IAA, zeatin, GA_3, and total sugar of flowers and fruit of wax apple at 6 stages that would fall smaller than those of retention and ACC content and starch was higher in flower and fruits that drop easily than retention. The kinetin content in the flower development that drop easily is smaller than the retention but in the fruit development the kinetin content is not significantly different between those that drop easily and retention.

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The authors declare no competing interests.

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1. Introduction

Water apple originates from the Southeast Asian region (Indonesia and Malaysia), then spreads to the islands of the Pacific and North and Central America. Generally, wax apple fruit is consumed as fresh fruit, but it can also be made for salad and preparations such as pickles, syrups, jellies, and cocktails. Water apple fruit is not only sweet and refreshing but has diversity in appearance. Types of water apple are *Syzygium aqueum* (water apple) and *Syzygium samarangense* (wax apple). The varieties of *Syzygium samarangense* include Delima, Lilin, Camplong, Cincalo, Citra, Kesuma, and Madu, (Kuswandi, 2008).

Corona Virus Disease (COVID-19) is originating from Wuhan, China began to spread throughout the world from January 2020 to 31 December 2020, so there were 83,264,353 cases, with 1,816,164 deaths from 218 affected countries (World Health Organization, 2020). No vaccine has been found to treat patients with the COVID-19 virus. According

to the Ministry of Health of the Republic of Indonesia (2020), prevention of viruses can be done by frequently washing hands, wearing masks, consuming vegetables and fruit, exercise, and adequate rest. One of the prevention efforts that can be done by consuming fruits that contain Vitamin C to enhance immunity. Every 100 g of wax apple fruit contains 22.3 µg of vitamin C (Asia-Pacific Association of Agricultural Research Institutions, 2014). Therefore wax apple is a tropical fruit that is cheap, easily obtained, and contains vitamin C which can be used as an alternative to increasing body immunity as a preventative measure against the COVID-19 virus.

The main problem in the cultivation of wax apple plants is the high drop of flowers and fruit. The high rate drop of flowers and fruit causes a few numbers of wax apple fruit that can be produced. Wax apple drop fruit rate reaches 52% (Khandaker *et al*., 2016). Fruit drop also occurs in other fruits, such as durian flowers reach 95 to 100% (Suparto and Sakhidin, 2013), lychee fruit is 90 to 97% (Stern *et al*., 1995), star fruit is 25 to 30% (Kurniawati and Hamim, 2009), mangosteen flower is 14.1% and mangosteen fruit is 70.1% (Rai *et al*., 2008).

Dropped fruit is a natural phenomenon that occurs in almost all types of fruit. Drop fruit, especially at the beginning of fruit growth, is a mechanism for regulating autoregulation in each plant (Davarynejad *et al*., 2009). Physiologically the loss of flowers and fruit correlates with the limited supply of photosynthate and nutrient adequacy (Iglesias *et al*., 2007), as well as hormonal regulation in the abscission zone (Bangerth, 2000). Flower and fruit drop are also influenced by endogenous hormone content in plants due to high ethylene concentrations and low concentrations of auxin and gibberellins. Auxin, gibberellins, and ethylene are hormones that are directly related to the process of flowers and fruit drop (Bangerth, 2000). Complex hormonal interactions occur during fruit development. Gibberellins and cytokines generally stimulate fruit growth and auxin as a growth stimulator and also as a fruit drop agent. Abscisic acid (ABA) and ethylene are also involved in the process of loss (Sakamoto *et al*., 2008). High ethylene concentrations, low concentrations of auxin and gibberellins, and high ABA concentrations in plants are the main causes of drop fruit (Iglesias *et al*., 2007).

The causes of flower and fruit drop of the wax apple due to imbalance of plant growth regulator as well as cultivation and environmental engineering factors, including pollination, fertilization, fruits set, lack of water and nutrition supply, pest attack, rainfall and wind (Khandaker *et al*., 2016). Research on the use of synthetic Growth Plant Regulators (GPR) to reduce the fruit drop of wax apple has been conducted by Khandaker *et al*. (2013), Khandaker *et al*. (2016), 50 mg l^{-1} Gibberellins (GA_3), GA_3 produces the lowest flower and fruit buds loss of 29% compared to controls (not given GA_3 36%). GA3 20 mg l^{-1} reduced fruit loss 32% compared to 52% control. 2-4-Dichlorophenoxyacetid (2,4 D) 5 mg l^{-1} reduces flower and fruit bud loss by at least 30% and 18% compared to 35% and 40% controls. Naphthaleneacetid Acid (NAA) 5 mg l^{-1} reduced the flower buds and fruits drop as lower as 28% and 30% compared to 30% and 52% controls. The causes of flowers and fruit drop of wax apple are because the content of endogenous hormones is not yet known, so the application of growth plant regulator given is not appropriate to reduce the flowers and fruit drop.

Singh *et al*. (2017) the results of research on the application of growth plant regulator on *Khasi* mandarin (*Citrus reticulata* Blanco) showed an increase in fruit retention and the number of fruit with the application of Urea 1% + 2, 4-D 15 ppm (45.4% and 244, 3 fruits/tree), application of NAA 5 ppm + 2,4-D 10 ppm (44.3% and 241.6 fruit/tree) compared to controls (17.3% fruit retention and 181 fruit/tree). NAA treatment 15 ppm increased the proportion of cape gooseberry cv. Aligarh is retention (71.40%) (Kaur and Kaur, 2016).

The purpose of this study was to obtain information on hormones that causes flower and fruit drop at several stages of the development of wax apple fruit and to obtain differences in the hormone content at how many stages of flower and fruit development are easily dropped and retention.

2. Materials and Methods

The research used an experimental method from May 2019 to April 2020. The experimental design used was a Completely Randomized Design (CRD) with the treatment being tried, namely flower and fruit retention and drop easily of wax apple at six stages fruit development: (1) Bud development (from 0 to 7 days before anthesis); (2) Anthesis (full bloom), from 0 to 7 days after anthesis; (3) Fruit set (the formation of finished fruit), from 7 to 14 days after anthesis; (4) fruit development, from 14 to 28 days after anthesis; (5) Fruit maturation, from 28 to 35

days after anthesis; (6) Fruit ripening (from 35 to 50 days after anthesis). Experiment with 4 replications and data analysis using t-test and advanced test DMRT (Duncan Multiple Range Test). Flower and fruit samples were taken from 46 wax apple plants, Kajongan Village, Bojongsari District, Purbalingga Regency, Central Java. The variables observed were auxin content (IAA), cytokinins (zeatin and kinentin), gibberellin (GA3), ACC (1-Aminocyclopropane 1-Carboxylic Acid), total sugar, and starch.

The samples used for the study were flowers and fruits from 6 stages of development of flowers and water guava. Plants are 5 years old, plant spacing 8 m x 9 m, with a height of ± 7 m and have been fruiting for the last 4 years. Temperature 25.8°C with 76% humidity, sunlight intensity 571 Lux outside the canopy, and under the canopy 529 Lux and soil pH 6.8. Organic cultivation of wax apple uses goat manure at a dose of 100 Kg per plant per year. Irrigation in the dry season with springs that are channeled from a hose from a pipe made by the garden. The results of soil analysis conducted at the Postharvest Center of the Ministry of Agriculture, inceptisol soil type, pH 6.14, organic C 0.769%, total N 0.082%, available N 0.025%, total K 55.960 ppm, K available 30.277 ppm, total P 45.189 ppm, and 1.321% organic matter.

The samples drop easily are taken from the flower and fruit which when the branches or twigs are shaken fall out and after the fall there is a black abscission layer on the flower stalk or fruit. The retention samples are wax apple flower and fruit which if the branches or twigs are shaken not fall off, are still attached to the plant, and usually, the flower stalk or fruit is still present. The sample is placed in an icebox containing blue ice and then taken to the Integrated Research Laboratory, Jenderal Soedirman University to be dried in a 2kxc bench top Vacuum Freeze Dryer. Wax apple samples for bud development, anthesis, and fruit set for 25 hours, and fruit development, fruit maturation, fruit ripening for 55 hours with a temperature of -70°C, and a pressure of 13,332.2 Pa The contents of auxin (IAA), GA_3, zeatin, kinetin, ACC, total sugar, and starch were carried out at the Chemical Laboratory, Center for Postharvest, Ministry of Agriculture, Bogor, West Java, Indonesia.

Procedure for measuring hormone content (auxin, cytokinin, and gibberellins) using HPLC (High-Pressure Liquid Chromatography) at a wavelength of 214 nm, sample temperature of 10°C and column temperature of 25°C, stationary phase using C-18. The formula for hormone content by HPLC is sample area divided by standard area multiplied by standard concentration (Harborne, 1973). Measurement of ACC content according to Lizada and Yang (1979), using GC (Gas Chromatography). The formula for ACC content in GC is sample area divided by standard area multiplied by standard concentration. Measurement of total sugar according to the Indonesian National Standard (1992) using the Luff Schoorl method. Starch calculation according to Horwitz and Latimer (2006).

3. Results

Table 1 shows that the fruit drop easily having lower IAA, zeatin, and GA_3 content than fruit retention in all phases of fruit development. The kinetin content at the bud development, anthesis, and fruit set stages that drop easily is lower than the retention, but the kinetin content at the fruit development, fruit maturation and fruit ripening stages is not significantly different in the fruit that drop easily dan retention. While the ACC content of fruit that drops easily is greater than that of retention.

Table 2 shows that the total sugar content at various stages of flower and wax apple fruit development in flower and fruit retention is higher than that of drop easily. The content of starch in fruit retention of various stages of development of the flower and fruit is lower than those that are of drop easily.

4. Discussion and Conclusions

The IAA content in wax apple flower and fruit which drops easily on bud development, anthesis, fruit set, fruit development, fruit maturation, and fruit ripening is lower than the retention flower and fruit. This is according to Rai *et al*. (2008). The IAA content of mangosteen flowers falling 3.37 ng g^{-1} is lower than the retention rate of 8.80 ng g^{-1} dry weight and IAA content in mangosteen fruit falling 0.83 ng g^{-1} is lower from the IAA content of 6.43 ng g^{-1} retention fruit. Sakhidin *et al*. (2011), the content of IAA in mango fruit loss is lower than that of retention. Gadung 21 and Lali Jowo cultivars that will fall age 6 and 9 days after anthesis are 5.96 and 3.86 10^{-1} µg g^{-1} sample fresh weight and 4.48 and 4.12 10^{-1} µg g^{-1} sample fresh weight. Mango fruit retention of 10.32 and 8.12 (10^{-1} µg g^{-1} sample fresh weight and

Table 1 - The content of several hormones at the stage of development fruit of wax apple var. Citra

Treatment	Flower and fruit development					
	Bud development	Anthesis	Fruit Set	Fruit development	Fruit maturation	Fruit Ripening
IAA (ppm)						
Retention	69.50 a	33.72 a	47.40 a	57.02 a	42.86 a	42.10 a
Drop easily	1.42 b	0.73 b	0.68 b	1.22 b	0.78 b	0.41 b
Zeatin (ppm)						
Retention	2.18 a	2.25 a	3.29 a	2.19 a	2.59 a	2.04 a
Drop easily	0.17 b	0.50 b	0.66 b	0.36 b	0.03 b	0.46 b
Kinetin (ppm)						
Retention	4.36 a	2.46 a	11.31 a	3.17 a	3.30 a	3.93 a
Drop easily	2.16 b	1.38 b	2.96 b	3.05 a	2.75 a	2.39 a
GA3 (ppm)						
Retention	79.65 a	68.08 a	90.47 a	68.25 a	63.45 a	57.72 a
Drop easily	28.63 b	16.13 b	20.14 b	26.69 b	17.57 b	8.97 b
ACC (ppm)						
Retention	19.18 b	17.26 b	14.21 b	14.13 b	13.31 b	12.14 b
Drop easily	48.39 a	38.09 a	37.99 a	35.75 a	34.14 a	30.92 a

The number followed by the same letter in the same column for each fruit development is not significantly different from the t-test at $p<0.05$.

Table 2 - Total sugar content and starch content on stages of wax apple flower and fruit development

Treatment	Flower and fruit development stage					
	Bud development	Anthesis	Fruit set	Fruit development	Fruit maturation	Fruit ripening
Total sugar content (%)						
Retention	6.87 a	8.72 a	11.38 a	23.35 a	28.93 a	32.59 a
Drop easily	5.80 b	7.60 b	8.60 b	20.61 b	26.36 b	28.66 b
Starch content (%)						
Retention	17.23 b	16.29 a	14.52 b	13.11 b	11.02 b	8.77 b
Drop easily	17.90 a	16.40 a	15.80 a	13.89 a	12.62 a	11.21 a

The number followed by the same letter in the same column each fruit development is not significantly different in the t-test at the level of $p<0.05$.

10.08 and 7.98 10^{-1} µg g^{-1} sample fresh weight. According to Kurniawati and Hamim (2009), star fruit with an application of 15 ppm 2, 4-dichlorophenoxy-acetic acid (2,4-D) contains 227 ppm IAA and 60 ppm GA_3 applications have 221 ppm IAA.

The formation of the absicission layer at the stem point that causes fruit drop is an imbalance of auxin, cytokinin, and gibberellins (Chen *et al*., 2006). Flower and fruit loss is also influenced by high ethylene concentrations and low IAA type auxin concentrations and low gibberellins. Auxin and ethylene are hormones that are directly related to the process of a drop of flowers and fruit (Bangerth, 2000).

Auxin is often reported to delay or to induce fruit, it increases cell enlargement rather than cell division. This observation might indicate that auxin is related to cell enlargement, an essential factor controlling fruit size during the rapid fruit growth phase. Enlargement of fruits treated with auxin seems to be due to cell expansion rather than cell division (Iglesias *et al*., 2007).

Table 1 shows that the content of zeatin type cytokinins and fruit kinetin that drop easily is smaller than retention. This is because cytokinins function to inhibit aging (Kieber and Schaller, 2018) so that the flowers and fruits of wax apple which will fall at several stages of development have a smaller zeatin content compared to the retention seen in table 1. The cytokinin content of both zeatin and kinetin at the stage of development flowers and fruit of wax apple was the highest at the fruit set stage. According to Chen (1983), the cytokinin content

increased in mango flowers maximum at 10 days after anthesis and decreased gradually up to 50 days after anthesis. This is consistent with Trueman (2011), that high cytokinin concentrations in macadamia fruit are retention. Cytokinins are produced in roots and young fruit (Pratima and Chawla, 2019).

Table 1 shows that the GA_3 content in several stages of flower and fruit development will fall lower than the retention of flowers and fruit. The GA3 content in fallen fruit was lower than that in retention (Bains *et al*., 1997). The role of GA3 is able to stimulate plant growth and flowering, increase flowering and reduce drop flower (Budiarto and Wuryaningsih, 2007). According to Kurniawati and Hamim (2009), gibberellin and auxin can support fruit retention by inducing the enzyme α-amylase to hydrolyze starch to sugar that is needed for fruit growth and development (Subiyanto, 1991). This can be seen in Table 1 that the increased auxin content also increases the gibberellin content in retention flowers and fruits compared to drop easily and in Table 2 flowers and fruits retention have a higher total sugar content than drop easily.

The ACC content of wax apple flowers and fruits that drop easily is higher than the retention of flowers and fruits are seen in Table 1. This is because the ACC which is an ethylene precursor makes the drop of flowers or fruits become higher (Wang *et al*., 2002). The mango ACC content that drops easily is higher than that of retention (Sakidin *et al*., 2011). The ACC content of Gadung 21 and Lali Jiwo cultivars which drop easily higher at the age of 3, 9, 12 days after anthesis are 26.81, 35.78, 43.41 (10^{-1} mg g^{-1}) sample fresh weights and 29.13, 29.35, 35.79 (10^{-1} mg g^{-1}) sample fresh weight. The ACC content of Gadung 21 and Lali Jiwo cultivars with age retention of 3, 9, 12 days after anthesis is 26.81; 35.78; 43.41 (10^{-1} mg g^{-1}) fresh sample weights and 10.13; 13.66; 11.94 (10^{-1} mg g^{-1}) sample fresh weights (Sakhidin *et al*., 2006).

Flower and ovary abscission occur in AZ-A (Abscission Zone) located between branches and flower stalks. Absence in the AZ-A zone starts from the fruit set period and this negative effect is regulated by the content of gibberellins in the ovaries. Fruitlet abscission during fruit drop in June at the end of the fruitset period on AZ-C which is located in the petals, between the flower disks and the ovarian wall, and highly dependent on carbohydrate availability. Sugar in mature leaves is transported for fruitlet growth and activates AZ-C (Iglesias *et al*., 2007).

According to Iglesias *et al*. (2007), the lack of carbon in fruitlets induces an increase in abscisic acid (ABA) and ACC (1-Aminocyclopropane 1-Carboxylic Acid) which are ethylene precursors. These precursors are then oxidized to ethylene (C_2H_4) and release of gases that cause fruit abscission. In contrast to the development of fruitlets, in the process of cooking the fruit accumulation of sugar in ripe fruit has a role to induce the activation of abscission before harvest. The balance between C_2H_4 as a process accelerator, and auxin (AUX) as an inhibitor, is one of the main factors in the regulation of ripe fruit abscission. Synthesis of auxin in young leaves and transported to adult fruit as an inhibition inhibitor that protects AZ from high C_2H_4 content. The role of the regulation of jasmonic acid (JA) in fruit reduction is thought to be mediated through the stimulation of C_2H_4 biosynthesis. The balance between AUX and ABA in mature fruits is also important in determining the sensitivity of AZ-C for abscission stimulus because ACC is an ethylene precursor (Wang *et al*., 2002).

Fruit abscission that occur during fruit development due to the active abscission zone. The process is induced by several environmental factors, competition in the use of assimilates, and internal hormone content. The abscission zone on the mango is located on the fruit stalk with a distance of several mm from the fruit concave (where the fruit is attached to the fruit stalk). From the biochemical and molecular aspects, abscission occurs due to the active enzyme ß-1,4-endoglucanase (EG) and polygalacturonase (PG). The two hydrolase enzymes are involved in damage to plant cell walls that are responsible for the drop of flowers and fruits. The specificity of the abscission zone in responding to organ drop depends on the sensitivity of the layer to ethylene (Bonghi *et al*., 2000).

Control of fruit growth and abscission in oranges is due to three regulatory factors: genetic, metabolic, and environmental. All three of these affect hormonal signals in citrus plants (Iglesias *et al*., 2007). Overall, these studies show that a complex set of hormonal interactions occur during fruit development. Thus, gibberellins (GAs) and cytokinins are generally considered positive fruit growth regulators while auxin has been reported to act as a growth stimulator and also as an abscess agent. Abscisic acid (ABA) and ethylene have been involved in several ways in an abscess. Concentrations of IAA and GA3 in fruit and fruit stalks that drop easily are lower than

those in fruit retention and fruit stalks, fruits that will fall out have high abscisic acid content. Drop fruit is also caused by an increase in ethylene production.

Table 2 shows that the flower and fruit drop had a lower total sugar content but a higher starch content than the retention. This is following Stopar *et al*. (2001), apples that drop easily have higher starch content and lower sugar content compared to retention. Mangoes that pre-abscission have a lower total sugar content and a higher starch than retention (Sakhidin *et al*., 2011).

Total sugar content in flower and fruit development of wax apple var. Citra of bud development, anthesis, fruit set, fruit development, fruit maturation, and fruit ripening stages 5.8, 7.6, 8.6, 20.16, 26.36, 28.66% lower in fruits that drop easily with fruit retention of 6.87, 8.72, 11.38, 23.35, 28.93, 32.59%. This is in accordance with Rai *et al*., (2008), carbohydrate content (total sugar) in leaves in flower branches and mangosteen fruit which is lower (40.3 and 52.3 mg g^{-1} leaf dry weight) compared to leaves in branches that are flower and fruit retention (41.2 and 59.1 mg g^{-1} leaf dry weight). The retention of glucose, fructose, and inverted sugar of wax apple fruit was as follows: 8.9%, 8.9%, 8.7% var. Giant Green, 9.83%, 9.9%, 9.6% var. Masam manis pink, and 9.61%, 9.6%, 9.3% var Madu red (Khandaker *et al*., 2011). This shows that the drop of flowers and fruit is associated with a low supply of carbohydrates. Limited carbohydrate sources affect the formation and development of fruit (Pawar and Rana, 2019).

Increasing the rate of photosynthesis is very important to produce carbohydrates during the development of fruit sets (Iglesias *et al*., 2002). The limited supply of photosynthate and nutritional status of plants can limit the number of flowers that develop into fruit that can be harvested (Rai *et al*., 2008). In oranges, carbohydrate deficiencies produce young fruit abscess by triggering an increase in ACC levels (Gómez-Cadenas *et al*., 2000). Auxin applied to trees that have yellow leaves due to low carbohydrate cannot prevent pre-harvest loss (Sakhidin *et al*., 2006).

Table 2 shows that the starch content in flower and fruit development stages of bud development, anthesis, fruit set, fruit development, fruit maturation, and fruit ripening 16.29, 14.52, 13.11, 11.02, 8.77% in fruit retention. The starch content in wax apple is getting smaller as the fruit develops. According to Mureşan *et al*. (2015), the starch content was reduced in the early stages of fruit development in the 3 apple varieties studied, then the starch concentration increased significantly from 35 to 65 days after anthesis. Starch concentration gradually decreases as the fruit ripens. This can be seen in Table 2 that the starch content during the development of the wax apple fruit is seen to decrease from flower buds to fruit maturity, and in fruits that drop easily the starch content is higher than that of retention.

It was concluded the content of IAA, zeatin, kinetin, GA_3, and total sugars at several stages of the flower and fruit wax apple which drop easily lower than that of retention and the ACC content and starch are higher in fruit which drops easily than retention. The endogenous hormone content of wax apple flower and fruit that drop easily are as follows IAA 0.41 to 1.42 ppm, zeatin 0.03 to 0.66 ppm, kinetin 1.38 to 2.96 ppm, GA_3 8.97 to 28.63 ppm, and ACC 30.92 to 48.39 ppm. The wax apple fruit that retention IAA content of 30.92 to 48.39 ppm, zeatin 2.04 to 3.29 ppm, kinetin 2.46 to 11.31 ppm, GA3 57.72 to 79.65 ppm, and ACC 30.92 to 48.39 ppm. The total sugar content of wax apple flower and fruit which drop easily from 5.8 to 28.66% is lower than the retention rate of 6.87 to 32.59%. The content of fruit starch that drops easily is 11.21 to 17.9% higher than the retention of 8.77 to 17.23%. Actions that can be taken to prevent the drop of wax apple fruit by giving natural endogenous hormones contained in other plant materials. This can be done after knowing the content of auxin, zeatin, kinetin, gibberellins, and ACC in wax apple fruit that drop easily or retention.

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Clonal propagation of *Argania spinosa* (L.) skeels: effects of leaf retention, substrate and cutting diameter

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Key words: Argania tree, rooting, semi-hardwood, vegetative propagation.

Abbreviations: IBA= Indole-3-Butyric Acid, FS= Fine sand substrate, PM= Peat moss substrate, FS/PM= mixture of fine sand and peat moss.

Abstract: To evaluate the rooting ability and growth performance in semi-hardwood cuttings of *Argania spinosa* under non-mist greenhouse conditions, our experimentation was conducted with three cutting diameters (0.1-0.3, 0.3-0.6 and 0.6-0.9 cm), four leaf retention treatments (leafless, 2, 4 and 8 leaves) and three different rooting substrates (fine sand, peat moss, a 1:1 mixture of fine sand/peat moss). Significant effects of cuttings diameter, leaf retention and rooting substrate on sprouting, rooting and survival ability from *A. spinosa* semi-hardwood cuttings were observed. Among all diameters tested, a diameter of (0.3-0.6 cm) showed maximum rooting and survival capacity, while cuttings with a diameter of 0.6-0.9 cm resulted in the greatest sprouting ability. Successful vegetative propagation was restricted to leafy stem cuttings. Moreover, it was observed that the highest rooting ability was reached in cuttings planted in fine-sand substrate. However, the highest sprouting ratio (85.0%) and survival rate (92.5%) were achieved in a mixture of sand and peat moss. Thus, argan trees vegetative propagation could be most effectively achieved using semi-hardwood cuttings with a 0.4 cm diameter and 4 leaves, planted in a fine sand substrate during the root initiation period and grown in a mixture of fine sand and peat moss for hardening.

1. Introduction

The Argan tree [*Argania spinosa* (L.) Skeels] is the only species representing the tropical Sapotaceae family in Morocco (Emberger, 1925). It is

a prodigious perennial thermophilic and xerophilic thorny species (Ehrig, 1974; Morton and Voss, 1987). The argan tree can be found in a wide array of environments where soils are ranging from heavy clay to poor and rocky desert soils, it grows in altitudinal ranges extending from sea level (along the Atlantic coast) up to 1500 m (Msanda, 1993). Moreover, it has the ability to survive in very dry conditions at very hot temperatures up to 50°C and low rainfall levels between 150 and 400 mm/year, shedding foliage and remaining in a state of dormancy for several years during prolonged drought (M'Hirit *et al.*, 1998). Recently, *A. spinosa* has attracted worldwide attention as a source of highly valued oil (Charrouf and Guillaume, 2008). It's becoming the most expensive edible oil in the world, as the present supplies does not meet demand (Lybbert *et al.*, 2011). Despite the ecological value and local economic importance of this species, its forest density has been divided by two during the twentieth century and the natural regeneration rate is very low and is worsened by climate changes and the negative impact of over exploitation (Kenny and De Zborowski, 2007). Besides, its cultivation is not sufficiently developed. In part, this is due to excessive utilization of kernels for oil production which hindered the conventional propagation through seed germination (Nouaim *et al.*, 2002); on the other hand, it is due to the slow progress in propagation methods, mainly because of the generally low capacity of this species to form adventitious roots (Nouaim *et al.*, 2002; Justamante *et al.*, 2017). Therefore, research is required to develop efficient vegetative propagation techniques for the production of more planting material and conservation of this species. The rooting efficiency and growth performance of cuttings in many species have been found to be dependent of the cutting thickness (Foster *et al.*, 2000). The rooting effectiveness in function of cuttings diameter could be explained by different factors (Foster *et al.*, 2000). The variation observed among different cutting diameters could be influenced by physiological conditions (nutrients, carbohydrates, auxin, phenolics, lignification etc.), their position within the branch or their ontogenetic aging (Kaul, 2008; Wendling *et al.*, 2014). Moreover, leaf retention was also reported to influence rooting behaviour of cuttings (Leakey and Coutts, 1989). The proportion of leaves retained by the cuttings during propagation is related to the percentage of cuttings that form adventitious roots (Tchoundjeu and Leakey, 1996; Mesén *et al.*, 1997a). Another factor which creates a suitable environment for rooting is substrate (Tchinda *et al.*, 2013). The importance of substrate type on the rooting capacity of cuttings is widely recognized as the starting point to be addressed for a successful vegetative propagation using stem cuttings (Hartmann *et al.*, 2002; Leakey, 2004). Cuttings of many species root successfully in a variety of 50 propagation substrates, but the rooting performance may be greatly influenced by the kind of substrate which is linked to the hydromorphic or xeromorphic status of the species (Hartmann *et al.*, 2002; Leakey, 2004). The present study aims to evaluate the effect of three independent factors (cuttings diameter, leaf retention and substrate) on sprouting, adventitious rooting and survival ability of *Argania spinosa* semi-hardwood cuttings to develop an efficient technique for achieving large-scale production of superior clonal stock plants and conservation of genetic resources. It is a continuation of our previous research on Argan vegetative propagation, which concerned the nutrient solution influence (Benbya *et al.*, 2018), and the effects of auxin type and genotype (Benbya *et al.*, 2019) on the multiplication process.

2. Materials and Methods

Experimental site and plant materials

Vegetative propagation experiment was carried out during the vegetative period of the tree (May to September) at non-mist greenhouse of the biotechnology unit of the Regional Center of Agricultural Research of Rabat (INRA-Morocco), under natural photoperiod and a mean temperature of 32±2°C. The semi-hardwood cuttings of *A. spinosa* were collected from selected adult mature trees, naturally growing at the Arboretum of Oued Cherat in the province of Bouznika, Morocco (33° 81' 96" N; 7° 11' 03" W; 45 m Altitude), which is located within 2 km of the Moroccan Atlantic coast and with an average annual rainfall of 460 mm/yr.

Preparation of cuttings and experimental establishment

Plant material was placed in cold dark storage at 4°C for 48h before planting. Then, semi-hardwood cuttings were screened for uniform length (10±0.5 cm) and were divided into three groups by diameter: 0.1-0.3 cm with 10 to12 nodes; 0.3-0.6 cm with 8 to 10 nodes and 0.6-0.9 cm with 6 to 8 nodes using calibrated electronic digital Vernier caliper. Given cuttings were prepared as multiple nodes, without

leaves (leafless); or with two leaves, four leaves or eight leaves. The tip of shoot was removed and the lower leaves of each cutting were removed. Before planting, cuttings were treated with aqueous solution of 0.2% (w/v) fungicide (Dithane 750 g/kg Mancozeb) for 10 minutes and subsequently washed with distilled water to remove excess fungicide. Then, the basal end (5.0 cm) of the cuttings was soaked for 5 min in a freshly prepared aqueous solution of auxins (17.12 mM of IBA). Prepared cuttings were immediately inserted in different polyethylene (PE) pots (1000 cc) containing three different substrates: sterilized sieved fine sand (FS) (pH of 7.3, pH-KCl method; water retention of 160 ml/l; organic matter content of 0.2%, Walkley & Black method); peat moss (PM) pH of 6; water retention of 800 ml/l; organic matter content of 20%); mixture of fine sand and peat moss (1:1 v/v) (FS/PM). The rooting period for the experiment was run for 12 weeks, and then cuttings were uprooted carefully without harming root with running water. Successfully rooted cuttings (at least one root with a length of at least 1 cm long) were transplanted into polyethylene pots (4500 cc) and were maintained for hardening at a spacing of 20 cm × 20 cm for 48 weeks. Given cuttings were regularly watered twice a week with tap water during the first twelve weeks; after a Hoagland & Arnon nutrient solution (Hoagland and Arnon, 1950) was used as irrigation source.

Data collection

The morphological characteristics assessments were realized after 12 weeks for the number of sprouts (NS), length of the longest sprout (SL, cm), sprouting percentage (SP, %), number of roots (NR), length of the longest root (RL, cm), and rooting percentage (RP, %), whereas the survival rate success (SR, %) was recorded at week 48 of the hardening phase.

Experimental design and treatments

The experiment was conducted in Randomized Complete Block Design (RCBD) with four blocks and three independent variables (cutting size, leaf retention and substrate) (Fig. 1A). For each treatment there were 32 cuttings (eight per block), randomly allocated in groups of two cuttings (forming four

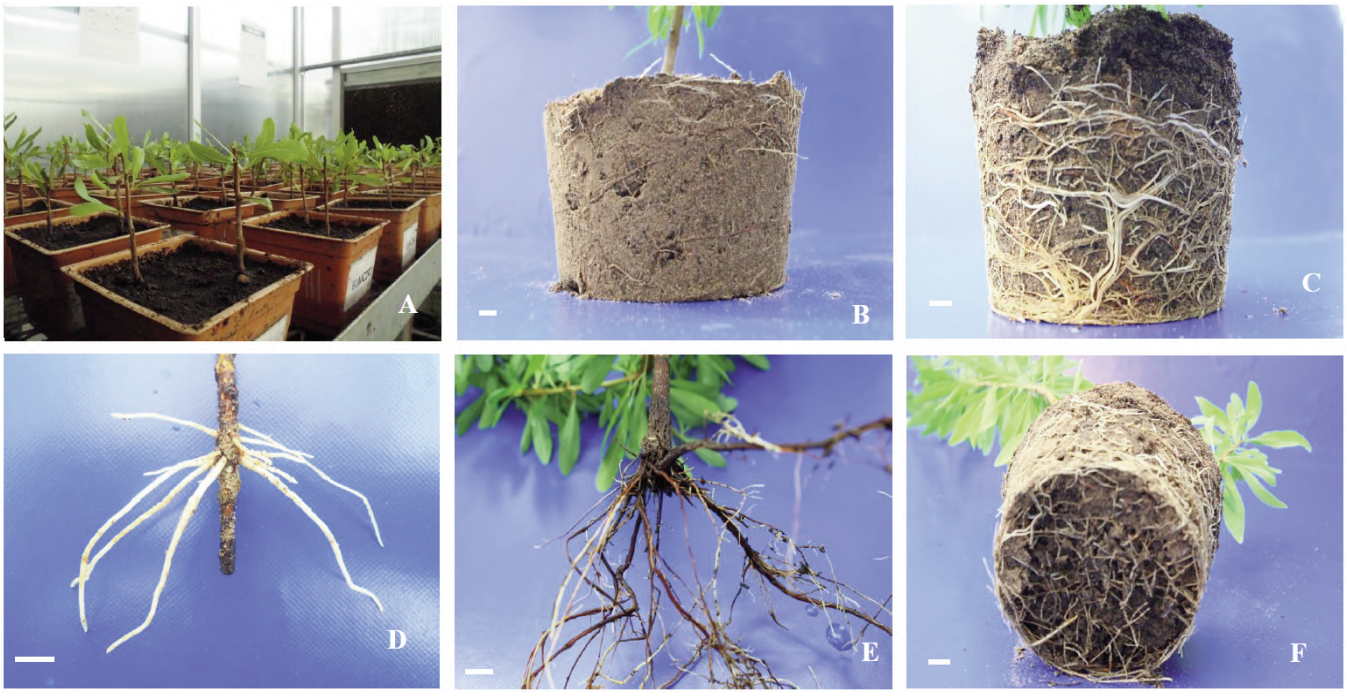

Fig. 1 - Clonal propagation of *Argania spinosa* semi hardwood cuttings. (A) Cuttings planted in peat moss substrate according to a randomized complete block design (RCBD) under non mist greenhouse conditions (24 weeks). (B) Rooted plant cutting set in sand substrate (2 years). (C) Well rooted plant cutting set in a mixture of sand and peat moss (1:1 v/v) substrate (3 years). (D) Cutting with developed root primordia from fine sand substrate (12 weeks). (E) Vigorous adventitious roots of semi hardwood cuttings grown in a mixture of sand and peat moss substrate (2 years). (F) Argan plantlet in a mixture of fine sand and peat moss substrate under non-mist greenhouse conditions. (3 years). Scale bars: 10 mm.

experimental units for thirty-six treatment combinations). The experiment was established with three cutting diameters (0.1-0.3 cm; 0.3-0.6 cm and 0.6-0.9 cm), four leaf number treatments (leafless, 2, 4, and 8 leaves) and three different substrates (FS, PM and FS/PM).

Statistical analysis

The main effects and interactions of cutting diameter, leaf retention and substrate were determined using a general linear model (GLM) procedure in SAS program version 9.1 (SAS Institute, Cary, NC) for all the evaluated parameters. The differences between the treatments were tested using Duncan's Multiple Range Test (DMRT) with at least 95% level of statistical reliance, and as a result, homogenous groups were acquired and interpreted. Collected data were subjected to simple linear regression analysis. All data were reported as means ± standard error (SE). Sprouting, rooting and survival percentage data were arcsine transformed to ensure normal distribution and homogeneity of variances.

3. Results

Effect of the cutting diameter, leaf retention and substrate on number of sprouts and sprouts length of the Argania spinosa cuttings

Cuttings with 0.6-0.9 cm diameter and four leaves set in FS/PM substrate showed the greater mean number of sprouts and the longest sprout of cuttings (1.81±0.10 cm and 17.25±0.96 cm respectively), while the lowest number of sprouts and the shortest sprout length occurred for cuttings with 0.3-0.6 cm diameter and eight leaves planted in FS substrate (1.06±0.09 cm and 8.19±1.11 cm, respectively). However, thinner cuttings (0.1-0.3 cm) and leafless cuttings for all diameters failed to produce any sprouts and are therefore not shown on the graphs (Fig. 2).

The mean number of sprouts per rooted cutting showed a progressive increase with increasing cutting diameter (r= 0.56***). A similar trend was also observed in sprouts length per rooted cutting (Table 1). Results showed that PM substrate as well as

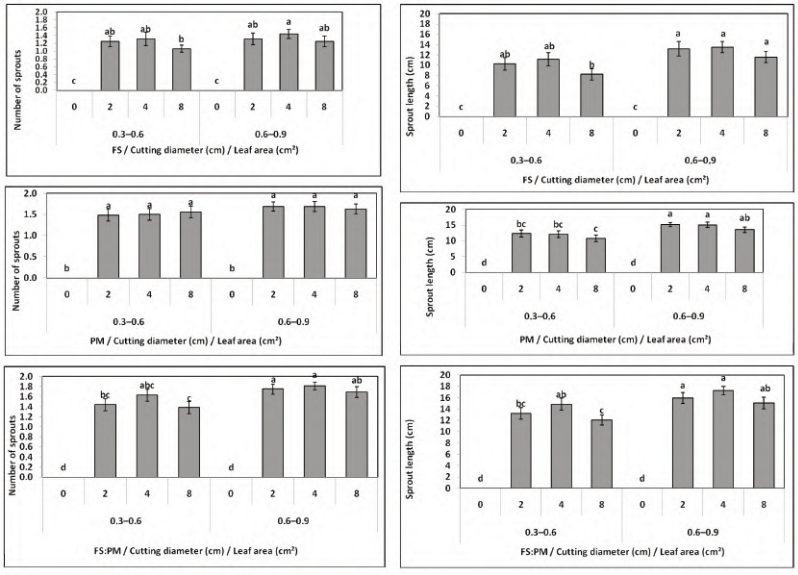

Fig. 2 - Effects of cutting diameters, four leaf number treatments (leafless, 2, 4, and 8 leaves) and three different substrates (FS, PM and FS/PM) on mean values of number of sprouts (NS) and length of the longest sprout (SL) per rooted cutting of *Argania spinosa*. Values followed by a common letter within each column are not significantly different at P<0.05 using the Duncan's Multiple Range Test (DMRT). A value represents mean ± standard error of means (n = 32).

Table 1 - Simple linear regression for cutting diameter and number of sprouts or sprout length (cm) of *Argania spinosa* cuttings

Dependent variable (Y)	Independent variable (X)	Correlation coefficient *(r)*	Regression equation	P-Value
Number of sprouts	Cutting diameter (cm)	0.56	Y= 0.59 X - 0.44	0.000 ***
Sprout length (cm)	Cutting diameter (cm)	0.58	Y= 5.42 X - 4.32	0.000 ***

***= Highly significant (P<0.001).
n = 32.

FS/PM substrate exhibited higher means number of sprouts compared to FS substrate. However, the FS/PM substrate showed the longest sprouts, followed with PM substrate, while FS exhibited the shortest sprout per cutting (Table 1). Over all the combined data for leaf retention treatments, it showed that with cutting diameter increase, there was an increase in the number of sprouts and sprout length (r=0.58***) (Table 1). Indeed, cuttings with 0.6-0.9 cm diameter showed the greater mean number of sprouts and sprout length of cuttings, followed by cuttings with 0.3-0.6 cm. Among leaf retention tested, cuttings with 4 leaves produced the longest sprouts compared with 2 and 8 leaves (Table 2).

Based on the analysis of variance (ANOVA), cutting diameter and substrate had a significant effect (P<0.05) on the mean number of sprouts, while leaf retention was not found significant on the number of sprouts per cuttings (Table 3). Moreover, cutting diameter, leaf retention and substrate had a highly significant effect (P<0.001) on the sprout length, whereas leaf retention had only significant effect on sprout length (Table 3). Neither mean number of the sprouts per cuttings nor the sprout length was significantly (P > 0.05) affected by the two-way and three-way interaction between the three factors tested (Table 3).

Effect of the cutting diameter, leaf retention and substrate on number of roots and length of the longest root of the A. spinosa cuttings

The highest number of roots and root length were observed in cuttings with 4 leaves, followed by 2 leaves though not significantly, while lowest number of roots and root length were mostly recorded in cuttings with 8 leaves. Best response in term of number of roots was obtained when cuttings were set in FS substrate with four leaves and diameters of 0.3-0.6 cm for which the mean number of adventitious roots was 45.06±1.14 (Fig. 1E), followed by cuttings with four leaves set in FS substrate with diameters of 0.6-0.9 cm (44.130±1.21). The greatest value of root length was recorded when cuttings were grown in FS substrate with 0.3-0.6 cm thickness and with four leaves (33.63±2.12), while cuttings set in a PM sub-

Table 2 - Main effects of cutting diameter, leaf retention and substrate on the number of sprouts and length of the longest sprout (cm) of *Argania spinosa* cuttings

Treatments	Sprouts number	Sprout length
Cutting diameter (cm)		
0.1-0.3	-	-
0.3-0.6	1.40 ± 0.05 b	11.65 ± 0.46 b
0.6-0.9	1.58 ± 0.05 a	14.46 ± 0.44 a
Leaf retention		
Leafless	-	-
2	1.48 ± 0.06 a	13.34 ± 0.58 ab
4	1.56 ± 0.05 a	13.98 ± 0.55 a
8	1.43 ± 0.06 a	11.85 ± 0.56 b
Substrate		
Fine sand (FS)	1.27 ± 0.07 b	11.30 ± 0.63 c
Peat moss (PM)	1.58 ± 0.06 a	13.13 ± 0.52 b
Mixture of FS and PM	1.62 ± 0.05 a	14.74 ± 0.51 a

Values followed by the same letter(s) are not significantly different at P<0.05 using the Duncan's Multiple Range Test (DMRT). A value represents mean ± standard error of means (n = 32).

Table 3 - Analysis of variance (ANOVA) for the effects of cutting diameter, leaf retention and substrate and their interactions on number of sprouts and length of the longest sprout cm per *Argania spinosa* cuttings

Source of variance	Dependent variable					
	No. of sprouts			Sprout length		
	df	F-value	P-value	df	F-value	P-value
Cutting diameter	2	7.36	0.0071 *	2	20.36	<0.0001 ***
Leaf retention	3	1.30	0.2736 NS	3	4.08	0.0179 *
Substrate	2	10.08	<0.0001 ***	2	10.15	<0.0001 ***
Cutting diameter × Leaf retention	6	0.03	0.9702 NS	6	0.9559	0.9559 NS
Cutting diameter × Substrate	4	0.39	0.6750 NS	4	0.01	0.9896 NS
Leaf retention × Substrate	6	0.35	0.8451 NS	6	0.23	0.9232 NS
Cutting diameter × Leaf retention × Substrate	12	0.20	0.9399 NS	12	0.03	0.9983 NS
Block	1	0.10	0.7534 NS	1	2.43	0.1200 NS

NS= Not significant (P>0.05); *= Significant (P<0.05); ***= Highly significant (P<0.001).
df= degrees of freedom.
×= Interaction between treatments.

strate didn't exceed 23 cm (Fig. 3; Fig. 1E). However, the incidence of necrosis was the greatest among thinner cuttings (0.1-0.3 cm) and leafless cuttings, indeed these cuttings failed to produce any adventitious roots and are therefore not on the graphs (Fig. 3).

The mean number of roots was dependent on type of substrate; cuttings grown in FS and FS/PM substrates rooted significantly better, whereas the lowest mean number of adventitious roots per cuttings was recorded in cuttings propagated in PM substrate (Table 4). Over the entire experimental period, the three substrates showed pronounced differences in the root length: cuttings planted in FS substrate were found to produce significantly longest adventitious roots, followed by FS/PM substrate then cuttings set in PM substrate (Table 4). Among the cutting diameters studied, (0.3-0.6) cm showed the highest number of roots and the longest root, followed by the cutting diameter (0.6-0.9) cm, while cuttings with (0.1-0.3) cm were the least performing and failed to produce roots (Table 4).

Regarding the ANOVA test values, the effects of cutting diameter, leaf retention and substrate were found highly significant (P<0.001) on the number of adventitious roots formed per cutting (Table 5). Moreover, cutting diameter and substrate had also a highly significant effect (P<0.001) on root length of cutting, while leaf retention had only a significant effect (P<0.05). Root length was significantly (P<0.05) affected by the interaction between cutting diameter and substrate, while the other interactions between the three factors studied were not significant on the number of roots and root length of cuttings (Table 5).

Table 4 - Main effects of cutting diameter, leaf retention and substrate on the number of roots and the length of the longest root (cm) of *Argania spinosa* cuttings

Treatments	Roots number	Root length
Cutting diameter (cm)		
0.1-0.3	-	-
0.3-0.6	38.38 ± 0.74 a	27.24 ± 0.73 a
0.6-0.9	35.61 ± 0.90 b	24.42 ±0.69 b
Leaf retention		
Leafless	-	-
2	36.95 ± 0.94 b	25.65 ± 0.93 ab
4	38.97 ± 1.04 a	27.47 ± 0.83 a
8	35.07 ± 1.03 b	24.36 ± 0.85 b
Substrate		
Fine sand (FS)	42.07 ± 0.66 a	30.76 ± 0.76 a
Peat moss (PM)	27.44 ± 0.82 b	18.25 ± 0.63 c
Mixture of FS and PM	41.48 ± 0.75 a	28.47 ± 0.65 b

Values followed by the same letter(s) are not significantly different at P<0.05 using the Duncan's Multiple Range Test (DMRT). A value represents mean ± standard error of means (n = 32).

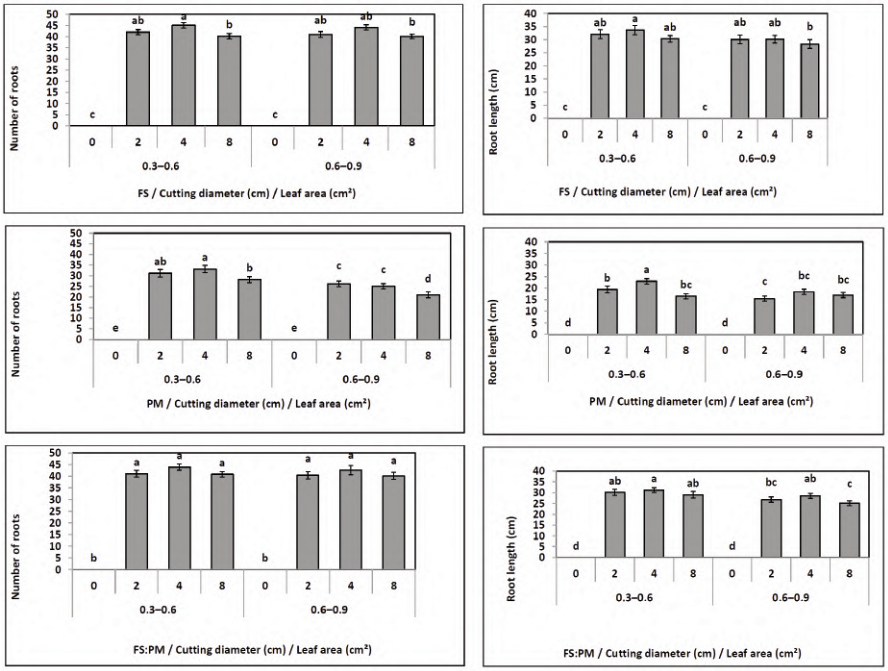

Fig. 3 - Effects of cutting diameters, four leaf number (leafless, 2, 4, and 8 leaves) and three different substrates (FS, PM and FS/ PM) on mean values of number of adventitious roots (NR) and length of the longest root cm (RL) per rooted cutting of Argania spinosa. Values followed by a common letter within each column are not significantly different at P < 0.05 using the Duncan's Multiple Range Test (DMRT). A value represents mean ± standard error of mean (n = 32).

Table 5 - Analysis of variance (ANOVA) for the effects of cutting diameter, leaf retention and substrate and their interactions on the number of adventitious roots and the length of the longest root (cm) of *Argania spinosa* cuttings

Source of variance	Dependent variable					
	No. of roots			Root length		
	df	F-value	P-value	df	F-value	P-value
Cutting diameter	2	11.27	0.0009 ***	2	13.29	0.0003 ***
Leaf retention	3	7.43	0.0007 ***	3	5.42	0.0049 *
Substrate	2	134.30	<0.0001 ***	2	98.88	<0.0001 ***
Cutting diameter × Leaf retention	6	0.18	0.8318 NS	6	0.46	0.6345 NS
Cutting diameter × Substrate	4	5.81	0.0034 *	4	0.11	0.8990 NS
Leaf retention × Substrate	6	0.72	0.5809 NS	6	0.30	0.8766 NS
Cutting diameter × Leaf retention × Substrate	12	0.14	0.9657 NS	12	0.58	0.6775 NS
Block	1	2.46	0.1178 NS	1	0.26	0.6133 NS

NS= Not significant (P>0.05); *= Significant (P<0.05); ***= Highly significant (P<0.001).
df= degrees of freedom.
×= Interaction between treatments.

Effect of the cutting diameter, leaf retention and substrate on sprouting percentage, rooting percentage and survival rate of the Argania spinosa cuttings

Although the thinner cuttings gave no sprouts, among the other different treatments, the highest sprouting percentage was 85.00 ± 3.76% when cuttings with 4 leaves and 0.6-0.9 cm diameter were planted in FS/PM substrate, while cuttings with 8 leaves and 0.3-0.6 cm diameter propagated in FS substrate gave the poorest sprouting percentage (61.50± 5.68%). However, leafless cuttings performed least and failed to produce any sprouts (Fig. 4). The proportion of cuttings forming new shoots during growth varied between the three substrates. Cuttings planted in PM and FS/PM substrates were the most successful in sprouting, while cuttings propagated in FS

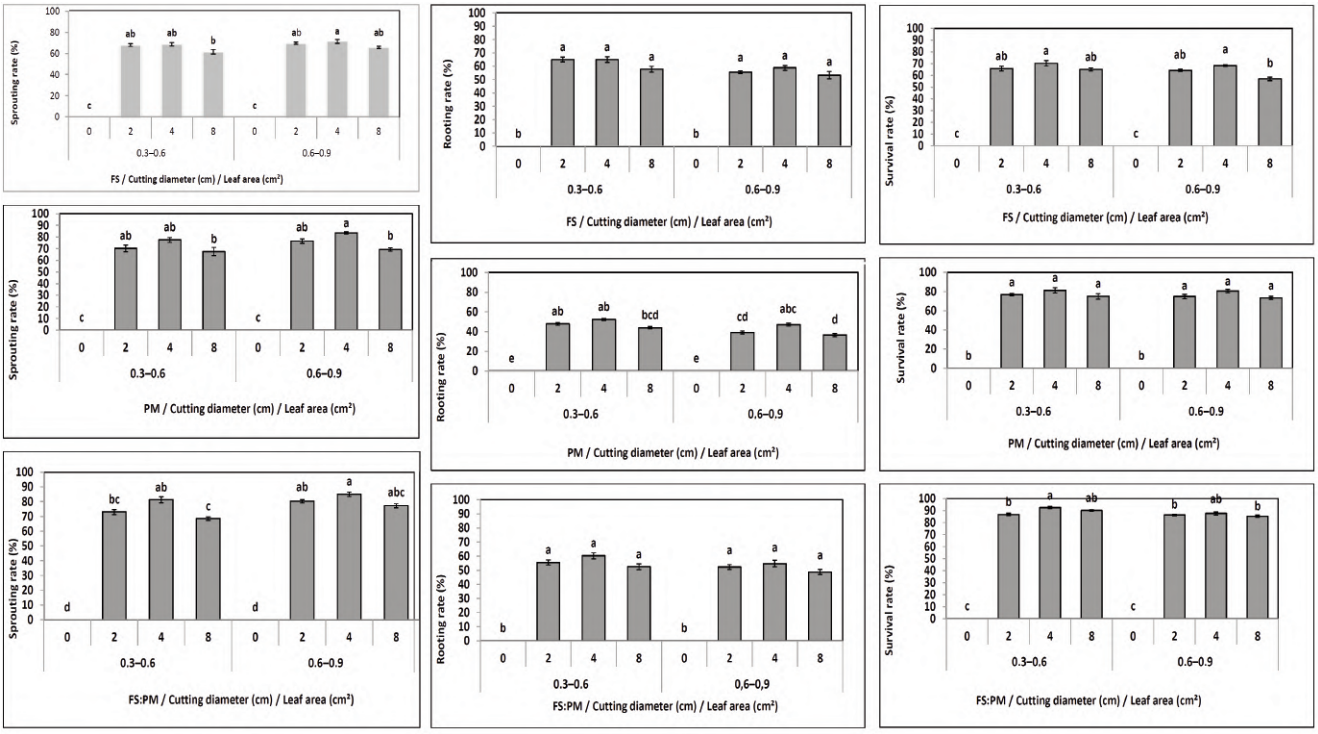

Fig. 4 - Effects of cutting diameters, four leaf number treatments (leafless, 2, 4, and 8 leaves) and three different substrates (FS, PM and FS/PM) on mean values of sprouting percentage (SP), rooting percentage (RP), and survival rate (SR) of *Argania spinosa* cuttings. Values followed by a common letter within each column are not significantly different at P<0.05 using the Duncan's Multiple Range Test (DMRT). A value represents mean ± standard error of mean (n = 32).

substrate showed the lowest sprouting ability (Table 6).

Results showed that cuttings with 0.3-0.6 cm diameter with two and four leaves set in a FS substrate showed maximum rooting: (65.00 ± 5.15% and 65.00 ± 5.87% respectively). Compared with the cuttings of 0.1-0.3 cm thickness and leafless cuttings which failed to root, all cutting diameters and leaf retention treatments enhanced the rooting percentage. Moreover, percentage of cuttings developing adventitious roots decreased when the leaf number exceeds 4 leaves (Fig. 4). The maximum rooting rates were obtained for cuttings grown in FS substrate, followed by FS/PM substrate, while the cuttings grown on PM substrate showed the lowest rooting capacity (Table 6). The survival rate ranged from 57.00 ± 4.53% for plantlets with 0.6-0.9 cm diameter and 8 leaves grown in FS substrate, to 92.50 ± 1.94% for plantlets derived from cuttings with 0.3-0.6 cm diameter and 4 leaves potted on substrate containing a mixture of fine sand and peat moss. However, cutting mortality was the greatest in thinner cuttings (0.1-0.3) cm and leafless cuttings which showed 100% mortality rate within forty-eight weeks (Fig. 4). The rooted plantlets were successfully hardened and acclimatized under non-mist greenhouse conditions (Table 6). These plants showed the best survival rate and performed better growth and development when they were grown in a FS/PM substrate compared with cuttings raised in PM and FS substrates (Fig. 1F).

The data revealed that the substrate caused significant ($P < 0.05$) variations in sprouting, rooting and survival percentage of the rooted cuttings, while cutting diameter and leaf retention were not significant on the survival rate (Table 7). However, there was no significant effect ($P>0.05$) of treatment interactions between the three independent factors on the three parameters studied.

4. Discussion and Conclusions

The present investigation revealed that cutting diameter (0.1-0.3, 0.3-0.6 or 0.6-0.9 cm), leaf retention (0, 2, 4 or 8 leaves) and substrate (FS, PM or FS/PM) were found to be important factors for successful adventitious rooting, sprouting and survival ability of *Argania spinosa* semi-hardwood cuttings.

This experiment on *A. spinosa* cuttings concerning cutting diameter indicated a significant effect on the sprouting, rooting and survival performances. Among the different cutting diameters tested, cuttings with 0.3-0.6 cm diameter tended to root better and develop more rooting capacity compared with thinner diameter (0.1-0.3 cm), which failed to produce any sprouts or roots. Moreover, larger cuttings (0.6-0.9 cm) showed the best sprouting ability but did not result in any significant improvement in root growth and development. The greatest rooting potential of cuttings with 0.3-0.6 cm diameter could be due to their good storage capacity of carbohydrates and

Table 6 - Main effects of cutting diameter, leaf retention and substrate on sprouting percentage, rooting percentage, and survival rate of *Argania spinosa* cuttings

Treatments	Sprouting (%)	Rooting (%)	Survival (%)
Cutting diameter (cm)			
0.1-0.3	-	-	-
0.3-0.6	70.69 ± 1.58 b	55.56 ± 2.22 a	78.14 ± 1.72 a
0.6-0.9	75.42 ± 2.52 a	49.55 ± 1.75 b	75.25 ± 2.20 a
Leaf retention			
Leafless	-	-	-
2	72.96 ± 1.87 ab	52.54 ± 2.03 ab	75.75 ± 1.04 ab
4	77.92 ± 1.93 a	56.33 ± 1.82 a	80.04 ± 2.16 a
8	68.29 ± 1.52 b	48.79 ± 1.90 b	74.29 ± 2.03 b
Substrate			
Fine sand (FS)	67.54 ± 1.94 b	59.21 ± 2.27 a	65.08 ± 2.37 c
Peat moss (PM)	74.08 ± 2.01 a	44.42 ± 2.19 b	76.96 ± 2.44 b
Mixture of FS and PM	77.54 ± 2.17 a	54.04 ± 2.41 a	88.04 ± 2.83 a

Values followed by the same letter(s) are not significantly different at $P<0.05$ using the Duncan's Multiple Range Test (DMRT). A value represents mean ± standard error of means (n = 32).

Table 7 - Analysis of variance (ANOVA) for the effects of cutting diameter, leaf retention and substrate and their interactions on sprouting percentage, rooting percentage and survival rate of *Argania spinosa* cuttings

Source of variance	Dependent variable								
	Sprouting (%)			Rooting (%)			Survival (%)		
	df	F-value	P-value	df	F-value	P-value	df	F-value	P-value
Cutting diameter	2	4.28	0.0434 *	2	6.70	0.0124 *	2	1.96	0.1668 NS
Leaf retention	3	5.93	0.0047 *	3	3.53	0.0363 *	3	2.80	0.0694 NS
Substrate	2	6.60	0.0027 *	2	13.98	<0.0001	2	41.37	<0.0001
Cutting diameter × Leaf retention	6	0.02	0.9849 NS	6	0.06	0.9444 NS	6	0.23	0.7918 NS
Cutting diameter × Substrate	4	0.22	0.8072 NS	4	0.17	0.8411 NS	4	0.13	0.8775 NS
Leaf retention × Substrate	6	0.27	0.8937 NS	6	0.12	0.9745 NS	6	0.29	0.8855 NS
Cutting diameter × Leaf retention × Substrate	12	0.15	0.9633 NS	12	0.09	0.9860 NS	12	0.14	0.9655 NS

NS= Not significant (P>0.05); *= Significant (P<0.05); ***= Highly significant (P<0.001).
df= degrees of freedom.
×= Interaction between treatments.

other reserves for adventitious root formation (Leakey and Storeton-West, 1992; Tchoundjeu and Leakey, 1996). The good sprouting response may be due to the presence of adequate sugar reserves such as fructose, glucose and sucrose (Gehlot *et al.*, 2015). Moreover, the level of endogenous auxins and other rooting cofactors might be lower in cuttings with a small diameter, which leads to reduced rooting percentage or even the absence of rooting (Wilson and Van Staden, 1999). The rooting efficiency of these cuttings could be also due to the lower content of mineral elements, especially nitrogen which is positively correlated with rooting (Budiarto *et al.*, 2006). In addition, the effect of cutting diameter on rooting capacity could also be attributed to the origin of a cutting within shoots, and to its position of the stock-plant (Leakey, 2004). Indeed, thin cuttings are produced from shoots which arise in sub-dominant positions of the mother plants which contain a low amount of auxin, grow slowly and stop growth early (Howard and Ridout, 1991). The lower rooting capacity of cuttings with larger diameters (0.6-0.9 cm) may probably be due to changes in the extent of lignification and the degree of secondary thickening along their stems (Girouard, 1969; Hartmann *et al.*, 1990). These results are consistent with other studies that found a positive effect of cutting diameter on sprouting, rooting and survival success of rooted cuttings. Palanisamy and Kumar (1997) showed that the higher rooting efficiency of *Picea abies* was obtained by cuttings of 0.3-0.4 cm diameter. OuYang *et al.* (2015) confirmed the highest rooting efficiency of *Picea abies* obtained by cuttings of 0.3-0.4 cm diameter. Moreover, Foster *et al.* (2000) observed that the cuttings of *Pinus taeda* with an average diameter of 0.2-0.3 cm tended to root better and develop more roots.

Concerning leaf retention, this study showed that successful vegetative propagation of *Argania spinosa* was restricted to leafy stem cuttings. Indeed, defoliated cuttings failed to produce any sprouts / roots and showed a mortality rate of 100% within forty-eight weeks after planting. This result could be explained by the direct influence of the presence of leaves on the primary shoots, because initial growth of shoots depends on assimilates supplied by leaves and also through their influence on the cutting's water status (Newton *et al.*, 1992; Van Labeke *et al.*, 2001). Moreover, the leaf retention could also exert a strong influence on root initiation and development because it allows post-severance carbon assimilation (Leakey and Coutts 1989; Hartmann *et al.*, 1990; Thomas and Schiefelbein, 2004). Leafy cuttings provide a continuous supply of photosynthates besides their reserves after implementing them into the substrate (Tchoundjeu *et al.*, 2002; Leakey, 2004). On the other hand, the inability of leafless cuttings to root has been associated with the rapid depletion of stored carbohydrates in stem tissues after excision from the stock plants (Hoad and Leakey, 1996; Druege *et al.*, 2000). In addition, the leaf retention may also influence the endogenous auxins of the cutting. Since rooting is stimulated by the level of auxins available, it is expected that adventitious rooting response of a cutting will be proportional to the number of leaves retained (Tchoundjeu and Leakey, 1996). In fact, our results showed that the ability of cuttings to develop adventitious roots decreased when the leaf number exceeds 4 leaves. This could be explained by the balance between the positive

effects of assimilate production through allowing sufficient photosynthesis and the negative effects of water loss via transpiration (Leakey and Coutts, 1989; Mesén *et al.*, 1997a). However, cuttings with eight leaves exhibited early root growth and delayed sprouting and subsequently a decreased rooting efficiency. Indeed, cuttings with a high number of leaves appear to suffer from water deficit and a consequent reduction in photosynthetic activity as reflected by higher transpiration rates and leaf shedding compared to cuttings with an optimal number of leaves (Leakey and Coutts, 1989; Newton *et al.*, 1992; Aminah *et al.*, 1997). Thus, the balance between photosynthesis, transpiration and nutrient transport is an important factor influencing rooting (Leakey and Coutts, 1989). The positive effect of leaf retention on cutting success has been reported across a wide range of species including *Triplochiton scleroxylon* (Leakey and Coutts, 1989), *Cordia alliodora* (Mesén *et al.*, 1997a), *Vitis vinifera* (Thomas and Schiefelbein, 2004), *Eucalyptus hybrids* (Trueman and Adkins, 2013), *Santalum austrocaledonicum* (Tate and Page, 2018).

Finally, the study of the substrate effect showed that a significant difference in sprouting and rooting as well as in survival ability was found between the three substrates tested. It was observed that the number of roots, root length and rooting percentage reached significantly higher values in cuttings set in the FS substrate compared to the PM substrate and to the mixture of fine sand and peat moss. This effectiveness of adventitious rooting by rooted cuttings set in FS substrate could be related to its optimal volume of gas-filled pore-space and oxygen diffusion rate which create an adequate aerated environment for increased transpiration and respiration and enhanced adventitious root formation (Andersen, 1986). Moreover, sand is a porous substrate that limits the development of microbial pathogens and where roots can be settled well without damage (Tchinda *et al.*, 2013). Though, the poor aeration and high water holding capacity of peat moss substrate is a fundamental problem for adventitious root formation, leading to enhance the rate of fungal infection and decay of cuttings before root initiation (Schmitz *et al.*, 2013). Furthermore, the higher water content in the peat moss substrate induces closing of the stomata and inhibits oxygen diffusion. This anoxia caused by oxygen deficiency or by accumulation of toxic substances, including bicarbonate and carbon dioxide, is suspected of being linked to the rooting problems due to tissue death, wilting, severe defoliation, and reduced water absorption and leaf water potential (Mesén *et al.*, 1997b; Drew, 1983; Veen, 1988). The results also showed that cuttings set in a mixture of sand and peat moss performed better than those set in sand or peat moss substrate alone, in terms of sprouting and survival rate. It has been suggested that the addition of peat moss to fine sand improved the water holding capacity and promoted mineral nutrients absorption (Leakey *et al.*, 1990). The uptake of water by cuttings is positively related to the water content of the substrate and this may enhance survival rate by reducing water deficits, leaf abscission and cutting necrosis (Newton *et al.*, 1992). A number of comparative studies between different substrates have indicated that cuttings set in a sand substrate performed better in term of rooting and survival capacity. Atangana *et al.* (2006) reported that the higher rooting percentages were observed in sand for *Allanblackia floribunda*. Gehlot *et al.* (2014) also recommended that higher rooting percentages of *Azadirachta indica* were recorded for cuttings planted in sand. Moreover, Adugna *et al.* (2015) stated that in *Vanilla planifolia*, the cuttings set in sand performed the highest rooting percentage.

In conclusion, the results of the present study clearly indicate that cuttings with thinner diameters (0.1-0.3 cm) and defoliated cuttings have failed to produce any sprouts or roots and showed a 100% mortality rate within forty-eight weeks after planting. The greatest mean number of sprouts, sprout length and sprouting capacity as well as survival success was performed by cuttings with diameter of 0.3-0.6 cm and 4 leaves set in a mixture of sand and peat moss. The best mean number of roots, root length and rooting ability was achieved by cuttings with diameter of 0.3-0.6 cm and 4 leaves in a fine sandy substrate. This study also reveals that *Argania spinosa* is amenable to clonal propagation using sand-based rooting substrate which could provide a promising prospect for the conservation of this endemic species. In addition, it is suggested that this low-cost propagation techniques could greatly facilitate the domestication and the development of superior tree crops as a commercial agroforestry species with genetically homogenous plant material. The cuttings used in the experiments were taken during the spring season. It is therefore recommended that further works should be done to determine the optimal period for cutting collections for successful clonal propagation of *A. spinosa* trees.

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The induction and development of somatic embryos from the *in vitro* cultures of *Catharanthus roseus* (L.) G. Don

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Key words: *Catharanthus roseus* (L.) G. Don, MS, PGR, somatic embryogenesis.

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Abstract: *Catharanthus roseus* is containing anticancer alkaloids of vincristine and vinblastine and is an important medicinal plant. Several studies have conducted on *in-vitro* culture of this plant. To optimize the somatic embryogenesis, a factorial based on CRD experiment with 10 replications was conducted. Root, hypocotyl and leaf explants grown in-vitro were transferred and cultured on MS media containing different combinations of 2,4-D, NAA and 2,4-D×BAP. The results revealed that in callogenesis, the interaction effects of root and hypocotyl explants×2,4-D and NAA as well as hypocotyl×(1 mg l^{-1} NAA+1 mg l^{-1} BAP) was superior than other treatments (p≤0.01). For calli fresh weight, hypocotyl×NAA and hypocotyl× (1 mg l^{-1} NAA+1 mg l^{-1} BAP) was the treatment of choice (p≤0.01). The calli produced were sub-cultured to attain the pre-embryos and somatic embryos. For the number of pre-embryos and somatic embryos; the interaction of hypocotyl×2,4-D was the most efficient treatment. Seemingly, the production of somatic embryos is accessible in this plant by the logical management of growth regulator combinations. Furthermore, the production and genetic engineering of the somatic embryos could be a promising trend in the subsequent production of high-valued metabolites from this plant.

1. Introduction

Catharanthus roseus (L.) G. Don, generally known as *Madagascar Periwinkle,* is a dicotyledonous plant with 2n=16 belongs to the family *of Apocynaceae*. *Catharanthus roseus* (L.) G. Don is an herbaceous plant that grows to a height of about 80 Cm and is native of Madagascar (Hogan, 2003). More than 130 types of alkaloids have been extracted from the vegetative parts and roots of this plant, which are used to treat several diseases (Aslam *et al.,* 2009). The most important alkaloids extracted from the shoots of this plant are vincristine and vinblastine, with a well-defined anti-cancer properties (Mujib *et al.,* 2012). The amount of these compounds in this plant is about 0.0005% of dry weight of the plant and their extraction is costly and time consuming (Barrales-Cureño *et al*., 2017). These problems have led the scientists to a new approaches of tis-

sue culture studies in *C. roseus* L. (Van Der Heijden *et al.,* 2004). The first study done on tissue culture of *Catharanthus roseus* (L.) G. Don was in 1977. Those, researchers were able to produce callus from the plant (Dhruva *et al.,* 1977). The production of shoots from callus has been successfully done in *Catharanthus roseus* L. (Ramawat *et al.,* 1987). Somatic embryogenesis is a process by which somatic cells differentiate into embryos, called somatic embryos, which are used as one of the practical in vitro techniques for plant micro-propagation (Von Arnold *et al.,* 2002). The first studies on somatic embryogenesis in the *Catharanthus roseus* (L.) G. Don was done in 1994, which were succeeded in producing somatic embryos by the anther culture (Kim *et al.,* 1994). Furthermore, by hypocotyl explant and 1 mgl^{-1} of 2,4-D (2,4-Dichlorophenoxyacetic acid), somatic embryos were obtained (Aslam *et al.,* 2004, 2006). Growth regulators (composition and concentration) and the plant genetic make-up play a role in the success of somatic embryos production. The phenomenon of genotype-dependent plant regeneration also exists in other plant species (Firoozabady and De Boer, 1993).

The physiological conditions, the explant growth stage and embryogenic tissue type affect the production of somatic embryos. The tissue that has the highest metabolism and the least differentiation rate may have the suitable embryogenesis potential (Mikula and Rybczynski, 2001). Auxins, especially 2,4-D, are among the most important plant growth regulators initiate the somatic embryogenesis (Choi *et al.,* 1999; Martin, 2003; Gulzar *et al.,* 2019). In medicinal herb *Spermatozoa hispida* L.; cytokinin, especially BAP (6-Benzylaminopurine), induced frequent somatic embryos (Deepak *et al.,* 2019). With medicinal herb, *Coccinia abyssinica;* 2,4-D and BAP combination helped to produce embryogenic callus, and BAP triggered the production of somatic embryos (Abate *et al.,* 2019). L-glutamine and L-alanine amino acids have had the most positive effect on somatic embryogenesis process (Ji *et al.,* 2011). Various sources of carbon; such as sucrose, fructose, and glucose, as energy sources and osmotic regulators; play an important role in the somatic embryogenesis initiation and frequency (Aslam *et al.,* 2011). MS has been employed as the most common culture medium for most of the plants (Ji *et al.,* 2011). Somatic embryogenesis, leading to the regeneration of intact plants, is an eminent step in the plant transformation. Successful and sustainable transformation requires that a single cell give rise to a whole intact plant. The Ideal transformation takes place through the direct somatic embryogenesisfrom the single cells to reach the intact plants (Aslam *et al.,* 2007).

The aim of the present study was to investigate the different explant types and the diverse plant growth regulators effects to induce somatic embryos and to study of the traits related to the somatic embryogenesis potential in *Catharanthus roseus* (L.) G. Don.

2. Materials and Methods

In vitro seed germination and seedlings production

The present study was conducted in the Research Laboratory, Department of Horticultural Sciences, University of Maragheh, Iran. The seeds were acquired from Pakan Bazr Isfahan Company. The seeds were immersed in water for one day before planting in the dark, and the next day, they were first treated with ethyl alcohol (70%) for one minute and then disinfected with 20% solution of sodium hypochlorite for 10 more minutes. Then, they were washed with sterile distilled water 5 times. The disinfected seeds were cultured in petri dishes containing 20 ml of MS medium (Murashige and Skoog, 1962) without plant growth regulators. In each petri dish; 10 disinfected seeds were cultured at 25-28°C. After emergence, the seedlings were transferred to the photoperiod conditions with 16 hours of light at 25°C and 8 hours of darkness at 20°C. After about 10 days, the 2-4 cm in length seedlings were suitable to continue the experiment.

Preparation of culture media

The culture medium was MS basic medium. Sucrose at a concentration of 30 g l^{-1} was used as a source of carbon. Then, MS culture medium salts were added. pH was set at 5.8. At the last stage, 6 gl^{-1} of agar was added and placed in an autoclave at 121°C for 20 min. Agar-free culture medium (liquid) was employed for the somatic embryos.

Experimental design

A factorial experiment based on completely randomized design (CRD) was planned with 10 replications.

Factor 1: Plant growth regulators, concentration of 1 mgl^{-1} 2,4-D, 2 mgl^{-1} NAA (1-Naphthaleneacetic acid), 1 mgl^{-1} NAA + 1 mgl^{-1} BAP

Factor 2: Explants (Root, hypocotyl and leaf).

Sampling and culture of explants in different treatments

The explants; root, hypocotyl and leaves were excised from the 2-4 cm seedling grown *in vitro*. Hypocotyl explants were cut into a length of about 1 cm and, in order to obtain embryogenic callus; they were cultured in the MS medium supplemented with the same treatments as subcultures. For calli proliferation; 3 weeks after the first date of cultivation, the same plant growth regulator treatments were applied to the subcultures. One month after the first subculture; embryogenic calluses were sub-cultured in MS medium supplemented with concentrations of 0.5, 1, and 1.5 m g l^{-1} BAP. Then, the resulting calli were transferred to MS medium supplemented with 7 g l^{-1} of agar, 30 g l^{-1} of sucrose, 1 mg l^{-1} glutamine and 3 g l^{-1} of polyethylene glycol with 6 replications. At the all stages of cultures and subcultures; the samples were kept in a growth chamber for 16 h light at 25°C and 8 h dark at 20°C.

Data mining and statistical analysis

The callus production percentage, the percentage of necrotic calli and callus weight were noted. After multiple sub-cultures and transfer to the embryogenesis culture medium, the number of pre-embryogenic mass formed were recorded. SAS was used to analyze the variance, and mean comparisons were done with Duncan's multiple range test at 5% probability.

3. Results

Callus production

Hypocotyls of *in vitro* germinated seeds were used as explant on MS medium supplementary with 2,4-D and NAA, which induced white to yellowish callus within 10 days of incubation. The embryogenic callus was white, granular, friable, fast growing within 3 weeks of culture. The number of explants that produced callus and the necrotic calli proportion showed the callogenesis percentage and the percentage of necrotic calli (Fig. 1).

The mean comparison showed that for the callus formation, the interaction between root and 2,4-D; hypocotyl and 2,4-D; root and NAA; hypocotyl and NAA; and hypocotyl and 1 mgl^{-1} NAA + 1 mgl^{-1} BAP were significant at 1% probability level. Figure 2 shows the significant effect of auxin use on the rate of callus production. In this study 2,4-D was very efficient in producing callus and embryos on *Catharanthus roseus*. Embryonic callus was not observed using leaf explants and hypocotyl explants had the highest embryonic callus.

Furthermore, for the callus fresh weight, the interactions of hypocotyl and NAA; and hypocotyl × 1 mg l^{-1} NAA + 1 mgl^{-1} BAP were significantly different from other treatments ($p \leq 0.01$) (Fig. 3).

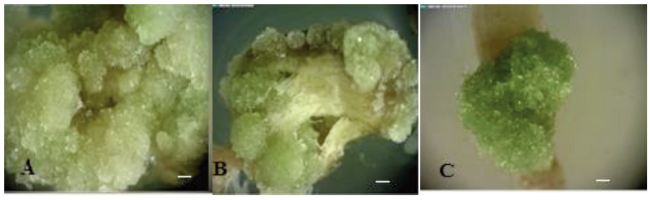

Fig. 1 - The effect of 2,4-D, NAA and BAP on hypocotyl explants of *Catharanthus roseus* and the formation of embryogenic callus (3 week after culture). Scale bars= 1.0 mm. (A) Embryogenic callus from the treatment with 1 mg l^{-1} 2,4-D; (B) Embryogenic callus from the treatment with 2 mg l^{-1} NAA, (C) Embryogenic callus from the treatment with 1 mg l^{1}- NAA + 1 mg l^{-1} BAP.

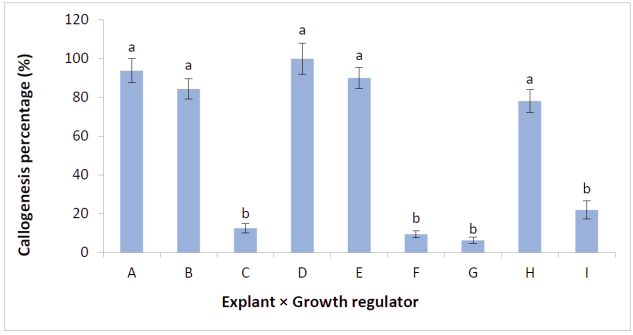

Fig. 2 - The interaction effects of explant×plant growth regulators on the callogenesis percentage of *Catharanthus roseus*. A= 1 mg l^{-1} 2,4-D×root; B= 1 mg l^{-1} 2,4-D×hypocotyl; C= 1 mg l^{-1} 2,4-D×leaf; D= 2 mg l^{-1} NAA×root; E= 2 mg l^{-1} NAA×hypocotyl; F= 2 mg l^{-1} NAA×leaf; G= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×root, H= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×hypocotyl; I= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×leaf).

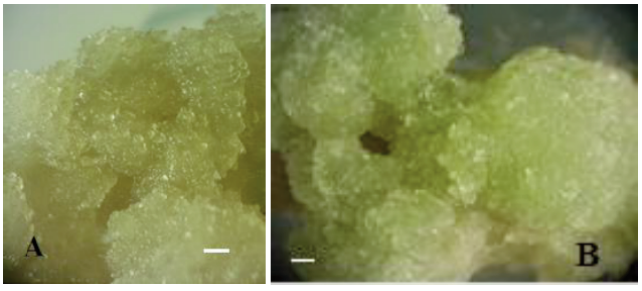

Fig. 3 - The effect of sub-culture of embryogenic calli from *Catharanthus roseus* hypocotyl influenced by different treatments (calli are produced 1 month after subculture). Scale bars= 1.0 mm. (A) Embryogenic calli produced by the treatment of 2 mg l^{-1} NAA; (B) Embryogenic calli produced by the treatment of (1 mgl^{-1} NAA+1 mg l^{-1} BAP).

The calli weight was obtained by weighting 10 samples. The results showed that the growth regulators and explants type and their interaction were significant on the weight of callus production, Treatment composition H and E with 0.85 and 0.81 g, had the most callus weight respectively. The interaction of hypocotyl and NAA; and hypocotyl x NAA + BAP were significant (p≤0.01) on callus fresh weight (Fig. 4).

Pre-embryonic and somatic embryogenic tissues

In this study, two different approaches were employed to increase the formation of embryogenic calluses. Both approaches were aimed at choosing the best combination and concentration of plant growth regulators. Different masses were observed on the embryogenic calli tissues and the pre-embryonic structures were formed evidently. The number of masess of possible pre- and somatic embryos formed were counted. Somatic embryos were isolated by liquied culture and shown with distinct roots and shoots (Fig. 5).

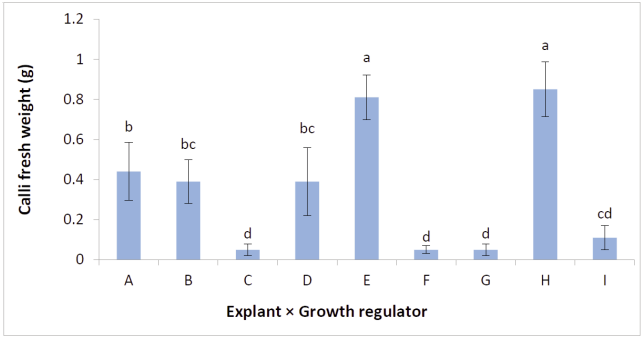

Fig. 4 - The interaction of explant×growth regulators on calli fresh weight at the *in-vitro* cultures of *Catharanthus roseus*. A= 1 mg l^{-1} 2,4-D×root; B= 1 mg l^{-1} 2,4D×hypocotyl; C= 1 mg l^{-1} 2,4-D×leaf; D= 2 mg l^{-1} NAA×root; E= 2 mg l^{-1} NAA×hypocotyl; F= 2 mg l^{-1} NAA×leaf; G= 1 mg l^{-1} NAA+1 mg l^{-1}BAP×root; H= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×hypocotyl; I= 1 mg l^{-1} NAA+1 mg l^{-1} BAP×leaf.

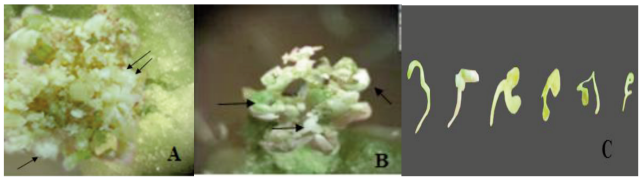

Fig. 5 - (A) Pre-embryo spots (bar= 1 mm); (B) somatic embryos formed on the calli derived from hypocotyl explants of *Catharanthus roseus* (bar= 1 mm); (C) Somatic embryos isolated from liquid cultures including root and shoot ends (bar= 5 mm).

The results showed that the effect of plant growth regulators and explants, and their interactions were significant (p≤0.01) on the number of pre-embryos and somatic embryos produced.

Furthermore, the interactions of hypocotyl × 2,4-D produced the highest number of pre-embryogenic and somatic embryogenic with mean 3.83 and 5.33 respectively in solid culture medium (Fig. 6).

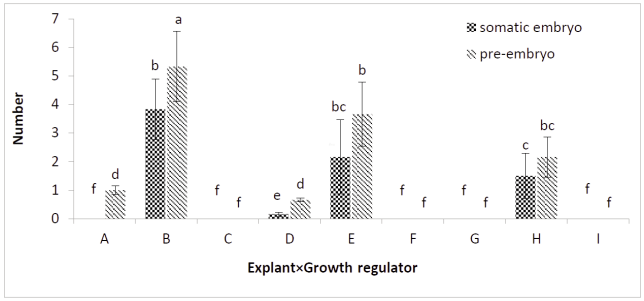

Fig. 6 - The interaction of explant×growth regulators on pre-embryos and somatic embryos number produced in *Catharanthus roseus in-vitro* culture. A= 1 mg l^{-1} 2,4-D × root; B= 1 mg l^{-1} 2,4D × hypocotyl; C= 1 mg l^{-1} 2,4-D × leaf; D= 2 mg l^{-1} NAA × root; E= 2 mg l^{-1} NAA × hypocotyl; F= 2 mg l^{-1} NAA × leaf; G= 1 mg l^{-1} NAA+ 1 mg l^{-1} BAP × root; H= 1 mg l^{-1} NAA + 1 mg l^{-1} BAP × hypocotyl; I= 1 mg l^{-1} NAA + 1 mg l^{-1} BAP × leaf.

4. Discussion and Conclusions

The most frequently used auxin in the studies on somatic embryogenesis is 2,4-D (Bhojwani and Razdan, 1996; Junaid *et al.*, 2006; Jushee *et al.*, 2007). The significant effects of 1 mgl^{-1} 2,4-D has been proven on callus formation and somatic embryogenesis on *Catharanthus roseus* and *Aconitum heterophyllum* (Giri *et al.,* 1993; Aslam *et al.,* 2004). This auxin stimulates cell division and plays an important role in the production of callus (Paramageetham *et al.,* 2004). Auxins alone or in combination with cytokinins are used for somatic embryogenesis induction and initiation (Wojcikowska and Gaj, 2016; Tanida and Shiota, 2019). More often, the use of cytokinins alone induces the production of non-embryogenic callus (Martin, 2004). In dicotyledonous plants, cytokinins are usually added to the culture medium along with auxins for the promotion of callus production (George *et al.,* 2008). In medicinal herb, *Asparagus racemosus* Willd with the embryonic explants cultured on MS- medium and supplemented with 1.5 mgl^{-1} 2,4-D + 0.43 mgl^{-1} kin; 74%

non-embryogenic callus was obtained (Chaudhary and Dantu, 2019). BAP is the major cytokinin used in the studies related to the somatic embryogenesis (Jimenez and Thomas, 2005). Singh *et al.* (2011) obtained 92% of non-embryogenic callus in *Catharanthus roseus* (L.) G. Don in MS culture medium supplemented with 1 mg l^{-1} NAA + 1 mg l^{-1} BAP with hypocotyl explants. Our results are consistent with the findings of Aslam *et al.* (2006) in which hypocotyl explants in MS medium supplemented with 1 mgl^{-1} 2,4-D, resulted in 85% of embryogenic callus. Moreover, they reached 73% of embryogenic callus by hypocotyl explant in the MS medium enriched with 1.5 mgl^{-1} BAP + 1 mgl^{-1} NAA; and 61.75% of embryogenic callus by hypocotyl explants in MS medium supplemented 1 mgl^{-1} NAA + 1 mgl^{-1} BAP as well as 85% of embryogenic callus by hypocotyl explant again in MS medium enriched with 1 mgl^{-1} 2,4-D (Aslam *et al.,* 2007). Studies have shown that 1 mgl^{-1} 2.4-D in *Ocimum basilicum* L., produced about 75% of embryogenic calli (Gopi and Ponmurugan, 2006). Also, the results of another study revealed that by culturing hypocotyl explants in MS medium containing 1 mg l^{-1} NAA +3 mgl^{-1} BA; 80% of non-embryonic callus was observed (Ren *et al.,* 2020).

Auxins and cytokinins form callus tissues by accelerating the division of plant cells (George *et al.,* 2008). In *Withania somnifera* stems cultured in MS medium with 1mgl^{-1} BAP + 1 mgl^{-1} NAA; calli were produced with an approximate weight of 0.22 g (Adhicari and Pant*,* 2013). In another study, internode explants of *Centella asiatica* L. on MS supplemented with auxins and cytokinins produced calli weighting up to 1.5 g (Martin, 2004).

2,4-D causes the rapid cell division and the polarization of cells (Jushee *et al.,* 2007). Our results are almost similar with the findings of Choi *et al.* (1999) On *Eleutherococcus senticosus* (75% of somatic embryos). Moreover, the findings of the present study are in line with the results of Martin (2003) on *Holostema adakodien*, which by using different explants and plant growth regulators; they clearly showed that hypocotyl explants and 2,4-D (1 mg l^{-1}) attained about 50% of somatic embryos. In another study, hypocotyl explants of *Eleutherococcus senticosus* in MS medium produced 89% of somatic embryos (Han and Choi, 2003). Also, our results are the same with Aslam *et al.* (2004) on *Catharanthus roseus* (L.) G. Don, whom described that hypocotyl and 2,4-D (1 m gl^{-1}) were the combination of choice. Also, in another study on *Catharanthus roseus* (L.) G. Don; the most somatic embryos were observed using hypocotyl explants and 2 mgl^{-1} 2,4-D. Auxins have synergistic effects with cytokinins and accelerate the cell division. 2,4-D may also add-up the endogenous levels of IAA in plant tissue, thereby creating dipoles within the cell and forming pre-embryonic structures (Mendez-Hernandez *et al.,* 2019). 2,4-D promotes the accumulation of ROS (oxygen reactive species), and stimulates the ethylene and abscisic acid biosynthesis in plant tissue and ultimately induces stress behavior in plant tissue. As a result, plant cells change or shift to form somatic embryos (Bharatia *et al.,* 2015; Wojcik *et al.,* 2020). 2,4-D application and availability in the culture medium probably results in the expression of cell differentiation genes and the demethylation of DNA. 2,4-D plays an important role in the somatic embryos induction and in the stages of maturation and development of somatic embryos. Whereas, 2,4-D, has an inhibitory role with the biosynthesis of a number of proteins and mRNAs (Krishnan and Siril, 2017). In our study, calli from leaf explants were unable to produce somatic embryos (Gulzar *et al.,* 2019). Otherwise, *Paeonia ostii* 'Feng Dan', *Asparagus racemosus* Willd, and *Cnidium officinale* Makino, the combinations of auxins and cytokinins produced reasonable somatic embryos (Adil *et al.,* 2018; Chaudhary and Dantu, 2019; Ren *et al.,* 2020).

Auxins had dominant effect on callus production of *Catharanthus roseus* (L.) G. Don. and the results showed that 2,4-D was much efficient than NAA. More somatic embryos were obtained from hypocotyl explants. Due to the medicinal importance of *Catharanthus roseus*, it is suggested that in the further studies, somatic embryogenesis behavior in this plant should be studied on a larger scale and that the possibility of somatic embryogenesis in a liquid culture medium using suspension culture should be tested in different ways. Therefore, it is possible to study the biosynthesis of secondary metabolites and valuable alkaloids *in vitro* conditions and to optimize the protocols to extract and purify the above mentioned metabolites.

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Genetic variability and relationship among different accessions of *Froriepia subpinata* Bail (Gijavash) an endangered medicinal plant from Iran revealed by ISSR and IRAP markers

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Key words: Endemic plant, genetic conservation, threatened species.

Abstract: The genetic variability of *Froriepia subpinata* Ledeb. Bail., an endangered Iranian endemic species, has been estimated with a total of 52 accessions using 20 markers including ISSR and IRAP. The results showed the polymorphic band produced by primers was 82.3%. The best mean values of genetic diversity parameters observed in ISSRs markers, being UBC873, UBC811, and UBC873 the best primers tested. The similarity range among accessions was 34.45% to 93.3%. The cluster analysis classified the accessions into five main groups that in totally, accessions with similarity in region generally were clustered in the same group. Overall, present study could provide elementary information for formulation of conservation strategies and invaluable elementary genetic information for next breeding or designing conservation programs.

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The authors declare no competing interests.

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1. Introduction

Froriepia subinata Ledeb. Bail. syn= *Buplerum subinatum, Froriepia nuda* is a biennial medicinal and aromatic plant, locally known as Gijavash, that belong to the Apiaceae family. It is a self-pollinated plant with white flowers and small achene fruits. Gijavash leaves are used in diet people and have antimicrobial, antifungal properties and high antioxidant activity (Salmanian and Sadeghi, 2012). This species is the only endemic threatened one of *Froriepia* genus in northern Iran. In this genus, somatic chromosome number ranged between 2n= 14 and 2n=16. Some of its phonological characteristics like irregular and delay germinations and also excessive and improper harvest have exposed it to mortality and annihilation (Mozaffarian, 2015).

Nowadays, due to increased human activities and excessive growth in residential area, plants habitats have been destroyed. So, investigation and study on each threatened plant for identifying best way to protect should be considered. Although pastures protection ways as well as molecular finger-

printing, micropropagation and other biotechnological methods and beneficial techniques (Glover and Abbott, 1995; Sudha *et al.*, 1998) are the most important method for conservation of rare and endangered plants. Therefore, genetic diversity of endangered species is the first step in conservations strategies and selection for domesticating process (Vicente *et al*., 2011).

Moreover, recently an increasing number of studies for plant conservation biology, especially in rare endemic species have demonstrated the value of genetic data (Gaudeul *et al*., 2000; Bellusci *et al*., 2008; González-Pérez *et al*., 2009).

One of the most important features for long-term survival and adaptation to environment conditions of population or species is genetic variation within their taxon (Frankham, 2010).Having information on genetic diversity of a plant species is very necessary for its conservation (Höglund, 2009; Frankham, 2010; Laikre, 2010) as losses of genetic diversity are likely to have consequences for plant fitness (Reed and Frankham, 2003; Dostálek *et al*., 2010). Preserving rare species endangered especially those which have restricted geographic distributions is main concern of scientist because of their habitat destruction and fragmentation. On the other hand, losing allelic richness or genetic diversity in fragmented populations due to their genetic drift and inbreeding depression have increased population differentiation (Buza *et al*., 2000; Tomimatsu and Ohara, 2003).

Thus, an accurate estimate on the level and distribution of genetic diversity of threatened and endangered species seems necessary for designing conservation programs (Smith and Wayne, 1996; Höglund, 2009). In addition, understanding the chance of species survival in the short-term, formulation of conservation strategy for long-term survival need to population genetic information (Cires *et al.*, 2013). Meanwhile, knowledge of population genetic structure can provide important information to understand the evolution of rare and endangered different species. For example, by identifying populations of greatest evolutionary potential as well as populations best suited for source material for *ex situ* preservation or reintroduction (Furches *et al.*, 2009).

According to the fragmentation distribution and the endangered status of this endemic species, there are no population genetic studies and conservation management plans. As an initial step in developing such plan, we have assessed the genetic variability of 52 natural populations of *F. subpinata* using inter simple sequence repeat (ISSR) and inter-retrotransposon amplified polymorphism (IRAP).These populations were naturally grown in their own habitat. To avoid from possibility pollination between different populations, the distance between the populations was considered and tried to elect population which had more difference between each other due to morphological and environmental characteristics.

ISSR and IRAP were chosen because of their advantages over other DNA polymorphism analysis methods, as they do not require prior sequence knowledge, cloning procedures or characterized probes. It is also generally accepted that they have a comparatively high reproducibility (Jones *et al*., 1997). Therefore, both techniques have been successfully used in plant population genetic studies, especially for endangered species (Li and Jin, 2007; Gong *et al.*, 2010; Noroozisharaf *et al*., 2015).

Froriepia subpinata is commonly used in traditional foods in Iran for its bioactive compounds and antioxidant potential. However, the destruction of its natural habitats by human activity has put a strain on its survival. Therefore, the study of genetic diversity among accessions collected from different areas of Iran would be very useful in the biodiversity management and conservation plans organization.

2. Materials and Methods

Plant Material

Fresh leaves of Gijavash (*F. subpinata*) accessions were gathered from 52 different localities of Guilan province, Iran. To accurate estimate the genetic variability, based on local people's knowledge and distribution of the plant, we elected 52 locations throughout several cities (Fig. 1).

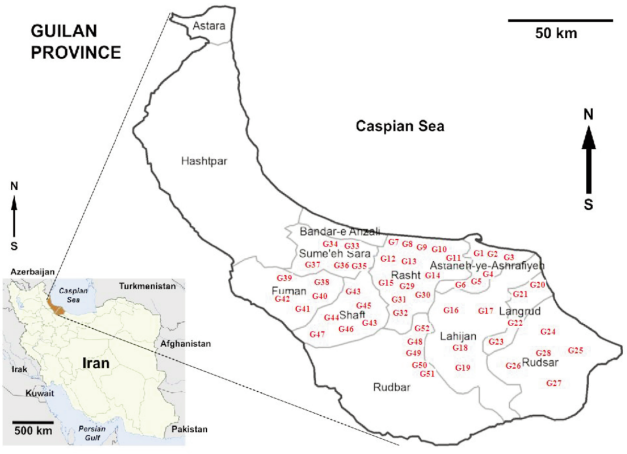

Fig. 1 - Geographical map of 52 *F. subpinata* accessions location collected from Guilan province, Iran.

All the accessions are listed in Table 1 with the location and the altitude of each one. Plants were randomly selected from two or three individuals of each site. To reduce the probability duplicate sampling fresh leaves were taken from each individual separated at least 3 m apart. Samples were immediately frozen in liquid nitrogen and kept at -80°C for genomic DNA extraction. In addition, voucher specimens were collected, dried by pressing in absorbent paper, stored at room temperature, and lodged at the herbarium of the University of Guilan.

DNA extraction

DNA extraction was carried out using the CTAB method described by (Doyle, 1990) with minor modifications as follow: approximately 50-70 mg leaf material was ground in liquid nitrogen, then 600 µL of hot (65°C) extraction buffer 2X (100 mM Tris HCl, pH 8; 20 mM EDTA; 1.4 M NaCl; 2% CTAB; 1% PVP) was added. Subsequently, an equal volume of cold chloroform/Isoamyl alcohol (24:1) was added and mixed by gentile inversion of the tube until a light green single phase emulsion is performed. In the next step, the emulsion centrifuged at 10000 rpm for 10 min. Then, the aqueous phase transferred into a clean tube and addition 100 µL of CTAB solution (10% CTAB, 0.7 M NaCl) and the extraction was repeated. This step may takes several times until no precipitate can be detected at phenol/aqueous layer interface. The aqueous phase is removed and the rest mixed with an equal volume of hot (65°C) CTAB precipitation buffer (50 mM Tris HCl, pH 8.5, 10 mM EDTA, 1% CTAB). The solution mixed gently and incubated every 3-5 min at room temperature for 30 minutes. The resulting CTAB/DNA complex is immediately platted by centrifugation at 12000 rpm for 10 min. The resulting pellet resuspended in 650 µL high salt buffer (10 mM Tris HCl, pH 8; 1 mM EDTA; 1 M NaCl) and the DNA precipitated by addition 1300 µL of cold 100% ethanol. The precipitation gently mixed and incubated every 3-5 min on ice for 30 min. then, it centrifuged at 12000 rpm for 10 min. The pellet was washed three times with 1 ml of cold 70% ethanol, and then dried at room temperature. Finally, pellet was resuspended in 100 µL TE buffer (10 mM Tris HCl, pH 8; 1mM EDTA). Extracted DNA was qualified using 1% (w/v) agarose gel electrophoresis. Afterwards, the DNA concentration and contamination rate was evaluated by NanoDrop spectrophotometers (Thermo Fisher scientific, 5225 Verona Rd, USA). For PCR reaction, only template of DNA was used which had a purity of 2 in a dilution of 15 ng/ml.

Table 1 - Different heat treatments used to enhance seed germination of *Cycas revoluta*

Accession name	Gathered site	Voucher number	Altitude (m)
G1	Poushal	APF53098	-14
G2	Reshvandeh	APF53099	-11
G3	Lashkam	APF54000	-20
G4	Loskehkelayeh	APF54001	-18
G5	Pasgahfarhad	APF54002	-5
G6	Kisom	APF54004	2
G7	Touchipaybast	APF54005	1
G8	Seghaleksar	APF54007	35
G9	Lakan	APF54008	62
G10	Selkisar	APF54011	90
G11	Aziz kiyan	APF54013	174
G12	Hasan kiadeh	APF54014	-22
G13	Kiashahr forest	APF54015	-22
G14	Kiashahr	APF54016	-24
G15	Koshkbijar	APF54017	-25
G16	Ghasabmahaleh	APF54018	-17
G17	Goharsara	APF54019	145
G18	Sheykhan bar	APF54020	124
G19	Toustan	APF54022	-12
G20	Salkuyeh	APF54024	-19
G21	Taleshmahaleh	APF54025	73
G22	Langrud1	APF54026	4
G23	Langrud2	APF54027	8
G24	Chafjir	APF54028	-25
G25	Sahnehsara	APF54030	-15
G26	Karaj posht	APF54031	-21
G27	Chinijan	APF54029	-21
G28	Rudsar	APF54032	-20
G29	Darehposht	APF54033	89
G30	Saravan park	APF54035	141
G31	Tekhsem	APF54036	114
G32	Saravan	APF54037	93
G33	Tahergurab	APF54038	24
G34	Ziabar	APF54039	31
G35	Shanderman	APF54034	49
G36	Sheykhneshin	APF54040	43
G37	Someehsara	APF54038	3
G38	Fuman1	APF54041	35
G39	Sehpiranpayin	APF54042	20
G40	Dobakhshar	APF54044	98
G41	Kohnehgurab	APF54043	14
G42	Fuman2	APF54046	41
G43	Shaft	APF54045	43
G44	Khartum	APF54047	71
G45	Mozhdeheh	APF54048	83
G46	Shah khal	APF54049	48
G47	Dastkhat Chamacha	APF54050	122
G48	Dozdak	APF54052	51
G49	Ezberem	APF54051	62
G50	Bidrun	APF54053	24
G51	Siyah Kal1	APF54054	37
G52	Siyah Kal2	APF54055	42

PCR amplification

PCR reactions were done in 1500 µL reaction volumes containing 750 µL of sterile double distilled water, 150 µL of Taq polymerase reaction buffer (10×), 1 mM $MgCl_2$, 150 µL of dNTPs (10 mM), 100 µL of each primer at 5 mM, 0.5 unit of Taq DNA polymerase, and 200 µL of plant DNA. The planning of thermal cycling was as follows: initial template denaturation at 94°C for 4 min, 35 cycles of denaturation 94°C for 1 min, annealing at 42-50°C (depending on primer used) (Table 2) for 1 min, extension at 72°C for 90s, and final extension at 72°C for 5 min.

The PCR products were loaded on 1.5% (w/v) agarose gel in 1× TAE buffer at voltage of 70 for 90 min. The gel's images were captured using the Biometra gel documentation system (Whatman Biometra, Gottingen, Germany). The produced fragments size in comparing to size marker was distinguished (GeneRuler 1 kb DNA ladder, SM0241, Fermentase, Ontario, Canada).

Data analysis

In all, 20 individual ISSR and IRAP primers with their combinations were used (Table 2). Only reproducible and well clear bands in the replications were considered as potential polymorphic markers. It was assumed that each band represented the phenotype at a single biallelic locus, because the ISSR and IRAP markers are dominant (Williams *et al*., 1990). Amplified fragments were scored for presence (1) or absence (0) of homologous bands. According to PCR banding patterns, a data matrix was created for each reaction. Polymorphism information content (PIC), Effective multiplex ratio (EMR) and Marker index (MI) were calculated (Smith and Wayne, 1996).

Effective number of alleles (Ne), Nei's gene diversity (Nei, 1972) and Shannon's information index (Shannon and Weaver, 1949) were estimated for total accessions using POPGENE software version 1.31 (Yeh, 1999). Similarity matrix based on simple matching coefficient was constructed from the ISSR and IRAP

Table 2 - Polymorphism detected with ISSR and IRAP marker in 52 germplasm accession

Marker type	No.	Primer name	Annealing temperature (˚C)	Sequence (5'-3')	No. of bands	No. of polymorphic bands	% of polymorphic bands
IRAP	1	TOC-1	48.8	TGTTGGGAATAGTCCCACA	9	7	77.77
	2	TOC-2	45.2	TGTTGAATAGTTCCACATT	7	7	100.00
Mean					8	7	88.88
ISSR	3	UBC808	47.4	$(AG)_8$ C	8	6	75.00
	4	UBC811	41	$(GA)_8$ C	10	9	90.00
	5	UBC812	41.2	$(GA)_8$ A	5	4	80.00
	6	UBC813	42.8	$(CT)_8$T	8	7	87.5
	7	UBC816	49.2	$(CA)_8$T	9	8	88.88
	8	UBC817	48.8	$(CA)_8$A	7	5	71.42
	9	UBC824	46.6	$(TC)_8$G	8	8	100.00
	10	UBC825	50	$(AC)_8$T	6	6	100.00
	11	UBC826	50	$(AC)_8$ C	8	6	75.00
	12	UBC873	45.8	$(AG)_8$CTT	7	7	100.00
Mean					7.6	6.6	86.78
ISSR+ISSR	13	UBC808+UBC817	45.2	$(AG)_8$C+ $(CA)_8$A	6	6	100.00
	14	UBC812+UBC813	41	$(GA)_8$A+ $(CT)_8$T	7	5	71.42
	15	UBC811+UBC813	42	$(GA)_8$C+ $(CT)_8$T	6	5	83.33
	16	UBC816+UBC817	46	$(CA)_8$T+ $(CA)_8$A	7	4	57.14
	17	UBC825+UBC826	47	$(AC)_8$T+ $(AC)_8$C	9	5	55.55
Mean					7	5	73.48
ISSR+IRAP	18	UBC817+TOC-1	45.8	$(CA)_8$A+ TGTTGGGAATAGTCC-	5	4	80.00
	19	UBC812+TOC-2	42.8	$(GA)_8$ A+ TGTTGAATAGTTCCA-	7	5	71.42
	20	UBC813+TOC2	44.2	$(CT)_8$T+ TGTTGAATAGTTCCA-	8	7	87.50
Mean					6.66	5.33	79.64
Total Mean					7.3	5.98	82.19
Total					147	121	82.31

data. It was used for the cluster analysis and construction of dendrogram through unweighted pair-group method using arithmetic average (UPGMA), performed by NTSYS-PC software (Rohlf, 2000). In order to evaluate fitness between the dendrogram and similarity matrix, the cophenetic correlation coefficient was calculated. Principal coordinate analysis (PCoA) was accomplished using GenStat (GenStat v12, VSN International Ltd, UK) on a similarity matrix.

3. Results

Twenty individuals ISSR and IRAP and their combinations (ISSR+ISSR; ISSR+IRAP) produced 147 distinguish-able fragments out of which 121 (82.31%) were polymorphic. The polymorphic rang was from 4 in UBC812 to 9 in UBC811 with an average number of 6.05 polymorphic bands per primer. The products number varied from 5 in UBC812 to 10 in UBC811. The mean of polymorphic band percent were 88.88, 86.78, 73.48 and 79.64 for IRAPs, ISSRs, ISSR+ISSR and ISSR+IRAP, respectively. The TOC-2, UBC824, UBC825, UBC873 and UBC808+UBC817 primers had the maximum of polymorphic bands (100%). The minimum of polymorphic bands produced by UBC825+UBC826 primer (55.55%) (Table 2).

The means of PIC value for the amplification products was 0.30 (Table 2). UBC816+UBC817 and UBC873 showed the lowest (0.19) and the highest (0.45) PIC values, respectively. The means of PIC for IRAPs, ISSRs, ISSR+ISSR and ISSR+IRAP were 0.27, 0.32, 0.27 and 0.26, respectively (Table 3).

On the whole, among the 20 used primers, maximum of the EMR, MI, *Ne*, *H* and *I* recorded in UBC811 (8.1), UBC873 (3.16), UBC825 (1.75), UBC825 (0.41) and UBC825 (0.60), respectively .Also it must be considered that the total mean of EMR, MI, *Ne*, *H* and *I* were 5.11, 1.59, 1.49, 028 and 0.43 respectively (Table 3).

Table 3 - Genetic diversity detected with ISSR and IRAP markers in 52 germplasm accessions

Marker type	No.	Primer name	PIC	EMR	MI	*Ne*	*H*	*I*
IRAP	1	TOC-1	0.26	5.44	1.46	1.50	0.29	0.43
	2	TOC-2	0.28	7	1.98	1.57	0.34	0.52
Mean			0.27	6.22	1.72	1.53	0.31	0.47
ISSR	3	UBC808	0.23	4.5	1.06	1.52	0.28	0.41
	4	UBC811	0.34	8.1	2.77	1.56	0.32	0.47
	5	UBC812	0.36	3.2	1.16	1.54	0.31	0.46
	6	UBC813	0.32	6.12	2.00	1.46	0.23	0.38
	7	UBC816	0.35	7.11	2.55	1.60	0.34	0.51
	8	UBC817	0.26	3.57	0.94	1.53	0.29	0.42
	9	UBC824	0.31	8.00	2.54	1.38	0.24	0.40
	10	UBC825	0.35	6.00	2.10	1.75	0.41	0.60
	11	UBC826	0.27	4.5	1.22	1.40	0.24	0.38
	12	UBC873	0.45	7.00	3.16	1.60	0.36	0.55
Mean			0.32	5.81	1.95	1.53	0.30	0.45
ISSR+ISSR	13	UBC808+UBC817	0.35	6.00	2.11	1.73	0.40	0.58
	14	UBC812+UBC813	0.22	3.57	0.78	1.40	0.24	0.36
	15	UBC811+UBC813	0.38	4.16	1.58	1.45	0.28	0.44
	16	UBC816+UBC817	0.19	2.28	0.44	1.25	0.16	0.26
	17	UBC825+UBC826	0.22	2.77	0.61	1.30	0.18	0.28
Mean			0.27	3.75	1.10	1.43	0.25	0.38
ISSR+IRAP	18	UBC817+TOC-1	0.23	3.2	0.76	1.55	0.31	0.45
	19	UBC812+TOC-2	0.26	3.57	0.93	1.29	0.18	0.29
	20	UBC813+TOC2	0.30	6.12	1.84	1.48	0.28	0.43
Mean			0.26	4.29	1.17	1.43	0.25	0.39
Total Mean			0.30	5.11	1.59	1.49	0.28	0.43

PIC= polymorphism information content; EMR= effective multiplex ratio; MI= marker index; *Ne*= effective number of alleles; *H*= Nei's gene diversity; *I*= Shannon's information index.

In addition, there was a significant correlation at P≤0.01 probability level between most of these indices, so that only between EMR and *Ne* as well as EMR and *H* were significant at P≤0.05 level of probability (Table 4).

Table 4 - Correlation between genetic diversity parameters

	PIC	EMR	MI	*Ne*	*H*	*I*
PIC	1					
EMR	0.59 **	1				
MI	0.81 **	0.93 **	1			
Ne	0.58 **	0.49 *	0.56 **	1		
H	0.64 **	0.52 *	0.61 **	0.98 **	1	
I	0.70 **	0.60 **	0.69 **	0.96 **	0.98 **	1

PIC= polymorphism information content; EMR= effective multiplex ratio; MI= marker index; Ne= effective number of alleles; H= Nei's gene diversity; I= Shannon's information index.
** P ≤0.01; * P≤0.05 according to Tukey test.

Principal coordinate analysis (PCoA) was constructed based on simple matching coefficient of similarity. The results showed that the first twelve principal coordinates account for 70.29% of total variation. The first and second extracted component accounted for 26.43% and 9.11% of the variation, respectively (Fig. 2).

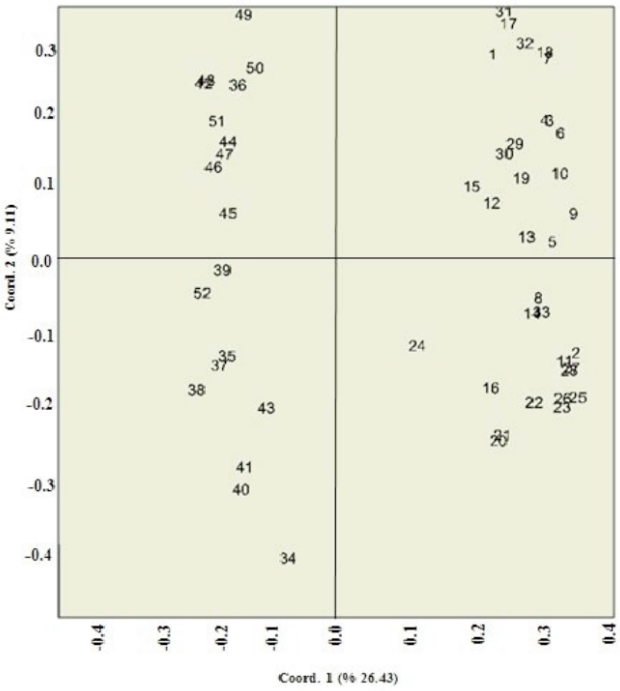

Fig. 2 - Principal coordinate analysis (PCoA). The plot shows the first two principal components (coord. 1 and coord. 2). Accessions codes are identified in Table 1.

To draw cluster analysis for 52 Gijavash accessions, the obtaining data from ISSR and IRAP analysis were used. Figure 3 presents the dendrogram of genetic relationships among the accessions as revealed by the UPGMA method. The 52 accessions of Gijavash classified into 5 main groups. The similarity coefficient range varies from34.45% to 93.27%. The highest similarity was related to G4 and G6 and the lowest similarity observed between G26 and G38 (Fig. 3). Also, high amount of calculated cophenetic correlation coefficient (r=95.2%) showed that UPGMA method was useful in the clustering plant accessions.

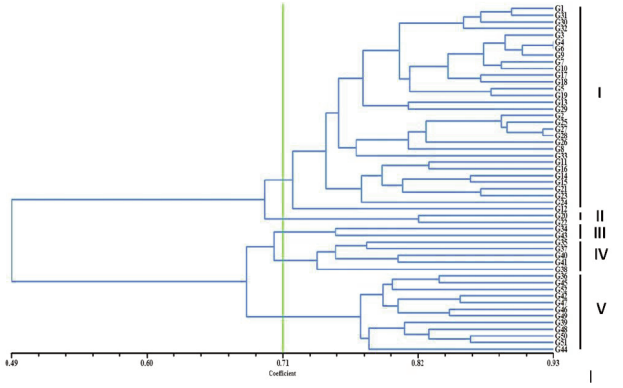

Fig. 3 - UPGMA dendogram of ISSR and IRAP analyses on 52 germplasm accessions of *Froripia subpinata* Ledeb. Bail based on simple matching coefficient.

4. Discussion and Conclusions

Using dominant molecular markers for assessing genetic diversity is usually similar and directly comparable (Nybom, 2004). So that, these dominant markers widely have been used for earning genetic information in large number of endemic and endangered species from different plant families (Jeong *et al.*, 2010; Brütting *et al.*, 2012; Cires *et al.*, 2013; Noroozisharaf *et al.*, 2015) and also for formulation and implementation conservation strategies, along with testing genetic relationships between species (González-Pérez *et al.*, 2009). We applied 20 ISSR and IRAP primers to examine the genetic diversity of 52 accessions from the natural distribution of wild *F. subpinata*. The results showed that a high genetic diversity has been achieved in this species (*H*= 0.28, *I*= 0.43) in comparison with the corresponding genetic coefficients of other endangered species (Hamrick and Godt, 1996; Nybom, 2004; Zheng *et al.*, 2008).

The results of ISSR and IRAP markers demonstrated similar overall trends for genetic diversity. Nevertheless, the genetic diversity indices from IRAP approximately are lower than those from ISSR due to IRAP tending to produce somewhat low estimates of within-population variation (Nybom, 2004).

According to the attributes of *F. subpinata* accessions (i.e. fragmented, endemic) it could expected that there should be low genetic diversity, but in general, it seems that the total genetic diversity based on ISSR and IRAP markers is similar to, or slightly higher, than most of those used by different authors in other plants like *Primula heterochroma*, *Bupleurum rotundifolium*, *Changium smyrnioide, Cycas guizhouensis*. Nei's genetic diversity accounts in other ISSR and IRAP studies ranged from 0.10-0.28 (Qiu *et al.*, 2004; Wu *et al*., 2004; Xiao *et al.*, 2004; Shao *et al*., 2009; Jeong *et al*., 2010; Brütting *et al.*, 2012; Noroozisharaf *et al*., 2015). Base on this result and high polymorphism rate (82.31%), our research has manifested the potential of ISSR and IRAP markers, reproducible and useful methods for classifying different accessions.

Principal coordinate (PCoA) showed that accessions were divided into two groups, (i) the first group of accessions who collected from East of Guilan Province and (ii) the second group belong to West of Guilan Province origination. Many biological factors can influence both the species genetic diversity and its distribution among populations. Among these, the geographic distribution has been considered as one of the most important (Hamrick and Godt, 1990).

In contrast, in another study the geographical range had no significant influence on genetic diversity (Nybom, 2004). Our finding may be related to self-pollination character of this plant, that cause, accessions with less distance from each other had more genetic similarity.

The result of cluster analysis (Fig. 2) also showed that accessions with same region had more similarity to each other, so that maximum of similarity (93.27%) was between G4 and G6, and also the lowest of similarity (34.45%) was between G26 and G38. The G26 and G38 accessions originated from the east (Rudsar city) and west (Fuman city) of Guilan province, respectively (Fig. 1) and it could confirm the relative between genetic similarity and geographic distance in this research.

Overall, present study could provide invaluable elementary genetic information for next breeding plan.

Genetic diversity of different Gijavash accessions was analyzed using ISSR and IRAP molecular markers for the first time.

Results revealed that using of ISSR and IRAP markers had high efficiency for differentiating among the various accessions. Among all used primers, the highest PIC value, EMR and MI was belonging to UBC873, UBC811 and UBC873, respectively. The maximum of *Ne*, *H* and *I* observed in UBC825. With respect to these findings the UBC873, UBC811, UBC873 and specially UBC825 were the most informative primers which could be used to determine the diversity of Gijavash accessions.

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Short note

Warm stratification improves embryos development and seed germination of *Cycas revoluta*

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Key words: *Cycas revoluta*, development, germination, treatment, warm stratification, zygotic embryos.

Abstract: The broad objective of this research is to study the effect of warm stratification on *Cycas revoluta* zygotic embryos length, seed germination and plant development. Four warm treatments were applied to seeds. Our results showed that seeds storage at room temperature or 30°C improved significantly zygotic embryos length. Moreover, time of germination was significantly reduced with the warm stratification. The highest percentage of germination was obtained with seeds warm treated at 30°C for 2 months while only 25% of seeds were able to germinate in the control. Regarding seedlings development, our results demonstrated that warm stratification did not affect plant development. No significant differences have been recorded in all the evaluated parameters except for root length. Taken together, these results underlined the beneficial effect of warm stratification on *Cycas revoluta* seed germination and plant development and proposed a new method to improve seed germination of *Cycas revoluta*.

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Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Author Contribution Statement
J.B, A.S and A.G.conceived and designed the experiment. J.B., S.B. performed experiments and collected the data. J.B., S.B., Z.T., Q.L.A., and R.L. **contributed** reagents/materials/analysis tools. J.B., S.B., A.S. and A.G. analyzed data and wrote the manuscript.

Competing Interests:
The authors declare no competing interests.

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1. Introduction

The sago palm (*Cycas revoluta L.)* is one of the widespread ornamental trees, grown in temperate, subtropical and tropical regions more precisely in Miyazaki and Kagoshima Prefectures in Kyushu District down to the Ryukyu Islands, Okinawa Prefecture in Japan (Zarchini *et al.*, 2011). Described as one of the most primitive species among the living cycads, *Cycas revoluta* has been used as an indoor and outdoor landscape plant for centuries (Stevenson, 1990; Jones, 1994).

Cycas revoluta is propagated either from seeds, which remain viable for only a short time, or from vegetative offshoots (Demiray *et al*., 2017). Germination of seed of *Cycas revoluta* is hard and time consuming (Zarchini *et al*., 2011). Seeds can take 3 to 9 months to initiate germination before they can continue to germinate for periods of a year or more. *C. revoluta* seeds also demonstrates rapid loss of viability and low mor-

phogenic potential, which hinder its conservation (NADERI *et al.*, 2015).

Breaking dormancy is the main problem faced by all *Cycas revoluta* (Frett, 1987). Several attempts have been made to overcome with *Cycas revoluta* seed dormancy problem. Priming treatments of seeds seems to offer a new way to increase seeds germination percentage. Mechanical and chemical scarification has been described widely as an efficient way to improve germination of the hard-seeds species of *Cycas* and some other species known for their hard-coated seeds (Frett, 1987; Rouhi *et al*., 2010). Indeed, several studies have reported a great responsiveness of Cycads seeds to various pretreatments, namely, scarification, depulping, exposure to chemical substances like potassium nitrate, gibberellic acid or sulfuric acid or soaking in hot water for specific period of time (Zarchini *et al*., 2011; Millaku *et al*., 2012). Warm stratification was also used to improve seed germination of many species such as *Sambucus* and *Symphoricarpos* (Baskin *et al.*, 2002). The present study aimed to improve the germination of *Cycas revoluta* seeds through different warm stratification treatments as a way to develop an efficient *in vivo* germination protocol for this ornamental species.

2. Materials and Methods

Plant material

Freshly harvested seeds collected from 50 years old female mature plants grown in Faculty of Sciences garden, University Mohammed V in Rabat (Morocco) were used in this study.

Zygotic embryos length measurement

Seeds were soaked in water for 48 hours in order to soften the sacrotesta; the orange external layer. The sacrotesta was then removed mechanically with a knife. Seeds were then flamed with ethanol for 2 minutes. Sclerotesta layer was mechanically eliminated. The megagametophytes were surface sterilized for 20 minutes by soaking in 30% dilution of NaOCl containing 2-3 drops of Tween-20, followed by 3-4 rinses with sterile distilled water. After surface sterilization, megagametophytes were pooled, longitudinally bisected and the zygotic embryo (ZE) was excised from each megagametophyte. ZEs length was measured and the mean was calculated from at least 20 biological replicates.

Warm stratification treatments and seeds cultivation

After removing the sacrotesta mechanically, equal samples of seeds were subjected to different treatments. Treatments consisted in seed storage at room temperature (18-20°C) or 30°C for 2 or 4 months depending on the treatment (Table 1). The warm stratification temperatures were chosen based on previous reports that underlined the beneficial effect of seed storage at 18°C and 30°C on seed's germination (Roh *et al.*, 2004; Baldos *et al*., 2014; Keun *et al*., 2016). Untreated seeds were cultivated immediately and referred as control treatment (T_0). Seeds of *Cycas revoluta* were then planted in bins containing sterilized soil at 2-5 cm depth. Cultures were incubated at 25±2°C, with a photoperiod of 16 hours of light and 8 hours of darkness and watered daily depending on soil moisture.

Table 1 - Different heat treatments used to enhance seed germination of *Cycas revoluta*

Treatment	Type of treatment	Time of application
T_0	Control/Untreated	0 hours
T_1	Room temperature (18-20°C)	2 months
T_2	30°C	2 months
T_3	Room temperature (18-20°C)	4 months
T_4	30°C	4 months

Germination and plant growth parameters recording

Weekly observations were performed and seed emergence was recorded after 10 months of cultivation. The data for the kinetic of germination and time of germination (days) were recorded. Percentage of germination was calculated after ten months of culture. Number of leaves, stip height and width, root length and the length of the most developed leaf were determined at the end of the experiment.

Statistical analysis

Zygotic embryos length, time of germination, number of leaves, stip height and width, root length and the length of the most developed leaf were compared using a fixed model of analysis of variance (ANOVA). For each parameter and condition, means were calculated based on at least thirty biological replicates. In case of significant difference between groups, a Tukey test was used for means separation, at risk of 0.05.

3. Results

Effect of warm stratification on zygotic embryos length

Zygotic embryos (ZEs) length was investigated in the different warm pre-treatments. Our results showed that all the applied treatments (T_1, T_2, T_3 and T_4) influenced ZEs length compared with the untreated seeds (T_0) (Fig. 1). ZEs length of seeds subjected to T_1, T_2, T_3 and T_4 was significantly enhanced, but no significant difference was observed between T_3 and T_4 seeds. Indeed, ZEs length increased by 134%, 276%, 300% and 342% in T_1, T_2, T_3 and T_4 warm treated seeds respectively when compared to untreated seeds.

Effect of warm stratification on seed's germination

As mean to gain more insight on the effect of warm storage on seed germination, time of germination (calculated starting from the first day of seeds cultivation), percentage of germination and its kinetic were determined for each treatment. The results were summarized in figures 2, 3 and 4.

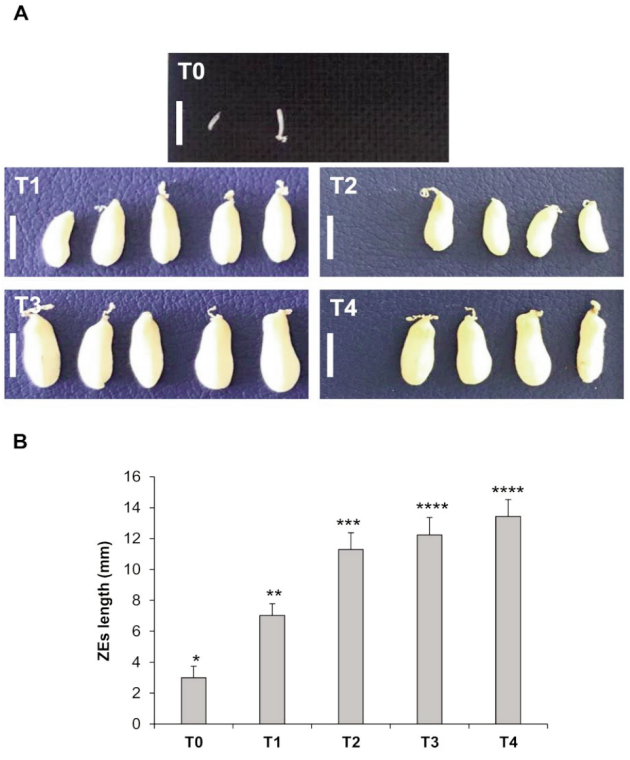

Fig. 1 - Effect of warm stratification treatments on *Cycas revoluta* embryos length. (A) Zygotic embryos isolated from seeds subjected to T0, T1, T2, T3 and T4. Scale bars=1 cm. (B) Zygotic embryos length in the different treatments. Bars are mean ± SD of at least thirty biological replicates. Asterisks indicate statistical difference according to Tukey test ($p<0.05$).

Effect of warm stratification on the time of germination

Minimum time of germination (163.33 days) was recorded in seeds stored at 30°C for 4 months followed by those stored at 30°C for 2 months (198.19 days) and those stored at room temperature for 4 months (202 days). Note that the difference between these three treatments was statistically insignificant. Untreated seeds (T_0) and those stored at room temperature for 2 months (T_1) took the maximum time duration for germination with an average of 294.4 days and 256.75 days respectively (Fig. 2).

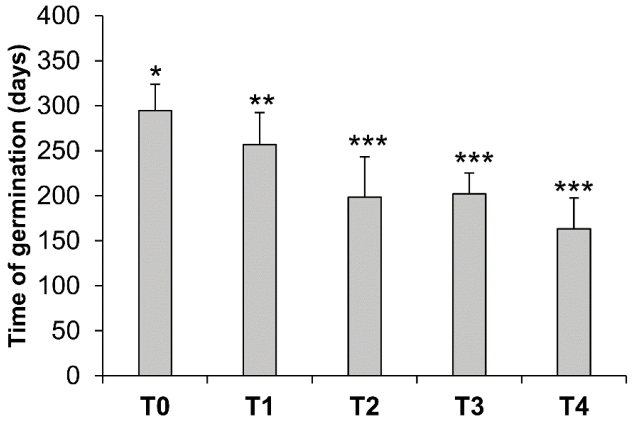

Fig. 2 - Effect of different warm stratification treatments on the time of germination of *Cycas revoluta* embryos Bars are mean ± SD of at least thirty biological replicates. Asterisks indicate statistical difference according to Tukey test ($p<0.05$).

Effect of warm stratification on the percentage of germination

Investigating the percentage of germination of seeds from the four different treatments and the untreated ones revealed that seed germination response varied among the different warm treatments. The highest percentage of germination (49.33%) was recorded in seeds stored for 2 months at 30°C while the lowest value of 8% was observed for seeds stored at room temperature for 4 months (Fig. 3). Untreated seeds showed although a percentage of germination around 25%.

Effect of warm stratification on the kinetic of germination

Investigating the kinetic of germination revealed a high variability between the different treatments applied (Fig. 4). Our data showed that seeds storage at 30°C for two or four months (T_2) allowed seeds to germinate faster. Indeed, seeds started to germinate

after four months of culture whereas, the first germinated seeds appeared after five months and 6 months respectively for T_1 and T_0. Seeds storage at room temperature for four months (T_3) delayed the germination by one month compared to untreated seeds (T_0). These data joined those related to time of germination.

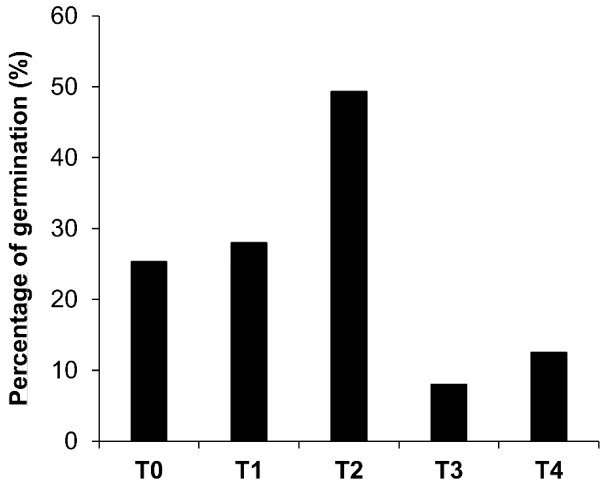

Fig. 3 - Effect of different warm stratification treatments on the percentage of germination of *Cycas revoluta* embryos.

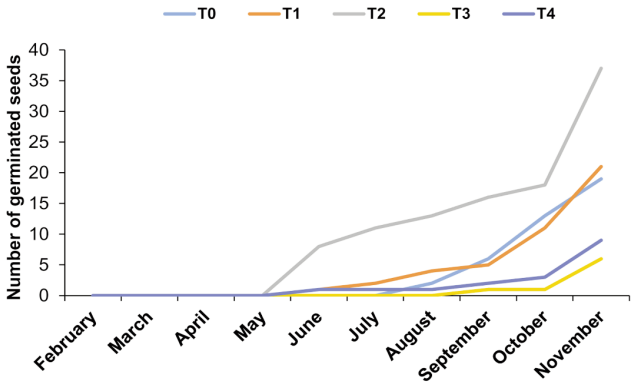

Fig. 4 - Effect of different warm stratification treatments on the kinetic of germination of *Cycas revoluta* embryos.

Effect of warm stratification on plant's growth and development

Seeds pre-treatment with temperature affected zygotic embryos length and their germination. These results prompted us to see whether the pre-treatment can influence seedlings growth and development. Several growth parameters namely, number of leaves per plant, stip height and width, root length and the length of the most developed leaf were evaluated. Table 2 summarized the results.

Data analysis had shown that seeds pre-treatment did not affect the number of leaves per plant. Seeds storage for 2 months at 30°C gave a maximum number of leaves per plant (1.50±0.59) while the lowest value was obtained with T_4 treatment (1.14±0.37). Note that these differences remained insignificant. Leaf length was also not significantly affected by the priming treatments. It was found that maximum leaf length was 57.33±3.05 cm in seedlings subjected to T_3 treatment while a minimum of 48.22±4.23 cm was recorded with T_1 treatment.

Stip height showed no significant difference between the different treatments. Stip width, on the other hand, displayed significant variations between the different treatments. A significant increase in stip width was observed in seedlings subjected to T_1 and T_2 as compared to the control (T_0) while non-significant changes was observed between the remaining treatments(T_3 and T_4).

Regarding root development, root length showed significant variations between the different treatments. Seeds storage for 2 months at 30°C resulted in a significant increase in root length compared to untreated seeds while a decrease in root length was observed with T_3 and T_4 treatments.

Table 2 - Effect of different priming treatments on growth parameters of *Cycas revoluta* seedlings

Treatments	Number of leaves/plant	Stip height (cm)	Stip width (cm)	Leaf length (cm)	Root length (cm)
T_0	1.31±0.47 a	3±0.47 a	1.81±0.19 b	50.63±6.24 abc	26.62±3.15 bc
T_1	1.20±0.42 a	3.92±0.32 a	2.10±0.24 a	48.22±4.23 bc	21.60±2.07 cd
T_2	1.50±0.59 a	3.77±0.45 a	2.15±0.22 a	55.91±4.25 ab	35.42±2.69 a
T_3	1.33±0.57 a	3.5±0.86 a	1.86±0.30 ab	57.33±3.05 a	31.66±2.31 ab
T_4	1.14±0.37 a	3.50±0.47 a	1.58±0.15 b	49±5 c	21±3 d

Values are mean ± SD of at least thirty biological replicates. Values with different letters indicate the statistical significance ($p<0.05$) according to Tukey test.

4. Discussion and Conclusions

Cycas revoluta is an ornamental tree that has been widely used as an indoor and outdoor landscape. However, despite its importance in landscape design, it is facing problems regarding its germination mainly due to the hard-coat (Ullah *et al.*, 2020). Different methods have been tested to overcome hardseededness. These include mechanical, chemical, and physical scarification treatments to make the seed coat permeable for water uptake. Several reports have shown that seeds pre-treatment with sulphuric acid (H_2SO_4), potassium nitrate (KNO_3) or GA_3 results in a better germination results. Indeed, *Zamia furfuracea* and *Cycas revoluta* germinations were assessed by Schutzman, using a chemical scarification with concentrated H_2SO_4 and then soaking them in gibberellic acid (GA_3) (Schutzman, 1984, 1989). However, these pretreatments are not always reliable with cycads (Dehgan and Yuen, 1983). For instance, Zarchini *et al.* (2011) have found that the use of sulphuric acid even at lower concentrations seems to affect negatively seed's germination while combined with hot water seems to improve *Cycas revoluta* seed germination.

It is well admitted that seeds storage in a warm environment usually results in relatively rapid embryos development (Baskin *et al.*, 2002; Merritt *et al*., 2007; Razavi and Hajiboland, 2009). However, no previous work has been conducted to study the effect of seed storage in warm conditions on *Cycas revoluta* germination and seedlings development. Thus, we investigated the effect of warm storage on zygotic embryos development, seeds germination and growth of young seedlings of *Cycas revoluta*. Our results showed that warm storage at 30°C for 2 months or 4 months speeded the germination when compared to the untreated plot (Fig. 2). Seeds storage at 30°C for 2 months improves also the germination percentage. Meanwhile, the prolonged storage at 30°C for 4 months reduced the germination percentage by half compared to the untreated plot which suggest that prolonged storage have an inhibitive effect rather than stimulating the germination (Fig. 3). Chen *et al*. (2007) have found that *Prunus campanulata* seeds required 4-6 weeks of warm followed by 8 weeks of cold stratification for maximum germination percentage. This finding was explained by the accumulation of high amounts of GA as a result of the cold stratification while GAs in warm stratified embryos were significantly low. Thus, *Prunus* warm stratified embryos failed to germinate since GAs content was very low. In *H. salicornicum* and *S. imbricate*, seeds germination was significantly improved when seeds were stored at 40±2°C for three months (El-Keblawy, 2013). Warm stratification for at least 1 month appeared to be essential for the germination of Japanese snowbell (*Styrax japonicus*) (Baskin, 2009). Indeed, Roh and Bentz (2003) found that without warm stratification, seeds were not able to germinate. Seed's dormancy in some orchids, mainly *Epipactis palustris* and *Goodyera pubescens,* could be overcame by a warm incubation of seeds followed by cold storage. This was explained by the fact that warm and cold stratification increased seeds permeability to water thereby softening the testa (Roh and Bentz, 2003). Thus, the increase observed in germination percentage could be attributed to the increase of seed's hard coat permeability caused by the warm storage which allow the removal of the physical barrier to water absorption. Besides the improvement of seeds germination percentage and the reduction of the germination time, warm storage improved significantly zygotic embryos length which suggest that warm scarification result in a better development of zygotic embryos. Regarding seedlings development, our data showed that warm scarification did not affect plant development (Fig. 5). Indeed, no significant differences have been recorded in all the evaluated parameters except for root length. Taken together, these results suggest that warm scarification improved seeds development and germination by prompting zygotic embryos length, increasing germination percentage and reducing time of germination.

Fig. 5 - *Cycas revoluta* plants obtained from pre-treated seeds with temperature (T1, T2, T3 and T4) and untreated seeds (T0).

This study aims to study the effect of seeds storage at different temperatures on *Cycas revoluta* seeds germination and development in order to develop an efficient *in vivo* germination protocol that can be used for mass production of this ornamental tree. Based on our results, we found that seeds storage at 30°C for 2 months or 4 months reduced time of germination. However, the highest percentage of germination was only assessed when seeds were stored at 30°C for 2 months. Taken together, this protocol represents a useful and potential method to improve commercial mass propagation of *Cycas revoluta*.

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