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CONTENTS

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Secondary metabolite changes in Maymars juniper cuttings (*Juniperus sabina*) under different treatments of propagation (IBA, substrate and harvest time of cutting)

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Key words: Antioxidant, flavonoid, IBA, phenol, rooting.

Abstract: The endemic Juniper of Maymars (*Juniperus sabina*) is one of the most valuable plants in forested areas. The objectives of this experiment were: I) to determine the best conditions for stem cutting propagation of this species, and II) to examine changes in some of the secondary metabolites during the four months (the first of each season): January, April, July, and October, after rooting of cuttings. The research was done with the treatment of five levels of Indole Butyric Acid, including: 0, 1000, 2000, 4000, and 8000 ppm in four rooting substrates, including perlite, perlite-cocopeat (1:1), pumice, and a mixed rooting substrate (sand, perlite, cocopeat, vermicompost, and potash; 1:1:1:1:1) in the four seasons of the year, with stem cuttings having an average length of 15 cm. The best treatment with more than 50% rooting was seen in April at levels of 4000 and 1000 ppm, and the best substrate was perlite cocopeat. Using lower levels of IBA led to a reduction in total phenol content in the cuttings during the rooting period. The flavonoid content of the cuttings varied across different seasons. Based on these results, we recommend this way of propagation for *Juniperus sabina* production. This propagation method takes less time in comparison with sexual propagation from seed.

1. Introduction

The genus *Juniperus* is one of the few conifers that act as a main tree in the natural ecosystems of the mountainous forests of the world. The

protective and valuable roles of various species of junipers in the management of forest erosion and water management are well known. Also, the role of junipers is important both in water storage and in soil conservation (Ali Ahmad Koruri *et al.,* 2011). They are great landscaping and ground cover species (Westerfield, 2012). Among the junipers, *Juniperus sabina*-Maymars is one of the most popular types of junipers. This species can be utilized for forest restoration on poor sites with low potential productivity, such as arid and semi-arid areas. In addition, Maymars is one of the most beautiful juniper species and is suitable for ornamental use (Piotto and Di Noi, 2003). Thus, information about the plant production of *Juniperus sabina* can be useful for forest managers and plant producers in many areas.

Berry extract of *Juniperus sabina* showed inhibitory activities against KB tumor cell lines (Sadeghi-aliabadi *et al.,* 2009). Fruit and leaves of junipers are commonly used as tea and pounded fruits are eaten to lower blood glucose levels in Anatolia. To evaluate antidiabetic and antioxidant potential and the chemical profile of *Juniperus sabina* L. in a study, phytochemical screening tests indicated the presence of flavonoids, tannins, terpenoids and carbohydrates in the extracts (Orhan *et al.,* 2017).

Maymars juniper is usually propagated by vegetative methods (Gheorghe *et al.,* 2010). To propagate plants via cuttings, the indole-butyric acid (IBA) growth regulator has been used as a treatment (Amri *et al.,* 2010). To produce junipers by stem cuttings, IBA has been used in previous studies (Henry *et al.,* 1992; Rifaki *et al.,* 2002). Research conducted by Rifaki *et al*. in 2002 on vegetative propagation showed the best concentration for the cuttings of junipers at 4000 ppm of IBA.

Phenolic compounds have effects on growth, development, propagation and plant defense (Croteau *et al.,* 2000). Measurement of internal compounds and their comparison during growth or rooting can be valuable factors in identifying internal barriers or enhancers of rooting in the cuttings, as there are no extensive resources available in this regard.

Phenolic compounds are a group of antioxidant agents (Choudhury *et al.,* 2013). Many scientists have reported the relationship between total phenol and antioxidant activities (Hariprasath *et al.,* 2015). In the propagation of varieties of blueberry, softwood cuttings and tissue culture, the interaction of genotype, propagation methods, and growth seasons significantly affected flavonoid content and antioxidant capacity. The interaction effect of the propagation method and genotype significantly affected total phenol and chlorophyll content. Also, the interaction between propagation method and growth season significantly affected the total flavonoid content (Goyali *et al.,* 2013).

Some studies have also revealed differences in rooting of cuttings as affected by substrate (Kentelky, 2011). Cocopeat and IBA were used to propagate *Juniperus excelsa* through stem cuttings, and they improved rooting ability (Esmael Nia *et al.,* 2006). Growth regulator and substrate are effective on the rooting of the cuttings of *Juniperus oblonga*, and proper substrate composition and the use of benzyl adenine increase the rooting of the cuttings (Khoshnevis *et al.,* 2012).

Roots uptake minerals and water from the soil (Chapin *et al.,* 1987). Higher numbers of adventitious roots could improve the root system's symmetry, stability, survival, and growth rate (Bryant and Trueman, 2015). Thus, rooting percentage is a good indicator of the growth strategies of root development and the capacity to endure water stress in Juniperus trees (Garcia Morote *et al.,* 2012).

Therefore, the present study is intended to investigate an efficient method of vegetative propagation of Maymars juniper using stem cutting and its effects on some of its phytochemical characteristics (phenolic compounds). We hypothesized that high level of phenolic compounds during rooting can be an indicator of the level of rooting in cuttings of *Juniperus sabina*, and that the percentage of rooting should be an indicator of rooting performance in cuttings. Thus, the objective of this research was to analyze the effects of five concentrations of IBA as treatment and four substrate types (perlite, perlite cocopeat, pumice, and mixed substrate) on the level of phenolic compounds and rooting performance in cuttings. The experiment was conducted in four months (February, mild climate; July, warm temperate climate; October, relatively cold weather; and January, cold weather) to determine the impact of harvesting time on the rooting capacity of cuttings.

2. Materials and Methods

Cutting preparation, treatment with indole butyric acid (IBA), and substrate composition.

The cuttings of *Juniperus sabina* were sampled from its natural habitat in the Chaharbagh mountains

of Gorgan, North Iran (Fig. 1), one of the main Mediterranean populations at higher altitude (2,700 m a.s.l.). Using a 30-year average, the mean annual temperature at the site is 9.2°C, and the mean annual precipitation is 429 mm. Extreme temperatures (summer and winter) range from 23°C to -5°C (data from Gorgan climatic station: 46° 06 N, 28° 00 W; 2,600 m a.s.l.). The crowns are approximately 2 x 2 m in length and width. The ring diameter of shrubs is 20.0 cm averagely, and the height is 1.5 m (these are old and horizontal shrubs). Generally, 20 male shrubs have been used for this experiment, and they are all growing in the same area with the same ecological environment. The experiment was conducted at Gorgan University of Agricultural Sciences and Natural Resources in winter, spring, summer, and fall of 2017. Stem cuttings were only collected from the upper crowns of male trees.

Fig. 1 - The worldwide distribution of different populations of *Juniperus sabina* (in grey) and the sampling area of stem cuttings (in a red circle) (Adams and Schwarzbach, 2016).

Cuttings were harvested in the morning. After harvesting, the stem cuttings were prepared to be 15 cm in length and 0.5-0.7 cm in diameter (Bohlenius *et al.,* 2017) for treating and cultivation in a greenhouse. Substrates were prepared, and cuttings were placed in the greenhouse equipped with an automatic system to control humidity (micro irrigation) and bottom heat. The average daily temperature during the experiment was 22°C, and the average relative humidity was 77%. The amount of light entering the greenhouse was varied based on the amount of natural light in each season.

For the treatment of stem cuttings, five levels of IBA were used: 0 or control, 1000, 2000, 4000, and 8000 mg L^{-1} (Control is a sample that is placed in the substrate without adding any treatment and is used to compare the effect of the treatments used on cuttings). The base of each cutting was placed in the aqueous solution of IBA for five seconds and then inserted into the substrate. The four used substrates were: I) perlite; II) mixed rooting substrate - a combination of sand (20%), perlite (20%), cocopeat (20%), vermicompost (20%), and potash (20%); III) perlite cocopeat (1:1), and IV) mineral pumice (each substrate about 10 Kg). For each treatment (combination of treatment and substrate), three replicates were prepared, with nine cuttings per replicate. Thus, a total of 540 cuttings in each season were cultivated.

Total phenol, flavonoids, and antioxidants of stem cuttings

Secondary metabolites were measured in both rooted and unrooted stem cuttings to detect differences in the internal compounds between cuttings that have the potential for rooting and others without this potential. For evaluating the treatments and to make comparisons between the chemical compounds in cuttings at the beginning of the sampling and the amount of increase or decrease between the time of planting and rooting (between the first and the end of each season), samples were taken from freshly harvested cuttings in each season (the first of each season with samples separately from the stem cuttings) and compared with the results at the end of the growing season.

In order to measure total phenol, antioxidants and flavonoids (at the end of each season and after harvesting the cuttings from substrate), in the first step, one gram of each plant sample, which was the bark of the stem of each cutting separately, was removed and powdered with liquid nitrogen, then placed in 10 cc of 80% methanol (Merk) in an Erlenmeyer flask, and after that, placed on a shaker for 24 h. The mixture was then filtered with filter paper and clean extracts were used to measure secondary metabolites in mg/g fresh weight (McDonald *et al.,* 2001). Then we began to assess the total phenolics, antioxidants, and flavonoids.

To measure total phenol, 20 µl of each of the above plant extracts were added to 1.16 µl of distilled water, 100 µl of folin (Merk) and 300 µl of sodium carbonate (20%), and they were mixed in a test tube (it is done for each plant sample separately) and then placed in a water bath at 45 °C for 30 min. After that, each sample was measured by a spectrophotometer (Unic-UV 2800 - 4 cells) at a wavelength of 760 nm. After drawing the standard graph (preparation of different concentrations with specific values of the control-different samples and readings with the spectrophotometer and then drawing on the curve) (Fig. 2), the phenol value of each sample was obtained (McDonald *et al.,* 2001).

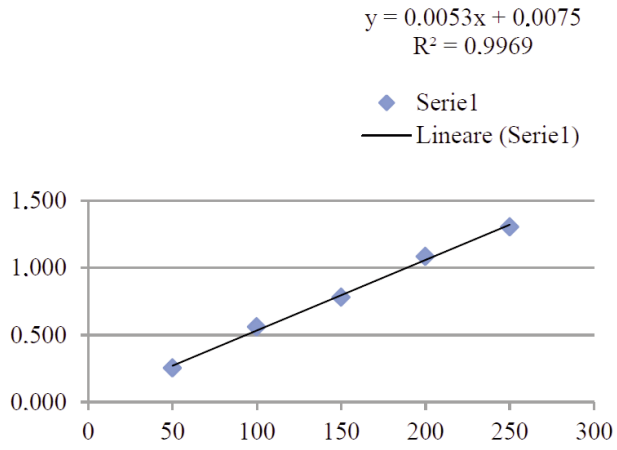

Fig. 2 - Standard graph of total phenol measurement.

To measure the flavonoids, 0.5 ml of each plant extract, 1.5 mg/L pure methanol (Merk), 0.1 ml of aluminum chloride, 0.1 ml of potassium acetate, and 2.8 ml of distilled water were combined and mixed in a test tube, and then all samples were placed in the dark for 30 minutes, and after that, they were measured by a spectrophotometer with a wavelength of 415 nm. After drawing the standard graph (preparation of different concentrations with specific values of the control-different samples with readings with the spectrophotometer and then drawing on the curve), the flavonoid value of each sample was obtained (Chong *et al.,* 2002) (Fig. 3).

To measure antioxidant activity, 1 ml of each plant extract was removed. In the next step, the amount of 0.0004 mg of DPPH was dissolved in 10 ml of methanol (Merk), and then 1 ml of this solution with 1 ml of each extract of the plant previously removed was combined, and finally, the antioxidant percentage was measured in a spectrophotometer with a wavelength of 517 nm (Miliauskas *et al.,* 2004).

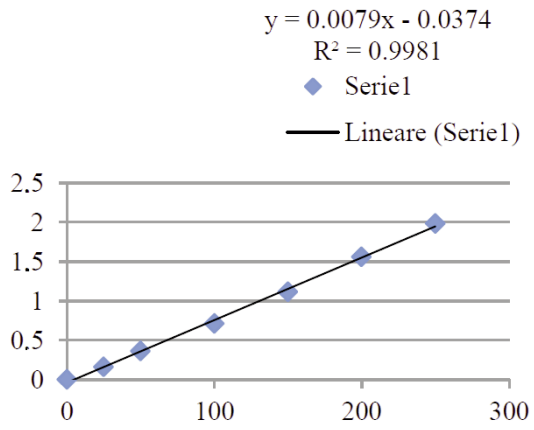

Fig. 3 - Standard graph of flavonoids measurement.

Rooting percentage

To determine the rooting percentage of each treatment, the roots were counted in all rooted cuttings (Fig. 4) in each treatment (three replications and each replication contained 9 cuttings; totally 27 cuttings) and then this number of cuttings was divided by 27 (some cuttings were unrooted and some of them were dried), (Negash, 2002).

Fig. 4 - Rooted cuttings.

Statistical analysis

A factorial arrangement of treatments (Hoshmand, 2006) was applied to analyze the effects of three main factors on five dependent variables. The first factor was “treatment” or concentration of IBA (five levels: 0, 1000, 2000, 4000, and 8000 ppm), the second was “substrate” (four levels: perlite, perlite-cocopeat, pumice, and mixed rooting substrate), and the third factor was “season” (four levels: January, April, July, and October). This represents a 5 x 4 x 4 factorial with 80 combinations of factor levels or treatments. The dependent variables were internal compounds of the cuttings (secondary metabolites in both unrooted and rooted cuttings) and the indicator of rooting performance (% of rooting). Therefore, in the dependent variables concerning chemical internal compounds, another level was added as treatment, secondary metabolites in fresh samples (stem cuttings not planted and prepared at the beginning of each season). This was done to compare the effects of treatments between cuttings not treated (at the beginning of each season) and treated cuttings at the end of each season.

SAS® statistical software (Neter *et al.,* 1996) was used to detect significant factors and to compare mean values between factors and levels of treatments. The comparison of the means was done using the PROC GLM procedure. We utilized Multifactor Analysis of Variance (a three-way ANOVA model) at a probability level of 5% ($p<0.05$). The analysis within season was performed by a two-way ANOVA (excluding season as a main factor in the complete model). In this research, we performed independent ANOVAs (not a mixed-design nor a repeated-measures ANOVA) because the measurements were indepen-

dent (we used different stem cuttings for each treatment and season).

A Fisher's Least Significant Difference (LSD) test ($p<0.05$) was used to determine the significant differences between treatments (Neter *et al.,* 1996). To apply this statistical method, it is desirable for data to be normally distributed. This is not the case with proportions, which have values that range between zero and one. In addition, errors must be independent and normally distributed with constant variance. To ensure these assumptions, a logarithmic transformation was used (Sabin and Stafford, 1990): for the percentage of rooting, the analyzed variable was [ln (r+0.5)], and r was the percentage of rooting (divided by 100). As this transformation requires numerical data above zero, a small number (0.5) was added to this variable before the transformation. The other dependent variables were normal and then distributed.

3. Results

Rooting performance

In Table 1, the p-values for the three principal effects (substrate, treatment with IBA and season, and their two-way interactions) and for the effects within each season (substrate, treatment, and their interactions) are represented. Effects must be considered significant when $p<0.05$. 540 stem cuttings in each season were planted. In January, five treatments rooted (99 cuttings), and 441 cuttings were unrooted. In April, 20 treatments rooted (502 cuttings) and 38 cuttings were unrooted. In July, four treatments rooted (89 cuttings) and 451 cuttings were unrooted. In October, four treatments rooted (102 cuttings) and 438 cuttings were unrooted.

As it is clear from figure 5, the best root-growing month is April. During spring, rooting was more than

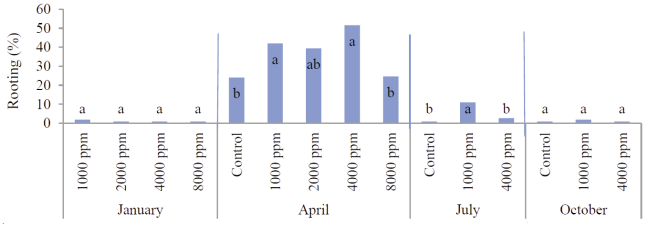

Fig. 5 - Mean values of rooting ability in rooted cuttings (percentage of rooting) within seasons and for the 5 treatments with indole butyric acid (IBA). The mean values for the same letter were not different at the 0.05 level according to the LSD test. Sample data = 540 cuttings for each month. For treatments not represented in the figure, all the cuttings dried. Error bars: LSD intervals.

50% at a level of 4000 ppm of indole butyric acid with no significant difference at the 1000 ppm level. Also, the minimum rooting percentage of the cuttings in this month was about 25% at the level of 8000 ppm of indole butyric acid and control treatment; however, it was higher than the rooting percentage of other months. In the study of the effect of different substrates on the percentage of rooting of the cuttings, the best substrate was seen in equal parts of perlite-cocopeat (v/v), with rooting at a maximum of 62% with a treatment of 1000 ppm (Fig. 6). And this substrate was one of the substrates that had the largest number and length of roots (Fig. 7 C and Fig. 8 C). Therefore, among the substrates used in this research to root the *Juniperus sabina*, the best substrate was perlite-cocopeat, with a maximum rooting percentage of 98. While the least rooting percentage of cuttings was seen in January, with less than 2% in all treatments, October is also not a good time for the reproduction of this plant. On the other hand, the most root number and root length was seen in April (Fig. 7 B and Fig. 8 B). So, the best months for rooting of cuttings of *Juniperus sabina* are April and May, and the best levels of IBA used were 4000 and 1000 ppm, Despite the fact that the largest number of roots was not seen in these treatments.

Table 1 - Results of the multifactor ANOVA to analyze the effects of the main factors on the rooting performance of cuttings across the four seasons

Variable	Effects	Growing season				Values
		January	April	July	October	
Rooting	Treatment	0.10	<0.001	0.0005	0.11	<0.0001
(log-transformed units)	Substrate	0.91	0.03	0.22	0.08	<0.0001
	Season	-	-	-	-	<0.0001
	Treatment x Substrate	-	0.24	-	-	<0.0001
	Treatment x Season	-	-	-	-	<0.0001
	Substrate x Season	-	-	-	-	<0.0001

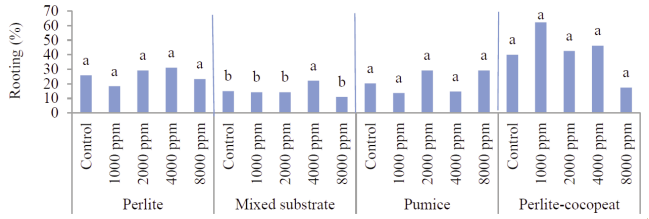

Fig. 6 - The mean values of rooting performance in rooted cuttings (percentage of rooting) within substrates and for the 5 treatments of indole butyric acid. The mean values with the same letter were not different at level 0.05 according to the LSD test. Sample data: 540 cuttings for each substrate.

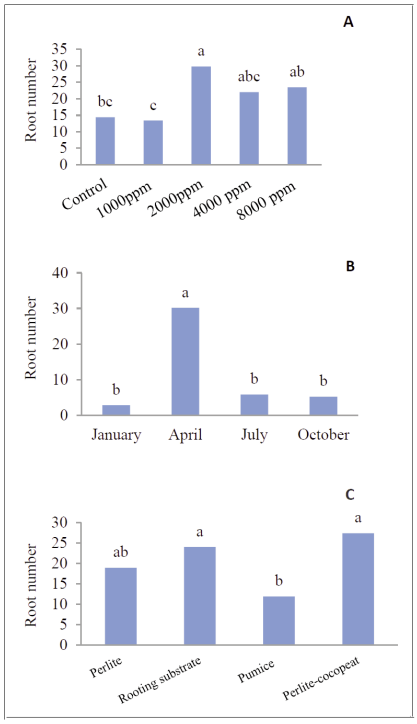

Fig. 7 - A, B, C The mean values of rooting performance in rooted cuttings (root number) within for the 5 treatments of indole butyric acid, 4 season and 4 substrates. The mean values with the same letter were not different at level 0.05 according to the LSD test. Sample data: 540 cuttings for each substrate.

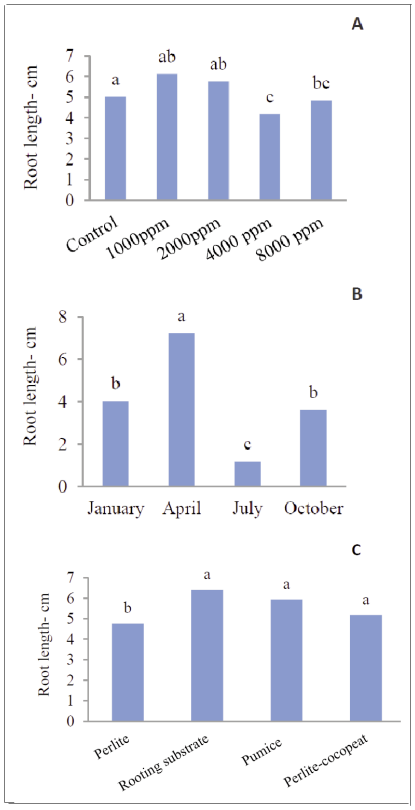

Fig. 8 - A, B, C The mean values of rooting performance in rooted cuttings (root length) within for the 5 treatments of indole butyric acid, 4 season and 4 substrates. The mean values with the same letter were not different at level 0.05 according to the LSD test. Sample data: 540 cuttings for each substrate.

Secondary metabolites concentration

The results of the main and interaction effects of different treatments are presented in Table 2. Based on the results, each of the measured factors has been interpreted and reviewed.

Phenol content

As it is shown in figure 9 A, among treatments in unrooted cuttings, the highest total phenol content

Table 2 - Results of a multifactor ANOVA used to examine the effect of major factors on the secondary metabolite composition of stem cuttings over four seasons

Factors	DF	Phenol		Flavonoid		Anti-Oxidant	
		Unrooted Cuttings	Rooted Cuttings	Unrooted Cuttings	Rooted Cuttings	Unrooted	Rooted Cuttings
IBA	4	16675.96 *	54417.29 **	19.35.41 ns	2034.93 NS	249.75 NS	813.69 **
Season	3	161064.55 **	19288.21 NS	84936.79 **	12674.40 **	10155.84 **	11007.76 **
Substrate	3	24699.73 **	14171.04 NS	2281.75 ns	1549.77 NS	337.35 NS	124.01 NS
IBA x Season	6	55862.72 **	93512.97 **	9142.79 **	9016.74 **	840.18 **	1411.13 **
IBA x Substrate	12	9947.75 NS	4875.10 NS	1489.16 NS	794.45 NS	118.72 NS	215.88 *
Season x Substrate	3	10173.14 NS	263.48 NS	2666.17 NS	0.00 NS	139.36 NS	6.48 NS
IBA x Season x	0	7509.96 NS	2371.40 NS	1690.41 NS	-	163.35 NS	38.92 NS
Error	66	5661.43	8792.53	1604.33	1818.08	170.08	90.61

In the table, the p-values for the three principal effects (substrate, treatment with IBA and season, and their two-way interactions) are represented. Effects were considered significant when p<0.05. 540 stem cuttings in each season were planted.* p<0.5.

(McDonald *et al.,* 2001) was observed with no significant difference in the fresh sample as well as in 4000 ppm and 8000 ppm of indole butyric acid treatments, and the lowest level was observed in control, 1000 ppm, and 2000 ppm treatments without any significant difference. A fresh sample was prepared with other cuttings at the beginning of each season and is used only to measure the internal composition of the plant at the beginning of the season; no treatment is performed on it. It was to compare the amount of internal compounds of the plant at the beginning of the cutting time and compare it with the amount of these compounds after maintaining the cuttings in the substrate to root (control is a sample that is placed in the substrate without adding any treatment and is used to compare the effect of the treatments used on cuttings).

Among the different substrates, the lowest amount of phenol content was found in stem cuttings that were planted in the mixed rooting substrate (Fig. 9 B). Among the unrooted cuttings, the lowest phenol content was observed in a fresh sample and a treatment of 1000 ppm in January (Fig. 9 C). Between rooted cuttings, in April, with the highest rooting percentage of cuttings, treatments of 4000 and 8000 ppm showed lower phenol content, and there was no significant difference between other treatments (Fig. 9 D).

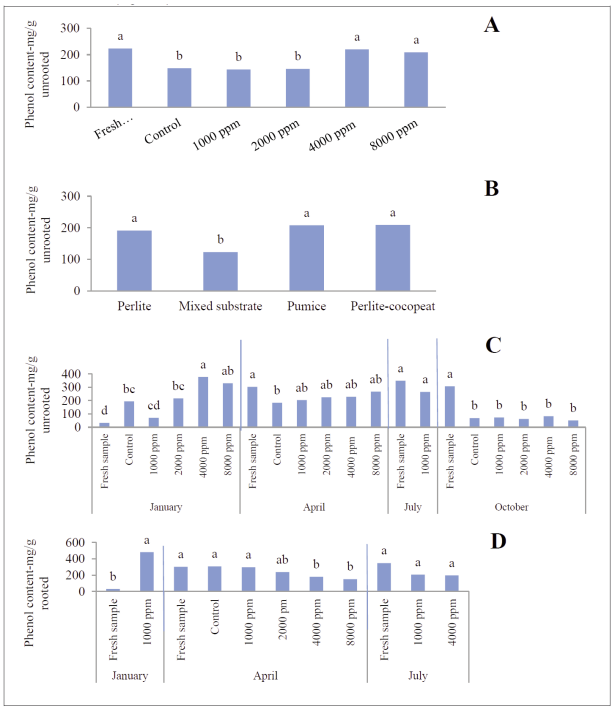

Fig. 9 - A, B, C, D Mean internal phenol content within seasons for the different treatments with IBA for rooted and unrooted cuttings (a, b, c, d). The mean values for the same letter were not significantly different at the 0.05 level according to the LSD test. 540 stem cuttings in each season were planted. For the treatments that were not represented in the figure, those cuttings have dried.

Flavonoid content

Among the unrooted cuttings in different seasons, the highest flavonoid levels were observed in January and July, and the lowest were seen in April and October (Fig. 10 A). The flavonoid content of *Juniperus sabina* differed during different seasons.

In rooted cuttings, flavonoid content was not significantly different in treatments applied in different months, and the overall amount of flavonoid was between 50 and 100 mg/g of fresh weight (Fig. 10 B).

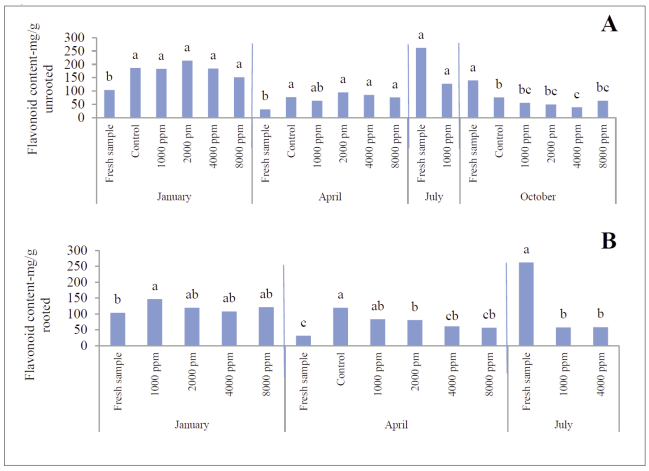

Fig. 10 - A, B. The mean internal flavonoid content within seasons for the different treatments with IBA for rooted and unrooted cuttings. The mean values for the same letter were not significantly different at the 0.05 level according to the LSD test. 540 stem cuttings in each season were planted. For treatments not represented in the figure, all the cuttings dried.

Percentage of antioxidant

In unrooted cuttings, the highest percentage of antioxidants was found in January and October with more than 70%, and the lowest was observed in July with a maximum of 20%. In April, an intermediate level of antioxidants was observed in unrooted cuttings (Fig. 11 A). In all months except July, the percentage of antioxidants in the first samples was about 70%, but in July it was about 20%. It should be noted that in July and January, the percentage of antioxidants increased after treating and planting the cuttings in substrate; this amount was unchanged in October (during fall) and it decreased in April (during spring), and its decline was also significant.

In rooted cuttings, the percentage of antioxidants in January was much higher April (Fig. 11 B). Among

different substrates, the lowest percentage of antioxidants in rooted cuttings was seen in mixed rooting substrate, and its maximum was seen in perlite substrate (Fig. 11 C). Pumice and perlite-cocopeat substrate showed a medium antioxidant percentage. It means in the lighter substrate, the antioxidant percentage was increased, and in the heavier substrate, the percentage of that was decreased.

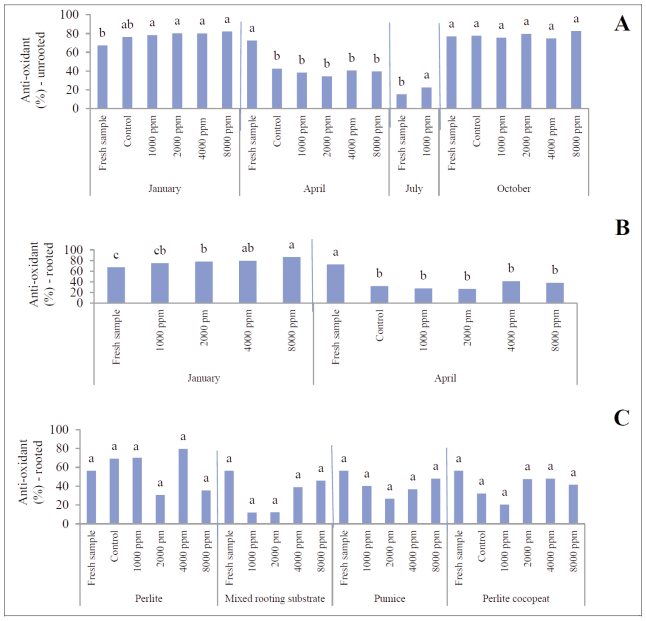

Fig. 11 - A, B, C. The mean internal antioxidant percentage within seasons for the different treatments with IBA for rooted and unrooted cuttings. Mean values for the same letter were not significantly different at the 0.05 level according to the LSD test. 540 stem cuttings in each season were planted. For treatments not represented in the figure, all the cuttings dried.

4. Discussion and Conclusions

Indole butyric acid is widely used at commercial level to root many species (Hartmann *et al.,* 1990; Negash, 2002; Esmael Nia *et al.,* 2006; Khoshnevis *et al.,* 2012). It slowly releases a source of indole acetic acid (Epstein and Ludwig-Muller, 1993). Current evidence indicates that indole butyric acid is naturally occurring in plants. Further stability of IBA in comparison with indole acetic acid during rooting experiments has been reported by Nordstrom *et al.* (1991), which is effective on decomposition and building. Part of the function of indole butyric acid is the direct effect of auxin (Ludwig-Muller, 2000; Poupart and Waddell, 2000). Although other functions are due to the conversion of IBA to IAA by b-oxidation (Epstein and Lavee, 1984; Zolman *et al.,* 2000; Bartel *et al.,* 2001).

Auxin can be increased for up to 24 hours after sampling (Tartoura *et al.,* 2004; Osterc *et al.,* 2009). Increasing root numbers after the use of indole butyric acid occurs in many woody plants (Jarvis, 1986). Adventitious roots on the cuttings were created by treating them with auxin growth regulators, especially indole butyric acid (Buchala and Schmid, 1979; Haissig *et al.,* 1992). This is consistent with the results of this research on its effectiveness on rooting. One possible explanation is that exogenous auxins can increase the amount of internal auxin in the direction of the onset of the formation phase of the rooting and then the root appearance (Metaxas *et al.,* 2004). With an increase in the presence of the cuttings in the substrate, the rooting rate of the cuttings also increases (Cope and Rupp, 2013). The use of indole butyric acid leads to an increase in rooting (Bielenin, 2003).

In our study, the best results were obtained from intermediate levels of IBA (1000-4000 ppm) without a significant difference between these treatments, and we hypothesize that IBA at 8000 ppm can damage the cuttings and reduce rooting. In *J. virginiana*, IBA concentrations up to 2000 ppm did not stimulate rooting beyond that obtained with 5000 ppm (Henry *et al.,* 1992). In general, IBA has been used for the rooting of *Juniperus* species with different treatment levels. For example, results were best at 8000 ppm of IBA in *Juniperus osteosperma* (Cope and Rupp, 2013), 5000 ppm of IBA in *J. virginiana* (Henry *et al.,* 1992), 1000 ppm to 9000 ppm in *Juniperus scopulorum* (Bielenin, 2003), Chowdhuri (2017), with 1000 to 3000 ppm in *Juniperus chinensis* and Tektas *et al.,* (2017) with 6000 ppm in Juniperus L. In the research on *Juniperus virginiana*, Henry *et al.* (1992) cited that in preliminary studies, IBA concentrations up to 20000 ppm did not stimulate rooting beyond that obtained with 5000 ppm. Thus, our results are more in agreement with Rifaki *et al.* (2002), which proposed a concentration of 4000 ppm of IBA in cuttings of *Juniperus excelsa*, and Esmaeil Nia *et al.* (2006), with 3000 to 6000 ppm in *J. excelsa*. Nevertheless, the novelty of our results is that the concentration of IBA we selected (1000 ppm) was lower.

Substrate characteristics are very important in rooting success. Several studies have shown that the substrate plays a significant role in the quality of root formation and the percentage of rooted cuttings. Proper air preservation is a necessary feature of a

good rooting atmosphere. Therefore, it seems that proper rooting substrate can maintain proper moisture to prevent the cutting ends from drying out and to provide enough air to facilitate rooting and prevent disease spread at the base of the cuttings. Surely there is an optimum temperature for substrate for root formation and growth, and rooting at low temperatures will not occur or will occur very slowly. It is also possible for the roots to appear and grow at very high temperatures in the substrate. Bottom-heat is useful for rooting only when the temperature is low (Couvillon, 1988) which is consistent with the results of this research. In our study, the percentage of rooting was more than 60% in substrate of perlite cocopeat. In an study of *Juniperus procumbens* the best substrate was 1.3 (v/v) vermiculite and 2.3 (v/v) perlite, with only 36% rooting (Hong-wei *et al.,* 2011). The results of our study was also better than the results obtained from Cuevas-Cruz *et al.* (2015) in Pinus, with 43.5% of rooting (substrate was a mixture of peat-perlite-vermiculite), Khoushnevis *et al.* (2012) with 28% of rooting in *Juniperus oblonga* (fine and harsh bed), Stuepp *et al.* (2014), with 16% of rooting in *J. chinensis* (fine grained vermiculite and carbonized rice hull 1:1) and Ayan *et al.* (2004), with 24% of rooting for *J. foetidissima*, 31.5% of rooting for *J. excelsa*, 38.42% of rooting for *Juniperus sabina* and 31.83% of rooting for *J. oxycedrus* (perlite).

Cutting time plays an important role in the success of rooting. Although many species are most rooted when cuttings are prepared in late spring or early winter before the wood has hardened, many other species have the best rooting when cuttings are taken at other times of the year. A good example of this is the *Juniperus horizontalis*, whose cuttings were most rooted when they were prepared between November and February compared to other times of the year (Ali Ahmad Koruri *et al.,* 2011). The result of this research showed that the best time for rooting *Juniperus sabina* to prepare the cuttings is April. Therefore, for this species, the best time to prepare cuttings and plant them is spring. This differs from Guerrero-Campo *et al.* (2006), who found the best rooting of several species of cuttings at different seasons and Chowdhuri (2017), who showed the best rooting time for *Juniperus chinensis* was summer. On the other hand, our result was in agreement with Fragoso *et al.* (2015) and Tektas *et al.* (2017), who respectively cited the best season for rooting of *Juniperus chinensis* and Juniperus L. as spring.

Apparently, the presence of secondary metabolites in plants acts as a defense (toxic) agent that inhibits proliferation and other growth-related actions (Singh Rattan, 2010), as shown in the results of this study. Although most of the phenolic compounds have a structural role in the cell wall, the major activity of these compounds is in defense of the plant; they have several roles in plants, but are mainly used for their great effects on growth, development, propagation, as well as plant defense against animals and pathogens (Croteau *et al.,* 2000). The presence and yield of secondary metabolites in plants, such as aromatic compounds and compounds in essential oils, may be affected in different ways, from formation to separation from plants. Rapid secondary metabolite induction occurs as a chemical mediator of plant rooting and defense (Metlen *et al.,* 2009), and the amount of secondary metabolites changed during the preservation of cuttings in the substrate. The rooting barrier of yew cuttings was identified by biological and organic methods. The results showed that the most important barrier to propagation in this plant was phenol content (Guangyou, 2000).

The maximum amount of total phenol in the leaves of common juniper was 315.33 mg/g (Ved *et al.,* 2017), which is consistent with the results of this study. In cherry leaf cuttings, GiSelA 5, auxin had no effect on phenol levels, so the same results were observed in the present study. Cuttings should have definite levels of different phenolic compounds to start the rooting induction phase, but the greater effect on rooting success is attributed to the effect of auxin level (Trobec *et al.,* 2004).

Phenolic compounds are a class of antioxidants (Choudhury *et al.,* 2013), and the level of internal antioxidants in plants is different (Rehman *et al.,* 2014). Many authors have reported an association between total phenol content and antioxidant activity (Hariprasath *et al.,* 2015). The main antioxidant activity is due to specific secondary metabolites, especially phenolic compounds and some terpenes (Marzouk *et al.,* 2007; Awaad and Al-Jaber, 2010).

Interactions among genotypes, propagation methods, and growing seasons significantly affect flavonoid content and antioxidant capacity (Goyali *et al.,* 2013), which is consistent with the results of this study. The amount of secondary compounds varied according to season and substrate, just as it did in the current study. The climate of the outdoor region during the three months of October, January, and

April increased the amount of antioxidants inside the plant, while in July, with a hot climate, it dropped dramatically. Growth regulators increase antioxidant activity (Dakah *et al.,* 2013), which contradicts the results of this research. Because in some cuttings treated with indole butyric acid, an increase in antioxidants was observed, and in other treatments, a decrease was observed.

The results of a study on one of the Iranian conifers showed that the antioxidant activity of the extracts ranged between 60 and 99% (Hariprasath *et al.,* 2015), which contradicts the result of the present study, which shows that the range of antioxidants in some treatments was less than 20%.

In the use of indole butyric acid for the propagation of *Juniperus sabina* through cuttings, the best rooting month (season) for cuttings was April, and the rooting percentage in this month was higher than in other months (more than 50%), while instead, the lowest rooting rate was seen in January. The best levels of indole butyric acid used were levels of 4000 and 1000 ppm, respectively. So, for the propagation of Sabina species, it is recommended to use these levels of IBA as a treatment for stem cuttings in April. Also, the best substrate used was perlite-cocopeat. Between rooted cuttings, in April, with the highest rooting percentage of cuttings, treatments of 4000 and 8000 ppm showed lower phenol content; flavonoid content was not significantly different in treatments applied in different months and the percentage of antioxidants in January was much higher than April.

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Physicochemical characteristic and internal browning of pineapple as affected by calcium and gibberellic acid dipping application

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Key words: Antioxidant, ascorbic acid, cell wall, citric acid, enzyme.

Abstract: Postharvest applications of calcium and gibberellic acid (GA) have proved to maintain optimal fruit quality and control decay during cold storage. This study evaluated the effect of calcium and gibberellic acid dipping application on pineapple physicochemical characteristics and internal browning. The experiment implemented two factors. The first factor relates to two dipping times (five and ten minutes) and the second factor related to four treatments, GA, Ca, mix GA-Ca, and control (no GA or Ca applied). Total soluble solids (TSS), total acidity (TA), TSS/TA ratio, sugar, citric and ascorbic acid content, together with internal browning severity and incidence were determined. The treatment of Ca, essentially using a dipping of five minutes delivered the best performance, having the lowest severity and incidence of internal browning (4.44 and 22.22%, respectively), the highest citric acid (0.61%), ascorbic acid content (405.18 mg kg^{-1}) and the lowest TSS/TA ratio (25.53). Meanwhile, the other treatments were considered less satisfactory, due to their highest internal browning severity and incidence, without a notable impact on the citric acid and ascorbic acid content, especially with a dipping time of ten minutes. In conclusion, dipping applications of calcium in postharvest can enhance pineapple quality and reduce internal browning.

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1. Introduction

Pineapple is an important crop worldwide, mainly exported as canned and fresh fruit (Hassan *et al*., 2011). Low acid hybrids are the pineapple cultivars more needed by the industry nowadays. These hybrids are attractive to consumers due to their more yellow shell colour, higher sugar content and uniformity (Chen and Paull, 2017; Paull and Chen, 2018; Cano-Reinoso *et al.*, 2021 a). Therefore, the control of the optimal

physico-chemical characteristics of these hybrids has become a primordial activity for the farmers.

To regulate the fruit decay, pineapple is commonly subjected to postharvest storage, where the deterioration of its physico-chemical properties is delayed, especially during long exportation periods (Hassan *et al.*, 2011; Paull and Chen, 2018; Cano-Reinoso *et al*., 2022 b). During cold storage, the fruit is affected by physiological disorders such as chilling injury and internal browning (De Freitas and Resender Nassur, 2017; Paull and Chen, 2018). These conditions highly endanger the shelf-life of the fruits. Because of that, several postharvest technics have been developed to extend the cold storage life of the horticultural products, mitigating the impact of any physiological disorders (De Freitas and Resender Nassur, 2017; Noichinda *et al*., 2017).

One of these technics is the dipping of the fruit employing calcium mineral sources. Calcium treatments during postharvest have been proved to maintain optimal fruit quality and enlarger the fruit cold storage life (De Freitas and Resender Nassur, 2017). In addition, calcium inhibits fruit softening by increasing the cell wall strength, which mitigates the cell breakdown, a normal phenomenon occurring during postharvest decay (Hocking *et al*., 2016; De Freitas and Resender Nassur, 2017). Besides, applications with this mineral have shown a positive impact on nutritional flavour, antioxidant capacity and reduction of internal browning (De Freitas and Resender Nassur, 2017). For example, in pineapple, postharvest calcium treatments have provided significant results in the internal browning reduction, the activity of oxidative enzymes like phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO), and an increase of the total phenols content (Youryon and Wongs-Aree, 2015; Youryon *et al*., 2018).

Another technique for controlling fruit decay during cold storage is by dipping applications using plant regulators like gibberellic acid (GA). This plant regulator has demonstrated outstanding results in reducing postharvest decay in fruits and vegetables due to its antagonist properties in ethylene sensing and production (Pusittigul *et al*., 2012; Mohamed *et al.*, 2016). Additionally, GA used during postharvest has delivered positive results improving the physicochemical attributes of fruits, especially those related to antioxidant accumulation and weight loss reduction during cold storage (Pusittigul *et al.*, 2012; Dong *et al.*, 2019). For example, in pineapple, GA employed during postharvest enhanced the shelf-life of the fruit up to 24 days, with an increase in its total soluble solids, total acidity, and percentage of weight loss (Pusittigul *et al*., 2012; Mandal *et al.*, 2015).

However, most of the calcium used on pineapple after harvest has been documented as calcium infiltrations or mixed with other techniques such as hot water treatments. At the same time, in the case of GA, more studies are needed to develop a correct characterization of its impact on pineapple quality. Because of that, few papers have been reported on postharvest calcium and GA employing dipping technics; moreover, they have studied their effect on low-acidic hybrids and possible synergy between them. Therefore, this study aims to evaluate the effect of calcium and GA dipping application on pineapple physicochemical characteristics and internal browning.

2. Material and Methods

Experiment design and treatments

The research was implemented in a pineapple packing house located in Lampung, Sumatra island of Indonesia, between January and February of 2020. In this experiment, the MD2 pineapple cultivar was used. This low acid hybrid is well known in the industry because of its outstanding qualities related to higher sugar and antioxidant content, more uniformity and yellowish shell colour than other acid hybrids (Bin Thalip *et al.*, 2015; Cano-Reinoso *et al*., 2021 b). The study was set using an experiment design with two factors. The first factor related to two dipping times (five and ten minutes), and the second factor concerning four treatments consisted of three solutions of, GA, Ca, mix GA-Ca, and C (Control - no use of GA and Ca). Three replications per treatment were used in this experiment. Furthermore, randomly fruit samples were picked from every treatment to be examined employing intervals of eight days.

All treatments, including the control used fungicide and wax before cold storage. The fungicide product implemented was Prochloraz in doses of 2 cc l^{-1}, while the waxing product applied was Sta-Fresh 2952 in doses of 74 g l^{-1}. Both fungicide and waxing, in that order, were used in dipping applications for ten seconds, just after the dipping of the fruits in GA or Ca. The calcium source used was Calcibor (Alba Milagro Internation, Lombardia, Italy) - (12.9% w/v CaO and 2.6% w/v B) in doses of 4 l 2000 l^{-1}; meanwhile, the GA product employed was ProGibb (40%

of GA_3 active component, Valent USA Corp., Walnut Creek, California - USA) in doses of 100 mg l^{-1}. The Calcibor doses implemented were prepared according to the producer recommendation; meanwhile, the GA doses were arranged based on the previous experiments of Mandal *et al*. (2015) and Dong *et al.* (2019). Concerning the mix GA-Ca treatment, it was employed a sequenced application (one solution of GA or Ca at time). Besides, the dipping times implemented in this experiment (five and ten minutes) were based on the previous studies of Pusittigul *et al*. (2012) and Islam *et al*. (2013).

The fruits were selected according to their weight and shell colour characteristics and arranged by their respective treatments inside cold storage for 40 days (Temperature: 7°C, relative humidity: 95%). The preference for fruit weight for the research was between 1.4 and 1.5 kg, with a shell colour where 10-20% of the area from the base already turned yellow. The MD2 pineapple is typically harvested and exported with the previously described weight and shell colour characteristics (Bin Thalip *et al*., 2015; Paull and Chen, 2018).

Determination of the total soluble solids (TSS), total acidity (TA) content

According to the procedure described in Shamsudin *et al*. (2020), the TSS and TA were measured in each fruit per replication of every treatment arranged. TSS was calculated employing a hand-held refractometer (MASTER-53 α, Atago, Japan), while the TA was measured by titration to pH 8.1 with 0.1 M NaOH using phenolphthalein as an indicator and expressed as a percentage of citric acid.

Sugar, citric acid and ascorbic acid (AsA) content

Pineapple sugar and citric acid were measured using the method described in Siti Roha *et al*. (2013), employing a High-Performance Liquid Chromatography (HPLC) - (Hitachi, USA) model L-2000 instrument with a Refractive Index detector model L-2490. A juice extracted from the fruit flesh adjacent to the core was employed for this procedure. Samples were obtained from each fruit per replication of every treatment implemented. For the sugar content, standard solutions of 500, 1000, 1500, 2000 mg l^{-1} of glucose, fructose and sucrose were prepared with the aim of developing a curve of sugar level; furthermore, in the case of the citric acid and AsA determination, the standard solutions were 1000 mg l^{-1} and, 200, 400, and 800 mg l^{-1}, respectively, having the same objective in mind. The standard solutions were dissolved in distilled water and filtered through a Millipore 0.45 µm membrane filter. Finally, the sugar, citric acid and AsA content was quantified, comparing the peak area by a chromatographic procedure.

The chromatographic condition for the sugar determination was:
Column: Purospher® STAR NH2 (250 x 4 (mm), 5 µm). Guard column: LiChocart® 4-4 / LiChrospher® 100 NH_2, 5 µm. Column temperature: Room temperature (22°C). Mobile phase: Acetonitrile: distilled water (80:20). Flow rate: 1 ml min^{-1}. Injection volume: 20 µL. Duration of analysis: 15 min.

Meanwhile, for the citric acid and AsA was:
Column: Purospher® STAR NH2 (250 x 4 (mm), 5 µm). Guard column: LiChocart® 4-4 / LiChrospher® 100 NH_2, 5 µm. Column temperature: Room temperature (22°C). Mobile phase: Pipette of 0.14 ml H_2SO_4 (0.0025 M) concentrated at 97% is introduced in a volumetric flask, then add 1000 ml of distilled water. Injection volume: 20 µl. Duration of analysis: 15 min.

Browning severity and incidence

The internal browning severity was calculated employing the following score classification and transformed into a percentage: 1 (No flesh browning, 0%), 2 (20% of browning in the flesh), 3 (40% of browning in the flesh), 4 (60% of browning in the flesh), 5 (80% of browning in the flesh) and 6 (100% of browning in the flesh). Figure 1 shows a schematic example of the previous score classification described. Furthermore, the incidence was measured by accounting the percentage of fruits affected by internal browning in each treatment during every observation.

Statistical analysis

Statistical analyses were performed using SPSS Version 22.0 software (SPSS Inc.; Chicago, IL: USA).

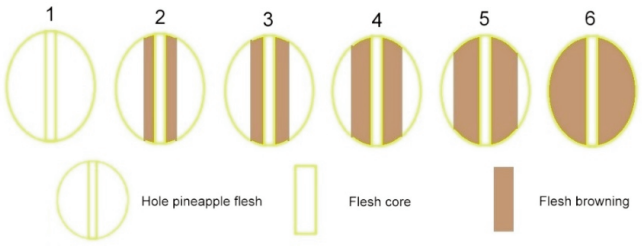

Fig. 1 - Scheme of the score classification used to determine the internal browning severity in the experiment during each observation. Score: 1 (0%), 2 (20%), 3 (40%), 4 (60%), 5 (80%), and 6 (100%).

All data were analyzed by two-ways ANOVA. Mean significant differences at $p<0.05$ were determined by Duncan's multiple range tests and Kruskal-Wallis test (in case of the internal browning data).

3. Results

TSS, TA, and TSS/TA ratio

There was no significant impact evidenced coming from the dipping time, the treatments implemented and the interaction between these two factors for the TSS, TA, and TSS/TA ratio. Concerning the TSS, the mean results exposed that the control had the highest value (16.93%), meanwhile the treatment of GA-Ca with a dipping of ten minutes provided the lowest outcome (14.47%). Regarding the TA, the mean results show that the treatment of Ca with a dipping of five minutes delivered the highest outcome, while the GA-Ca treatment with five minutes dipping had the lowest one (0.61 and 0.53%, respectively). On the other hand, the mean outcomes for the TSS/TA ratio delivered the highest value in the treatment of GA with five minutes dipping (38.17%), and a most inferior result in the treatment of Ca also with a dipping of five minutes (26.16%). The TSS, TA and TSS/TA ratio mean values corroborated the previous results described concerning the single factors influence and their interaction; there was no a clear positive or negative trend in the mean values when the dipping times were increased or reduced and combined with the treatments administrated (Table 1).

Sugar content

In terms of pineapple sugar after cold storage of 40 days, the fructose, glucose and sucrose content did not demonstrate significant differences coming from the dipping time, the treatments administrated, and their interaction. Concerning the mean values of these two factors, just the sucrose exposed significant differences. In this case, the treatment of GA with ten minutes dipping had the highest result, and the same treatment but with dipping of five minutes, the most inferior outcome (8.25 and 5.97%, respectively). Despite this particularity, for the mean results of the fructose, glucose and sucrose content, it was difficult to determine a positive or negative tendency coming from the rise or reduction of the dipping times and their combination with the treatments used, reaffirming the individual factors impact and their interaction outcomes (Table 1).

Citric acid and AsA content

The citric acid and AsA did not present a representative impact from the dipping time and the treatments studied; however, the interaction between these two factors was significant, impacting the mean outcomes among these variables after 40 days of cold storage. For the citric acid, the lowest outcomes were obtained in the treatments of GA and Ca

Table 1 - Results of the single and interaction effect from treatments and dipping times with their respective combined mean values on the TSS, TA, and sugar content, after 40 days of cold storage

	TSS (%)	TA (%)	TSS/TA	Glucose (%)	Fructose (%)	Sucrose (%)
			Studied factors (single and interaction effect)			
Treatment	-	-	-	-	-	-
Dipping time	-	-	-	-	-	-
Treatment x Dipping time	-	-	-	-	-	-
			Mean values (Treatment vs. Dipping time)			
GA 5 min	16.13 ± 0.48 ab	0.39 ± 0.06 b	43.32 ± 6.71 a	3.35 ± 0.22 a	3.41 ± 0.21 a	5.97 ± 0.56 b
Ca 5 min	14.80 ± 0.00 bc	0.62 ± 0.13 a	25.53 ± 4.48 b	2.48 ± 0.63 a	2.77 ± 0.56 a	7.72 ± 1.03 ab
GA-Ca 5 min	15.20 ± 0.70 bc	0.56 ± 0.04 ab	27.82 ± 4.02 ab	3.25 ± 1.02 a	3.49 ± 0.99 a	6.77 ± 1.50 b
GA 10 min	15.27 ± 0.27 bc	0.50 ± 0.10 ab	33.01 ± 5.88 ab	2.71 ± 0.18 a	2.99 ± 0.19 a	8.25 ± 0.45 a
Ca 10 min	14.67 ± 0.18 bc	0.59 ± 0.11 ab	26.80 ± 4.96 ab	3.01 ± 0.12 a	3.28 ± 0.11 a	7.11 ± 0.62 ab
GA-Ca 10 min	14.47 ± 0.29 c	0.50 ± 0.06 ab	29.91 ± 4.78 ab	2.06 ± 0.86 a	3.23 ± 0.43 a	6.54 ± 0.75 b
Control	16.93 ± 0.84 a	0.58 ± 0.04 ab	29.46 ± 2.57 ab	2.97 ± 0.22 a	3.27 ± 0.28 a	7.77 ± 0.09 ab

* Results marked with (+) indicate that the P-value ($p < 0.05$) for the single or interaction can be considered to draw conclusions; meanwhile, results marked with (-) indicate that the P-value ($p > 0.05$) for the single or interaction are not significant to draw conclusion in the text. Mean values ± SE in each column followed by the same lower-case letters are not statistically different by Duncan's multiple range test ($p < 0.05$). Treatments: GA, Ca, mix GA-Ca, Control - no use of GA and Ca. Dipping time: 5 and 10 minutes.

(GA: 34% - five minutes; Ca: 37% - ten minutes). Moreover, the treatment of Ca but using a dipping of five minutes had the highest outcome (0.61%). No notable differences were observed between the mean results of the treatment GA-Ca in both dipping times; however, those outcomes were higher than the control (Table 2).

Concerning the AsA, similar with the previous variable, when the Ca treatment was implemented with a dipping of five minutes the highest result was obtained (405.18 mg kg^{-1}). Besides, the most reduced outcome was determined in the same treatment but with a dipping of ten minutes (111.38 mg kg^{-1}). On top of that, a high remarkable mean result was observed in the treatment of GA with ten minutes dipping (208 mg kg^{-1}). In this case, not all the mean outcomes of the treatment GA-Ca in both dipping times provided a positive increase, as the control obtained a superior value, essentially when five minutes dipping was used (Table 2). Compressing the previous information, it was noticed that for the citric acid and AsA, Ca treatments caused a rise in their values employing a short dipping of five minutes, contrary effect detected on the GA treatments, which demonstrated positive increases with a long dipping of ten minutes. This situation was not clearly observed in the GA-Ca treatments.

Internal browning

The monitoring of internal browning after 40 days of cold storage provided significant differences coming from the treatments implemented but not from the dipping times, and the interaction among these factors. In the mean outcomes it was possible to observe that samples employing the treatments of Ca obtained the lowest severity and incidence of internal browning, more remarkable when a dipping of five minutes was implemented (severity: 4.44%, incidence: 22.22%), compared with the other treatments and the control. On the contrary, samples using the treatments of GA and GA-Ca had a more superior internal browning severity and incidence, more evidenced with dipping times of ten minutes, suggesting a negative effect of the GA, concerning this variable (severity: 42%, incidence: 100%, for the GA treatment with ten minutes dipping) (Table 2).

4. Discussion and Conclusions

TSS, TA, and TSS/TA ratio in the fruit

TSS in pineapple low acid hybrids should be minimal as 12%, although some authors have recommended higher values close to 14% (Paull and Chen, 2015, 2018; Cano-Reinoso *et al.*, 2021 b). These val-

Table 2 - Results of the single and interaction effect from treatments and dipping times with their respective combined mean values on the citric acid, ascorbic acid (AsA), and internal browning, after 40 days of cold storage

	Citric acid (%)	AsA (mg kg^{-1})	Browning severity (%)	Browning incidence (%)
	Studied factors (single and interaction effect)			
Treatment	-	-	+	+
Dipping time	-	-	-	-
Treatment x Dipping time	+	+	-	-
	Mean values (Treatments vs. Dipping time)			
GA 5 min	0.34 ± 0.03 b	170.79 ± 48.88 b	37.78 ab	88.89 ab
Ca 5 min	0.61 ± 0.10 a	405.18 ± 64.31 a	4.44 c	22.22 c
GA-Ca 5 min	0.53 ± 0.14 ab	148.11 ± 63.87 b	37.78 bc	88.89 ab
GA 10 min	0.52 ± 0.04 ab	208.00 ± 22.56 b	42.22 ab	100 a
Ca 10 min	0.37 ± 0.03 ab	111.38 ± 36.12 b	11.11 bc	66.67 bc
GA-Ca 10 min	0.55 ± 0.05 ab	186.03 ± 66.56 b	28.89 bc	100 a
Control	0.44 ± 0.04 ab	157.46 ± 32.30 b	42.22 ab	77.78 ab

* Results marked with (+) indicate that the P-value ($p < 0.05$) for the single or interaction can be considered to draw conclusions; meanwhile, results marked with (-) indicate that the P-value ($p>0.05$) for the single or interaction are not significant to draw conclusion in the text. Mean values ± SE in each column followed by the same lower-case letters are not statistically different by Duncan's multiple range test, and Kruskal-Wallis test (for the internal browning data) ($p<0.05$). Treatments: GA, Ca, mix GA-Ca, Control - no use of GA and Ca. Dipping time: 5 and 10 minutes.

ues were assessed in the results obtained after 40 days of cold storage, knowing that it is common the reduction of TSS content through the postharvest life of pineapples (Lu *et al.*, 2011; Hu *et al*., 2012). Therefore, as all treatments provided an optimal performance, it is possible to infer that no harmful impact on this variable was received, even with the application of different dipping times. In addition, the lowest result observed in the treatment of Ca-GA with ten minutes dipping can be attribute to the time implemented. Typically, GA influences cell enlargement, this process generates more soluble solids as sugars, which can be assimilated in the cell (Wang and Irving, 2011; Gupta and Chakrabarty, 2013). Furthermore, this situation can cause more Ca^{2+} ions to crosstalk, creating cell wall channels, with the objective of maintaining optimal membrane stabilization (Gupta and Chakrabarty, 2013, Hocking *et al*., 2016; Cano-Reinoso *et al*., 2022 a). Nevertheless, during this process several reactive oxygen species (ROS) are released, and those can interfere and destroy organic molecules presented as soluble solids (Wang and Irving, 2011; Gupta and Chakrabarty, 2013). Because of that, a longer dipping time could be a trigger for more ROS production, decreasing the TSS available in the cell structures, and as a consequence the more reduced level observed in this treatment.

On the other hand, values of TA between 0.4 and 06% are recommended for optimal quality in MD2 pineapple; nevertheless, higher percentages have been reported in former studies (Chen and Paull, 2017; Paull and Chen, 2018; Cano-Reinoso *et al*., 2021 b). The outcomes of this research delivered values among this ideal range for almost all the treatments applied in both dipping times. Previous experiments using calcium as a postharvest treatment in pineapple had not presented any remarkable impact on TA (Pusittigul *et al*., 2014; Youryon *et al*., 2018). Nonetheless, in some fruits like apricots (Hajilou and Fakhimrezaei, 2013) and apples (Shirzadeh *et al.*, 2011), postharvest calcium applications increased the TA content. Calcium may positively affect the accumulation of organic acids through its impact on fruit metabolism, which are highly associated with the TA level. Calcium has been proved to delay fruit senescence by regulating the opening of stomata, creating a reduction of the degrading of organic acids, main source of fruit respiration (Van Meeteren and Aliniaeifard, 2016; De Freitas and Resender Nassur, 2017). This fact can explain why the treatment of Ca with five minutes dipping had the highest results. Five minutes could be the ideal time to produce the most positive impact on the fruit metabolism. However, this dipping time could cause a negative influence when the treatment of GA was administrated. GA has been detected to reduce the TA degrading in postharvest of pineapple (Mandal *et al*., 2015; Dong *et al.*, 2019). Nevertheless, as mentioned before, GA tends to ROS production, this dipping time could cause that the ROS level overcame and interfere the organic acid production to maintain a stable fruit metabolism, causing the lowest content with this treatment.

Furthermore, the TSS/TA ratio for MD2 pineapple should range between 18 and 25, although current authors have suggested values close to 30 (Chen and Paull, 2017; Paull and Chen, 2018; Cano-Reinoso *et al*., 2021 b). Numbers more superior than 30 are considered not suitable for an ideal consumption (Ding and Syazwani, 2016; Paull and Chen, 2018). The most elevated and inferior TSS/TA ratio values observed in the treatments of GA and Ca were more associated with the impact of their TA content, as this displayed more remarkable differences among both treatments with five minutes dipping than the TSS.

Pineapple sugar content

Fructose, glucose, and sucrose are the major sugars present in pineapple. Sucrose is the most accumulated during fruit ripening, ranging around 7-9%, and essentially responsible for the sweet taste in low acid hybrids like MD2 (Nadzirah *et al*., 2013; Lu *et al.*, 2014; Paull and Chen, 2018). Pre-and postharvest applications with GA have shown an increase in the sugar content of pineapple (Mandal *et al*., 2015); also in fruits like mango (Islam *et al.*, 2013), and peaches (Çetinbaş and Koyuncu, 2013). Few studies have reported a dipping time ideal for GA (Pusittigul *et al*., 2012; Islam *et al*., 2013). Five minutes have been suggested as optimal to generate a positive effect on sugar content in pineapple; nevertheless, results of this experiment suggested another outcome, as this dipping time delivered the lowest result. In pineapple, sucrose phosphate synthase (SPS) and sucrose synthase (SS) are the main enzymes responsible for the sucrose accumulation, while the invertase enzyme (IE) mainly responsible for its hydrolyzation (Chen and Paull, 2017; Paull and Chen, 2018). The five minutes dipping with the treatment of GA may affect theses enzymes through the cold storage, essentially promoting a superior activity of IE, causing

more sucrose hydrolyzation and conversion into fructose and glucose, including other organic sugars. Contrary process evidenced with this treatment but employing ten minutes dipping, obtaining the highest value. This dipping time could generate a more pronounced activity of SPS and SS, having a more elevated sucrose content and reduced fructose and glucose mean outcomes. More emphasis in these enzyme activities under GA applications for future experiments is recommended.

Pineapple citric acid and AsA

In hybrids like MD2 the citric acid value should be between 0.4 and 07% for ideal consumption (Lu *et al*., 2014; Paull and Chen, 2018; Cano-Reinoso *et al*., 2022 b). Values within that range were obtained in most of the treatments implemented. Citric acid is the predominant organic acid in pineapple, impacting its taste (Chen and Paull, 2017; Paull and Chen, 2018). This acid is considered a plant antioxidant, which has been detected to improve the tolerance to disease attacks and delaying fruit senescence in pre- and postharvest studies (Patrignani *et al*., 2015; Yang *et al.*, 2019). The citric acid of this experiment are highly related to the TA results described previously. This acid accounts for most of the TA content in pineapple (Chen and Paull, 2017; Paull and Chen, 2018). As mentioned before, calcium can impact organic acids accumulation positively, like the citric acid, by regulating the opening of stomata, causing a reduction of its metabolizing process during fruit respiration, essentially with short dipping times. This information can clarify why the treatment of Ca with five minutes dipping obtained the highest result. Nevertheless, as mentioned in the TA outcomes, this dipping time could not be sufficient to provide an adequate production of ROS that does not alter the citric acid metabolization, primordially when GA is used, reason why this treatment had the lowest outcome. Furthermore, this lowest value could be attribute also to its impact on the organic acid metabolizing enzymes. A high activity of the aconitase enzyme (ACO) has been associated with the citric acid reduction in pineapple low acid hybrids (Saradhuldhat and Paull, 2007). Therefore, it could be possible to infer that a short dipping time together with the treatment of GA can produce a superior activity of ACO during cold storage. On the contrary, an opposite situation can occur when same dipping time was used but with the treatment of Ca. The more reduced picks of citric acid content observed in the figure 2 could be linked to the high ACO activity during postharvest. However, this phenomenon should be examined in detail for future studies.

On the other hand, AsA is considered a soluble vitamin and the most representative antioxidant compound in pineapple (Kongsuwan *et al*., 2009; Akram *et al*., 2017; Noichinda *et al.*, 2017). In MD2, a value higher than 300 mg kg^{-1} has been established as ade-

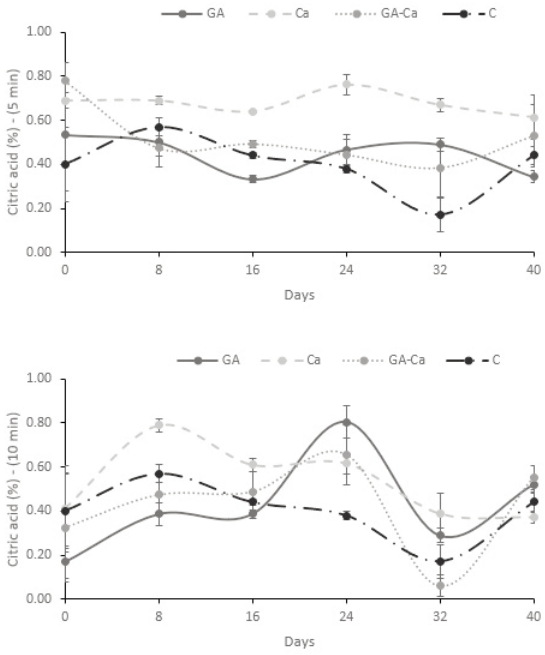

Fig. 2 - Effects of the treatments applied using both dipping times (5 and 10 min) on the citric acid content during cold storage. Treatments: GA, Ca, mix GA-Ca, C (Control-no use of GA and Ca). Values are the mean three replicates, and vertical bars represent ± SE.

quate to have a longer shelf-life, especially during cold storage (Lu *et al.*, 2014; Paull and Chen, 2018; Cano-Reinoso *et al.*, 2022 b). AsA increase in fruits has been associated with a higher activity of the enzyme ascorbic peroxidase (APX) (Akram *et al.*, 2017). Furthermore, more superior Ca^{2+} ion assimilation in fruits' primary cell wall matrix has been linked also to a higher production of AsA (Sadak *et al.*, 2010; Farouk, 2011). These facts can explain why treatment of Ca obtained the highest AsA content, due to the synergy between Ca^{2+} ion influences on the cell wall and the AsA generation. Besides, the five minutes dipping could be the ideal time to have an adequate ROS level that does not interfere with the AsA metabolization, similar with the situation described for the TA. However, an opposite phenomenon could have occurred in the same treatment but with ten minutes dipping; the ROS production could have overcome and reached inadequate levels, causing a

disturbance in the AsA production and Ca^{2+} ion assimilation, reason why this treatment obtained the lowest outcome. Figure 3 shows how at 24 days of cold storage there is a break point in the trend of both dipping times. At this moment, the decay symptoms and senescence process of the fruit could have been more intense, and this situation could have provoked a hypertensive response (HR). HR are plant physiology mechanisms activated under stress conditions characterized by antioxidants production to cope with stressfully circumstance (Goñi *et al*., 2017). Moreover, a more pronounce activity of APX could have been encouraged during that period. In the case of the GA, despite its positive effects reported on AsA and Ca assimilation (Mandal *et al*., 2015; Dong *et al.*, 2019), the dipping times employed together with its mix with Ca could have mitigate these characteristics, and as a consequence, no any superior AsA level observed with GA treatments.

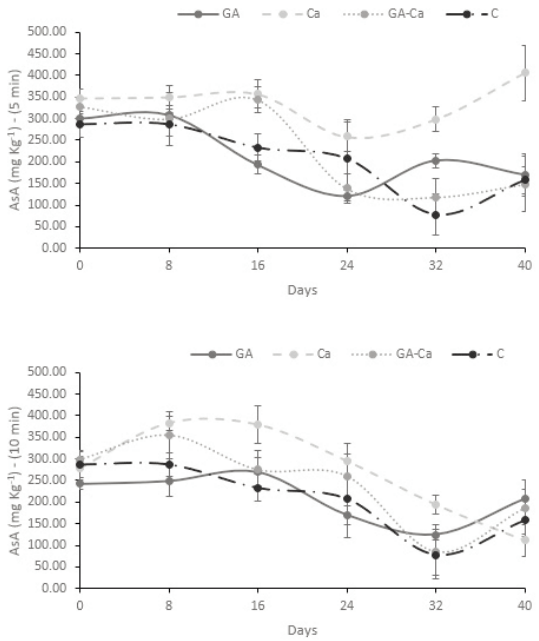

Fig. 3 - Effects of the treatments applied using both dipping times (5 and 10 min), on the AsA content during cold storage. Treatments: GA, Ca, mix GA-Ca, C (Control- no use of GA and Ca). Values are the mean three replicates, and vertical bars represent ± SE.

Internal browning

Internal browning in pineapple has been linked to an increase in the activity of the phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) enzymes during cold storage (Youryon *et al.*, 2018; Paull and Chen, 2019). Postharvest calcium treatments have shown a reduction of PAL and PPO associated with a more elevated activity of their antagonisms enzyme, catalase (CAT), peroxidase (POD), and superoxidase dismutase (SOD); besides, the already mentioned superior antioxidant capacity (Pusittigul *et al*., 2012; Youryon *et al*., 2018; Cano-Reinoso *et al.*, 2022 b). These antagonism enzymes can interfere in the production of ROS like hydrogen peroxide H_2O_2, and the superoxide radical O_2^-. This ROS inhibition creates a reduction of the membrane lipid peroxidation, giving more consistency to the cell wall matrix, which cause a longer shelf-life. Furthermore, Youryon *et al.* (2018) reported that Ca^{2+} ions could bind to the negative charge molecules associated with PAL and PPO, suppressing their metabolic disturbances. This previous information can help clarify why the treatments of Ca caused a low severity and incidence of internal browning, and why this circumstance was also linked to the highest AsA and citric acid results, especially with five minutes dipping. This dipping time can be ideal to maintain a low ROS, PAL, and PPO activity during postharvest.

On top of that, results show that there may be a negative impact in all treatments employing GA, due to their high severity and incidence of internal browning, primordially with ten minutes dipping. Pusittigul *et al*. (2012) demonstrated that an elevated endogenous concentration of GA in pineapple fruit can enhance the activity of PAL and PPO. Therefore, it is important to control the gibberellins level in the fruit, especially under the use of exogenous GA applications. This information suggests that the treatments with GA, essentially with long dipping times, like ten minutes, could not only facilitate these enzyme activities, also encourage superior ROS concentration. However, more studies are recommended for the future, especially to clarify GA interaction with Ca impacting the occurrence of internal browning.

In conclusion, calcium and GA dipping application affected the pineapple physicochemical characteristics and internal browning. The treatment of Ca with dipping time of five minutes delivered the best results. This treatment caused the lowest severity and incidence of internal browning, together with the highest AsA, citric acid content, and lowest TSS/TA ratio, after 40 days of cold storage. Also, this treatment had the closest values to an ideal fruit quality. On the other hand, the control, the treatments using GA, and its mix with calcium, in both dipping times, did not provide satisfactory results, primordially because those delivered a high severity and

incidence of internal browning and did not cause a remarkable enhancement in the fruit antioxidant content. Finally, the results described here can be consider preliminary, since the majority of the current investigations concerning the employment of Ca and GA as postharvest treatments on pineapple have not yet clarified what the optimal doses and dipping time to be implemented are; therefore, further studies are recommended.

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Impact of cultural conditions on germination of olive (*Olea europaea* L.) somatic embryos and plantlets development from the Algerian cultivar Chemlal

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Abstract: The *in vitro* propagation techniques are currently a commercial alternative for the production of plants with good quality in several plant species, including the olive tree (*Olea europaea* L.). Somatic embryogenesis is the process practically used for the application of several biotechnological tools of improvement and *in vitro* plant regeneration via the germination of somatic embryos. Our work aims to evaluate the effect of the chemical and hormonal composition of the culture medium on the germination of olive somatic embryos (cv. Chemlal) as well as the micropropagation of the obtained plantlets before their acclimatization to natural conditions. The results indicated that the production of olive plants by somatic embryogenesis depends strongly on the genotype of the somatic embryos (cell line) and more on the culture conditions, particularly the presence of growth regulators. Indeed, a solid OM medium supplemented with hormones (BA and IBA) permitted an advanced root emergence and germination allowing the production of well-developed plants with several leaves. In addition, an OM medium supplemented with Zeatin and IBA allowed better reactivity of micro-cuttings producing well-developed shoots with several emitted roots which facilitates their further acclimatization to natural conditions.

1. Introduction

The olive tree (*Olea europaea* L.) a diploid dicotyledonous species of the *Oleaceae* family, includes several cultivars selected and multiplied initially by farmers mainly for the size of their fruits and the oil content (Besnard *et al.,* 2018). Conventional methods of breeding and multiplication represent an important solution to the crop problems, especially the increasing demand for plants needed for new orchards. However, these

techniques have become unable to achieve significant results because of the long juvenile period of the species (Rugini *et al.,* 2020) as well as the recalcitrance of certain main cultivars, such as 'Chemlal' in Algeria, to semi-hardwood cuttings (Fabbri *et al.,* 2004). In this context, *in vitro* propagation techniques are currently a commercial reality for the multiplication of several olive cultivars (Rugini *et al.,* 2020) due to the sanitary quality and the confirmed genetic stability of the regenerated plants (Lopes *et al.,* 2009) despite some phenotypic changes and somaclonal variations observed after several subcultures (Leva, 2009; Bradaï *et al.,* 2016, 2019).

Somatic embryogenesis is a morphogenetic process through which somatic cells produce a bipolar structure morphologically similar to the zygotic embryo called a 'somatic embryo' able to develop into a whole plant (Neumann *et al.,* 2009). Actually, this process has become a common technique for *in vitro* regeneration allowing the application of several biotechnological tools of improvement and conservation such as genetic transformation, *in vitro* selection and cryo-preservation of various species and interesting genotypes (Sánchez-Romero, 2021). Most of the established works on somatic embryogenesis in olive consider three main steps starting with the establishment of embryogenic cultures combining the induction and proliferation of calli, followed by a phase of expression and development of structured embryos ready to be converted into whole plants. However, few works have been done about the germination conditions of olive somatic embryos (Mazri *et al.,* 2020) although regeneration of plantlets has frequently been achieved by introducing the embryos under photoperiod on a standard culture medium based on the chemical compositions 'MS' (Murashige and Skoog, 1962) or 'OM' (Rugini, 1984). In addition, the low conversion rates remain the great obstacle of the process in many species, including in olive tree, are caused mainly by deficiencies in the development and maturation of the used embryos (Merkle *et al.,* 1995; Sánchez-Romero, 2019) but also by unfavorable conditions to their germination (Bradaï *et al.,* 2016).

The objective of our study is to evaluate the effect of the chemical and hormonal composition of the culture medium on the germination of somatic embryos of the main Algerian olive cultivar 'Chemlal'. In addition, the micropropagation of the obtained plantlets as well as their acclimatization to natural conditions were tested.

2. Materials and Methods

Establishment of the embryogenic cultures

The embryogenic cultures used were induced from radicles of zygotic embryos of the cultivar 'Chemlal' according to a modified method of Cerezo *et al.* (2011). Radicles isolated after disinfection of the seeds extracted from the stones of mature olives were cultured on solid OMc medium (Cañas and Benbadis, 1988) supplemented with 0.5 mg l^{-1} of Zeatin and 5 mg l^{-1} of indole-3-butyric acid (IBA) for three weeks. Subsequently, the explants were transferred to the same OMc medium without Zeatin and containing 0.5 mg l^{-1} of IBA for four weeks. Finally, the obtained calli were maintained by monthly subcultures on a solid ECO basal medium (Cerezo *et al.,* 2011) supplemented with 0.1 mg l^{-1} of Zeatin, 0.1 mg l^{-1} of Benzylaminopurine (BA) and 0.05 mg l^{-1} IBA in addition to 0.55 g l^{-1} of glutamine and 1 g l^{-1} of casein hydrolyzate. All the media were supplemented with 20 g l^{-1} of sucrose, 50 mg l^{-1} of Myo-Inositol and solidified with 6 g l^{-1} of agar after adjusting the pH to 5.74 with NaOH or HCl (1 N). The cultures were incubated in total darkness at a temperature of 25±2°C.

Pro-embryos exceeding 2 mm in size were isolated from calli of two embryogenic lines (C2 and C3) after one month of culture in suspension on a liquid ECO medium supplemented with hormones in darkness with stirring at 100 rpm. These immature embryos were transferred for maturation on solid ECO free-hormones medium and supplemented with 1 g l^{-1} of activated charcoal for two months in total darkness at 25±2°C.

Germination of somatic embryos

Mature embryos with a perfectly bipolar or cotyledonary structure, were germinated individually in test tubes over two different media: OM (Rugini, 1984) and MS (Murashige and Skoog, 1962) solidified with 6 g l^{-1} of agar. In addition, the effect of a hormonal combination consisting of 1 mg l^{-1} BA and 0.1 mg l^{-1} IBA was tested. The two media were supplemented with 20 g l^{-1} of sucrose, 100 mg l^{-1} of Myo-Inositol. At least 16 mature somatic embryos were incubated for more than eight weeks under a 16 h light photoperiod (35 µmol m^{-2} s^{-1}) at a temperature of 25±2°C. Germination, root emergence, shoot

length as well as the number of leaves per obtained plantlet after eight weeks of culture were observed.

Micropropagation of shoots and acclimatization of regenerated plantlets

In order to maintain the maximum number of plants in culture; micropropagation of the shoots resulting from the germination of somatic embryos was applied. Therefore, the shoots were divided into uni-nodal micro-cuttings and cultured on two different culture media: OM and DKW (Driver and Kuniyuki, 1984) as modified by Revilla *et al.* (1996). The two culture media were supplemented with 20 g l^{-1} of sucrose, 100 mg l^{-1} of Myo-Inositol in addition to hormonal balances composed of 2 mg l^{-1} of BA or Zeatin combined with 0.1 mg l^{-1} of IBA. The different media were solidified with 6 g l^{-1} of agar. At least 16 micro-cuttings having two leaves were cultured individually in test tubes containing 15 ml of the micropropagation medium for eight weeks under a 16 h light photoperiod at a temperature of 25±2°C. The reactivity of the micro-cuttings and the number of reactive buds, shoot length, number of leaves as well as the number of roots emitted were observed.

The obtained plants showing an acceptable length with several leaves and well-developed roots were acclimatized in the laboratory under a photoperiod for about two months on a humidified mixture of sand/potting soil/perlite at a rate of 2/2/1 (v/v/v). Subsequently, the reactive plantlets were transferred to natural conditions under greenhouse on a substrate rich in organic matter and frequently irrigated before being permanently planted in the field.

Data analysis

Statistical analyses of the data (Analysis of variance and tests) were carried out using the "XLSTAT" program version 2016.02.27444. In case of a significant difference, the separation of means was performed by Fisher's LSD (Least Significant Difference) test. The percentages were analyzed by the chi-square test. The results were presented as a mean ± standard deviation or as a percentage relative to the total of introduced explants. A significance level of 5% was considered in all analyses. The letters in the tables indicate homogeneous groups.

3. Results

Germination of somatic embryos

From the first days under photoperiod, the white-opaque somatic embryos of olive showed greening of their stem part and yellowing of the root part (Fig. 1 A) followed by its elongation preceding an increase in size of the two cotyledonary leaves and their separation each one from the other (Fig. 1 B) before the emergence of a small shoot (Fig. 1 C). Indeed, the germination capacity of embryos and the plants development varied from one cell line to another and were significantly influenced by the culture conditions, particularly the presence of growth regulators (Table 1). Thus, more embryos of the C2 line germinated on OM medium while the germination of the two lines was similar on the MS medium. However, the presence of hormones allowed a significant improvement in the embryos germination of both lines. In fact, the best germination rates were obtained on OM medium supplemented with BA and IBA (OM_1) with 56.3 and 37.5% respectively for C2 and C3 while the low germination rate of 25% was recorded on MS without hormones (MS_0) (Table 1).

In addition, embryos of C2 germinated with root emergence from the first week of culture (Fig. 1 A) while no reactivity was observed before two weeks for C3 embryos. Likewise, the germination and the root emergence were faster on the OM medium than

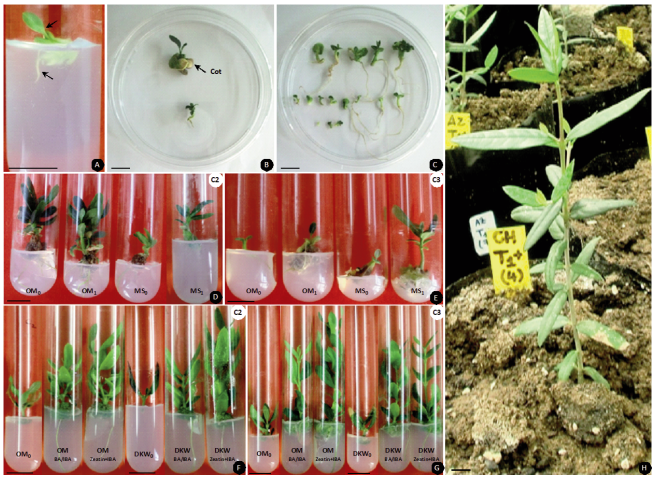

Fig. 1 - Germination of mature somatic embryos from two lines of embryogenic olive callus, cv. Chemlal, and micropropagation of the obtained shoots after 8 weeks of culture on different culture media. (A) Somatic embryo germinated on solid medium. (B) Swelling of cotyledonary leaves of embryos. (C) Different stages of embryos germination. (D and E) Plantlets obtained after germination. (F and G) Plantlets obtained after the micropropagation of shoots obtained from germination of somatic embryos. (H) Acclimated plantlets. (→: the arrows indicate the emergence of the root, shoot and the two cotyledonary leaves. Bar corresponds to 1 cm. Cot: Cotyledons).

Table 1 - Effect of the chemical composition (OM and MS) of the culture medium and the presence of hormones (without hormones '0' or with hormones '1') on germination and root emergence of somatic embryos of two lines of embryogenic olive calli, cv. Chemlal, after eight weeks of culture

Germination medium	Germination rate (%)		Average time of germination (Days)		Average time of root emergence (Days)		Average length of plantlet (cm)		Average number of leaves/plantlet	
	C2	C3	C2	C3	C2	C3	C2	C3	C2	C3
OM_0	50.0 b	31.3 d	16.8±3.8 b'	21.0±0.0 de'	12.3±3.5 a''	17.5±4.9 cd''	1.6±0.1 **b**	0.6±0.1 **d**	6.0±1.6 **a'**	2.0±0.0 **c'**
OM_1	56.3 a	37.5 c	14.0±4.4 a'	18.7±4.0 bc'	10.5±4.0 a''	15.4±3.1 b''	2.3±0.8 **a**	0.9±0.2 **c**	6.3±1.6 **a'**	2.3±0.6 **c'**
MS_0	25.0 e	25.0 e	24.5±4.9 f'	21.7±1.2 e'	21.0±0.0 e''	18.7±4.0 d''	0.8±0.1 **cd**	0.8±0.1 **cd**	2.5±0.7 **c'**	2.0±0.0 **c'**
MS_1	31.3 d	31.3 d	21.0±0.0 de'	19.3±4.7 cd'	17.5±0.7 cd''	16.3±4.0 bc''	0.9±0.1 **c**	1.9±0.1 **b**	2.5±0.7 **c'**	4.5±0.7 **b'**

The different small letters of the same format in columns indicate the homogeneous groups of a significant difference at level of 5%.

on MS, especially in the presence of growth regulators which accelerated significantly germination of embryos. Therefore, the embryos emitted their roots after 10.5 and 15.4 days and germinated after 14 and 18.7 days on the OM_1 medium respectively for C2 and C3 (Table 1). Consequently, early germination and rooting of C2 embryos resulted in well-developed plants with an average length of 1.6 and 2.3 cm with 6 and 6.3 leaves per plantlet respectively on OM_0 and OM_1 (Table 1, Fig. 1 D) while the MS_1 medium was more beneficial for the C3 plants reaching 1.9 cm in length with 4.5 leaves (Table 1, Fig. 1 E).

Micropropagation of shoots and acclimatization of regenerated plantlets

Reactivity of micro-cuttings and shoot development. The micropropagation of shoots resulting from the germination of somatic embryos was significantly influenced by the callus line as well as the chemical and hormonal composition of the culture medium (Table 2). In fact, micro-cuttings of the C2 line were more reactive than those of C3 regardless of the culture conditions, although the presence of hormones was essential for the development of the shoots given the low reactivity recorded on the control media. In addition, the best result of reactivity (100%) of the explants of both lines was obtained with the balance Zeatin/IBA and also the C2 cuttings on the combination BA/IBA in presence of which only 62.5 and 87.5% of the C3 cuttings reacted respectively on OM and DKW (Table 2). Elsewhere, the DKW medium in particular supplemented with hormones (Zeatin/IBA) accelerated the bud reaction and improved the number of reactive buds per explant while the cuttings introduced particularly on the free-hormones OM medium reacted late with less sprouted buds (Data not shown).

The development of the obtained shoots depended directly on the culture conditions and was significantly influenced by the reactivity degree of the cut-

Table 2 - Effect of the micropropagation medium (OM and DKW) and the presence of hormones (BA/IBA or Zeatin/IBA) on the reactivity, development and rooting of shoots from uni-nodal micro-cuttings obtained after germination of somatic embryos of two lines of embryogenic olive calli, cv. Chemlal, after eight weeks of culture

Composition of the micro-propagation medium	Hormonal combinations	Reactivity and shoot development						Rooting			
		Reactivity rate (%)		Average length of shoot (cm)		Average number of leaves/shoot		Rooting rate (%)		Average number of roots	
		C2	C3	C2	C3	C2	C3	C2	C3	C2	C3
OM	Control	25.0 d	12.5 d	0.7±0.3 g	0.9±0.0 fg	1.5±0.7 d'	2.0±0.0 cd'	0.0 e'	0.0 e'	0.0±0.0 f''	0.0±0.0 f''
	BA/IBA	100.0 a	62.5 c	2.0±0.7 de	1.9±0.2 de	2.9±1.0 cd'	3.1±0.9 cd'	31.3 d'	31.3 d'	1.0±0.0 e''	1.5±0.7 bc''
	Zeatin/IBA	100.0 a	100.0 a	3.7±1.0 b	5.2±2.1 a	6.9±3.2 b'	9.9±3.1 a'	62.5 a'	43.8 c'	1.5±0.0 bc''	1.7±0.6 b''
DKW	Control	18.8 d	12.5 d	0.5±0.0 g	0.7±0.0 g	2.0±0.0 cd'	2.0±0.0 cd'	0.0 e'	0.0 e'	0.0±0.0 f''	0.0±0.0 f''
	BA/IBA	100.0 a	87.5 b	1.7±0.3 ef	3.1±1.0 bc	3.4±1.1 c'	2.7±0.4 cd'	43.8 c'	31.3 d'	1.3±0.6 cd''	1.5±0.7 bc''
	Zeatin/IBA	100.0 a	100.0 a	2.7±0.2 cd	3.3±1.4 bc	6.6±1.1 b'	6.8±2.4 b'	56.3 a'	50.0 b'	1.3±0.5 d''	2.0±0.8 a''

The different small letters of the same format in columns indicate the homogeneous groups of a significant difference at level of 5%.

ting particularly the number of reactive buds. In cuttings with two active buds; one shoot regularly grown more than the other (Fig. 1 F and G). Moreover, the cuttings cultured on OM medium supplemented with hormones especially Zeatin/IBA and which sprouted early, produced well-developed shoots of 3.7 and 5.2 cm in length with 6.9 and 9.9 leaves respectively for C2 and C3 while the shoots obtained on the control media were the least developed, with less than 1 cm of length and a maximum of 2 leaves per plantlet (Table 2, Fig. 1 F and G).

Rooting of developed shoots. The presence of hormones in the culture medium was essential for the formation of a basal callus on the micro-cuttings before the emission of roots, while the chemical composition contributed more in the development of the induced calli due that the majority of calli generated on OM were generally larger compared to those obtained on DKW (Data not shown, Fig. 1 F and G).

Root emission was significantly influenced by the genotype (cell line) as well as chemical and hormonal composition of the propagation medium (Table 2). Indeed, more rooting was observed with micro-cuttings of the line C2 particularly on the OM medium supplemented with Zeatin/IBA allowing 62.5 and 43.8% rooting with 1.5 and 1.7 roots emitted by cutting respectively for the two lines while the chemical composition of DKW was more beneficial to C3 explants with 50% of rooting and an average of 2 roots per plantlet (Table 2). However, the appearance of roots occurring from the 3rd to the 7th week of culture was often faster on DKW compared to OM medium especially in the presence of BA with IBA. Furthermore, the substitution of BA by Zeatin in the added hormonal combination reduced the rooting time by more than a week in the cuttings of both lines, which improved the length of the emitted roots (Data not shown). Therefore, the plantlets showing well-developed shoots and roots were easily acclimatized in the laboratory and exhibit normal growth and phenotype even after transfer to natural field conditions (Fig. 1 H).

4. Discussion and Conclusions

Germination of somatic embryos

Germination of olive somatic embryos was frequently achieved on media based on the chemical formulations OM and MS with a reduced concentration of mineral salts, similar to those used for the culture of zygotic embryos (Sánchez-Romero, 2019). Rugini (1988) indicated that germination and development of olive plantlets from somatic embryos is faster on OM medium than on MS. Indeed, Rugini and Caricato (1995) observed the germination of embryos of cultivars 'Canino' and 'Moraiolo' after 1 to 2 weeks on OM free-hormones medium, whereas Shibli *et al.* (2001) didn't note reactivity in embryos of the cultivar 'Nabali' before two weeks of incubation on MS medium, which agree with our results indicating a faster germination on OM medium compared to MS one.

Several studies reported low rates of embryo germination varying with genotype but depending more on the quality of the used embryos. Therefore, Jafarzadeh-Bajestani *et al.* (2011) obtained less than 6% of germination on MS medium with embryos of the cultivar 'Zard' while a desiccation step for three days improved conversion to 50% and formation of rooted plantlets with 6 to 8 leaves. Furthermore, culturing embryos of the cultivar 'Picual' on a cellulose acetate semi-permeable membrane during the first month of maturation (Cerezo *et al.,* 2011) allowed an adequate dehydration, good structuring of the embryos and a significant improvement in germination and quality of the obtained plantlets. Therefore, the low germination capacity observed in this study was probably due to the use of embryos directly after their maturation without passing a desiccation phase allowing the synchronization of their germination (Merkle *et al.,* 1995).

Germination of olive somatic embryos was often achieved in the absence of hormones, although Rugini (1995) recommended the addition of Zeatin to solid MS medium to boost emergence. Thus, embryos of the cultivars 'Chetoui', 'Chemlali' and 'Arbequina' (Trabelsi *et al.,* 2003) and 'Picholine Marocaine' (Brhadda *et al.,* 2008) germinated easily on OM and MS media supplemented with 0.5 mg l^{-1} of Zeatin and resulted to well-developed plantlets, while a free-hormones medium rich in sucrose induced cell proliferation of the embryos. Conversely, Toufik *et al.* (2017) observed that the presence of growth regulators, in particular Zeatin alone in the OM-based medium, was not essential for the germination of embryos of the cultivar 'Dahbia' and can even inhibit the root emergence and cause a strong explant necrosis although the addition of NAA with GA3 allowed up to 45% conversion from mature embryos according to Mazri *et al.* (2020). Therefore, the inclusion of growth regulators especially cytokinins to the germination

medium is directly determined by the genotype and degree of maturity of the embryos (Merkle *et al.,* 1995). In this sense, Bradaï *et al.* (2016) indicated that well-matured and structured or cotyledonary embryos of 'Picual' germinated easily in the absence of hormones, unlike globular embryos whose germination varies between 30 and 70%.

The regenerated plants in this study showed a normal phenotype (phyllotaxis, leaf shape, etc.) during their *in vitro* maintenance as well as an easy acclimatization to natural conditions. Leva (2009) indicated that plantlets regenerated by somatic embryogenesis show a stable phenotype similar to that of the mother plants. Moreover, despite their fragility, these plants acclimatize easily to natural conditions and show normal growth under greenhouse and good development after transfer to the field.

Micropropagation of shoots and acclimatization of regenerated plantlets

The *in vitro* multiplication of plants regenerated by somatic embryogenesis was rarely practiced because their acclimatization was usually carried out directly after germination (Bradaï *et al.,* 2016). In fact, the DKW medium (Driver and Kuniyuki, 1984) as modified by Revilla *et al.* (1996) and supplemented with a hormonal balance rich in cytokinins was often used although the chemical formulation of OM medium containing Zeatin was commonly recommended for the rapid stimulation of axillary buds of several olive cultivars (Lambardi *et al.,* 2013) due to its nutritional content, particularly in microelements (Rugini *et al.,* 2020). Cerezo *et al.* (2011) indicated that the micro-cuttings taken after germination of embryos of the cultivar 'Picual' respond easily on DKW medium supplemented with BA and IBA by developing shoots of about 1.4 cm in height with formation of a basal callus often accompanied by the emission of one or more roots. Nevertheless, Bradaï *et al.* (2016) observed that shoots multiplication from somatic embryos was influenced more by the age of the cell culture than by its genotype (callus line). These authors obtained more developed rooted shoots from young cultures while less sprouted buds with older lines giving small shoots often without roots. According to Bhojwani and Razdan (1996) *in vitro* shoots need to reach a minimal size to root easily as small plants may not survive during the acclimatization period. Therefore, our results confirm the importance of the genotype, the significant effect of the DKW chemical formulation and the presence of Zeatin in addition to an auxin for the multiplication and rooting of shoots resulting from the germination of somatic embryos.

In conclusion, our study is a contribution to the optimization of the *in vitro* regeneration of olive tree by somatic embryogenesis and describes for the first time an efficient regeneration of whole plants without morphological abnormalities in the main olive cultivar in Algeria 'Chemlal' via embryogenic cultures induced from juvenile material, radicles of zygotic embryos. The obtained results show that the development of plantlets by germination of somatic embryos depends strongly on the genotype and the chemical and hormonal composition of the used culture medium. A solid OM medium supplemented with hormones allowed a faster germination resulting in well developed shoots. Subsequently, multiplication of the shoots on OM or DKW media containing Zeatin with IBA generated whole and rooted plantlets easily acclimatized to natural conditions.

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AHS
Advances in Horticultural Science

Maintaining postharvest quality of bell pepper (*Capsicum annuum* L. cv. California Wonder) using cactus (*Opuntia stricta* L.) mucilage coating

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Key words: Ascorbic acid content, edible coating, fresh weight loss, fruit vegetable, shelf life.

Abstract: Bell pepper (*Capsicum annuum* L.) experiences significant qualitative and quantitative loss during postharvest. This study aimed at providing an alternative postharvest handling technology for bell pepper. The factor studied was cactus (*Opuntia stricta* L.) mucilage coating at four levels: 0% (distilled water), 1, 2, and 3%. The fruits were stored under ambient conditions (25 ± 2°C temperature and 65 ± 2% relative humidity) until senescence. Weight loss and total soluble solids content were determined at an interval of 3 days whereas iron and ascorbic acid content were determined at an interval of 4 days. Shelf life elapsed when fruit lost 25% of their initial weight on average. Cactus mucilage coating reduced weight loss by up to 21.64%, maintained total soluble solids by up to 14.93%, iron by up to 6.46%, ascorbic acid by up to 19.46% and extended shelf life by up to 6 days. Cactus mucilage coating at 1% was the best treatment and therefore can be used by bell pepper growers, retailers, and consumers to maintain postharvest quality and extend shelf life of bell pepper.

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

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The authors declare no competing interests.

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1. Introduction

Postharvest losses in horticultural produce in developing countries is as high as 45% due to poor postharvest handling (Kitinoja and Kader, 2015); and is even higher in Sub-Saharan Africa (SSA) (Kitinoja and Kader, 2015). In bell pepper (*Capsicum annuum* L.), losses of 28.6% and 38.7% have been reported during dry and wet seasons, respectively in Nigeria (Tsegay *et al*., 2013). A short shelf life, even under the most favourable conditions is a major postharvest limiting factor in bell pepper handling (Ilić *et al*., 2017). Since bell pepper is a non-climacteric fruit, its senescence is mainly accelerated by excessive water loss through respiration.

There is increasing interest in edible fruit and vegetable coatings to extend postharvest life. Cactus [*Opuntia ficus-indica* (L.) Mill.] mucilage has potential in postharvest preservation of horticultural commodities such as minimally processed cactus pear fruits (Liguori *et al*., 2021),

mango (*Mangifera indica* L.) (Abera *et al.*, 2019) and papaya (Oluwaseun *et al*., 2014). According to Oluwaseun *et al*. (2014), papaya fruits that were dipped for 30 seconds in cactus mucilage + glycerol and in pure cactus mucilage recorded a significantly lower weight loss, lower increase in fruits' TSS content and higher ascorbic acid content as compared to uncoated fruits at the end of storage period at a temperature 27 ± 2°C and RH of 55-60%.

Low temperature storage and Modified Atmosphere Packaging (MAP) have been successfully used in maintaining quality and extending shelf life of bell pepper (Manolopoulou *et al.*, 2012; Bayogan *et al*., 2017). The most effective method has been rapid cooling after harvest followed by storage at low temperature and high relative humidity (Bayogan *et al*., 2017). Bell pepper being a tropical fruit, suffers chilling injury at temperatures below 7°C, which favors development of fungal diseases (Ilić *et al.*, 2017). The cost of purchasing, installing and running a cold storage facility is also high and unaffordable for most small-scale bell pepper growers, retailers and consumers in developing countries hence rendering the technology untenable.

Modified Atmosphere Packaging (MAP) using plastic bags has also been used for a long time for maintenance of quality of bell pepper. However, their use may trigger development of anaerobic microorganisms (Manolopoulou *et al*., 2012). These together with the restricted use of plastics in several countries due to environmental pollution has made the technology unreliable. Therefore, the objective of this study was to determine the effects of cactus mucilage coating, an alternative postharvest treatment, on postharvest quality and shelf life of bell pepper.

2. Materials and Methods

Experimental materials

Bell pepper, cv. California Wonder, fruit was produced at the Horticulture Teaching and Research Field of Egerton University, Njoro, Kenya under white agro net covers. The University lies at a latitude 0°23' South, longitude 35°56'East; and is 2,227 m above sea level (Jaetzold *et al.*, 2006). Fruit was harvested at mature green stage (Díaz-Pérez *et al*., 2007), packed in plastic buckets, and taken to the laboratory. Average minimum temperature, maximum temperature and relative humidity of the laboratory site was 11°C, 24.5°C and 64.7%, respectively (Egerton Meteorological Weather Station, 2020), where fruit free from bruises and blemishes were selected and used for the study. Cactus (*Opuntia stricta* L.) stems were also harvested from the field at Egerton University, packed in plastic buckets and transported to the laboratory.

Extraction of cactus mucilage and preparation of cactus mucilage treatments

Cactus mucilage was extracted at room temperature (25 ± 2°C) using the method described by Sepulveda *et al*. (2007). Cactus stems were washed using 2% volume per volume (v/v) sodium hypochlorite (NaClO) to remove dirt and for disinfection. Stems were peeled and chopped into small pieces using a sharp knife. Distilled water was added to the chopped pieces in a ratio of 1:1 (w/v) (200 g of the chopped pieces in 200 mL distilled water) and blended for 3 min using a blender (PPS SB-4171, Sayona, China) to obtain slurry which was gravity filtered through muslin cloth. The filtrate was precipitated using 20% isopropyl alcohol in a ratio of 1:1 (v/v) (1 L of the filtrate in 1 L of 20% isopropyl alcohol). The precipitated filtrate was centrifuged for 10 min at 2,683 × g using a centrifuge (DL-5-D). The supernatant was drained off and precipitates at the bottom of the Eppendorf tubes dried in a forced air oven at 70°C for 4 h to obtain dried cactus mucilage. To obtain 1, 2 or 3% mucilage solution, 1, 2 or 3 g, respectively, of the dried cactus mucilage was weighed using an electronic weighing balance (Denver Instrument XL-1810) and dissolved in 80 mL of distilled water. To each solution, 2 mL of glycerol plasticizer was added, volume made to 100 mL mark using distilled water and blended for 3 min to obtain complete dispersion. The solutions were centrifuged for 10 min at 2,683 × g using a centrifuge (DL-5-D) to obtain a supernatant; which was used to coat fruit. The different concentrations of cactus mucilage coating (1%, 2% and 3%) were chosen for the current study based on past research that were done on effects of cactus mucilage on other fruits and fruit vegetables (Alikhani, 2014; Zegbe *et al.,* 2013)

Treatments application

Before treatment application, all fruits were disinfected by washing for 5 min using 0.5% (v/v) NaClO (Lerdthanangkul and Krochta, 1996). This was followed by air drying of fruit at room temperature (25 ± 2°C) until the disinfecting solution on fruit skin was completely dry. The fruit were dipped in 1 litre of

cactus mucilage solutions for 5 min based on the treatments (Alikhani, 2014) after which the excess coating was allowed to drain off. The fruits were air dried until the cactus mucilage on skin was completely dry allowing formation of a layer of coating on the fruit surface. Control fruit were dipped in distilled water for 5 min, removed and allowed to air-dry at room temperature (25±2°C) until distilled water on fruit skin was completely dry. After treatments application, all fruit were stored on plastic trays under ambient conditions (25 ± 2°C temperature and 65 ± 2% relative humidity) until they senesced.

Experimental design

The experiment was a single factor experiment arranged in a randomized complete block design, with 3 replications. Blocking was done against different harvesting times; harvesting of the 3 blocks was done at 1 month interval. In total, there were 12 experimental units with each experimental unit represented by a plastic tray containing 30 fruits.

Data collection

Data collection commenced immediately after treatments application and continued until fruit lost 25% of their initial weight (Sibomana *et al*., 2015). Data collection was done on fresh weight loss and total soluble solids (TSS) at 3 days intervals; and iron and ascorbic acid content at 4 days intervals. Three fruits per experimental unit, were selected at random at the onset of the study, marked and used for data collection throughout the study for non-destructive variables which were fresh weight loss and shelf life. On the other hand, three fruits per experimental unit were also randomly selected from the remaining fruits and used to collect data for the destructive variables (TSS, iron and ascorbic acid content). Data for each destructive variable was collected from the three fruits with a new set of fruits used on each sampling date. The variables were determined as described below.

Fresh weight loss

The fresh weight (g) of the three selected fruits per experimental unit was measured using an electronic weighing balance (Denver Instrument XL-1810) immediately after treatment application (before storage). The same fruits were thereafter weighed at 3 days intervals until they lost 25% of their initial total weight. Progressive % fresh weight loss was determined using the formula by Moneruzzaman *et al.* (2008). Average % fresh weight loss of the 3 fruits was calculated and recorded as average % weight loss per fruit for the time period (Moneruzzaman *et al*., 2008). The shelf life of the fruit on the other hand was determined by counting the number of days the fruit took from harvesting to lose 25% of their initial weight (Sibomana *et al*., 2015).

Total soluble solids content

Total Soluble Solids (%TSS) content was determined using a portable hand-held refractometer RHB-32/ATC (YHEQUIPMENT CO., LIMITED, Shenzhen City, China) as described by Opiyo and Ying (2005). A small piece of pepper fruit was cut, squeezed and the juice obtained dropped onto a refractometer and readings taken. Average %TSS of the 3 fruits was calculated and recorded as average %TSS per experimental unit for the time period.

Iron content

Iron (Fe) content was determined using an Atomic Absorption Spectrophotometer (model 210 VGP, Buck Scientific, Norwalk, CT) following Jones and Case (1990). Dried ground sample (1 g) was weighed into crucibles and ashes were obtained in a furnace at a temperature of 550°C for 2 h. The ash was cooled to room temperature (25 ± 2°C), transferred into a 100 mL beaker and 10 mL of the digestion mix added. Distilled water (50 mL) was added. Activated charcoal (1 g) was added to obtain a clear sample and stirred. The contents were gravity filtered through Whatman No.5 filter paper into a 100 mL volumetric flask. The filtrate was filled to the mark with distilled water. Into a cuvette, 10 mL of filtrate was pipetted and absorbance read at 248 nm. Iron standard solutions of 0, 5, 10, 15, 20 and 25 µg/g were prepared from iron sulphate. Into a cuvette 10 mL of each standard was pipetted and absorbance read at 248 nm, and a standard curve developed. The amount of iron was calculated against the standards, converted to µg/g and expressed using the formula of Okalebo *et al.* (2002).

Ascorbic acid content

Ascorbic acid (Vitamin C) was determined by titration with 2, 6-dichloro-phenol-indophenol dye following a standard procedure (AOAC, 1990). Using an electronic weighing balance (Denver Instrument XL-1810, USA), 10 g of fruit sample was weighed. The weighed fruit sample was extracted in 20 mL 5% oxalic acid using a mortar and pestle, and then gravity filtered through cotton wool. Ascorbic acid standard solution was prepared by dissolving 0.05 g of pure ascorbic acid in a small volume of 5% oxalic acid solution and then diluting to 250 mL with the same oxalic

acid solution. Ascorbic acid standard solution (10 mL) was titrated with 0.005% indophenol solution to a persistent slight pink colour end point and 10 mL of oxalic acid as a blank. The amount of ascorbic acid corresponding to 1 mL of indophenol solution was calculated. Into a 50 mL flask, 10 mL of the gravity filtered sample extract was pipetted and made to the mark with the 5% oxalic acid solution. The standard indophenol solution was used to titrate 10 ml of the filtrate to a slight pink end point. Vitamin C content was calculated following Obel *et al.* (2019).

Data analysis

All the data were subjected to analysis of variance (ANOVA) in SAS (ver. 9.0, SAS Institute Inc., Cary, NC). Significant means at F-Test were separated using Tukey's Honestly Significant Difference (SAS, 2010).

3. Results

Effect of cactus mucilage coating on fresh weight loss and shelf life of bell pepper fruit

Cactus mucilage treatments significantly reduced fresh weight loss of bell pepper fruits from 3 DAH (days after harvest) to the end of storage (Fig. 1). At 3 DAH, fruits coated with 1, 2 and 3% cactus mucilage recorded a significantly lower weight loss as compared to a higher weight loss observed for the control treatment (distilled water) (Fig. 1). A similar trend was observed at 6 DAH and 9 DAH. At 12 DAH, weight loss of fruits coated with 1% cactus mucilage was significantly lower as compared to weight loss of fruits coated with 0, 2 and 3% cactus mucilage (Fig. 1). Fruits coated with 1% cactus mucilage treatment recorded a significantly lower weight loss as compared to the control fruits at 15 DAH (Fig. 1)

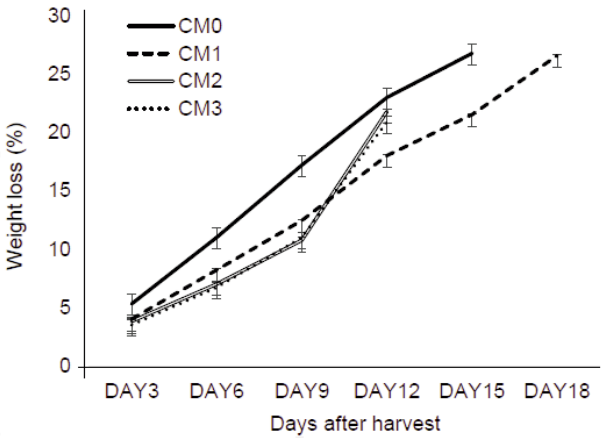

Fig. 1 - Effect of cactus mucilage coating on weight loss of bell pepper. Error bars indicate ±SE of the mean. CM0 is fruit coated with 0% cactus mucilage, CM1 is fruit coated with 1% cactus mucilage, CM2 is fruit coated with 2% cactus mucilage and CM3 is fruit coated with 3% cactus mucilage.

Application of cactus mucilage treatments significantly extended shelf life of bell pepper fruit by up to 6 days during storage (Fig. 2). Fruits coated with 1% cactus mucilage recorded a significantly longer shelf life, followed by a shelf life recorded for the control fruits with the shortest shelf life recorded for the fruits coated with 2 and 3% cactus mucilage (Fig. 2).

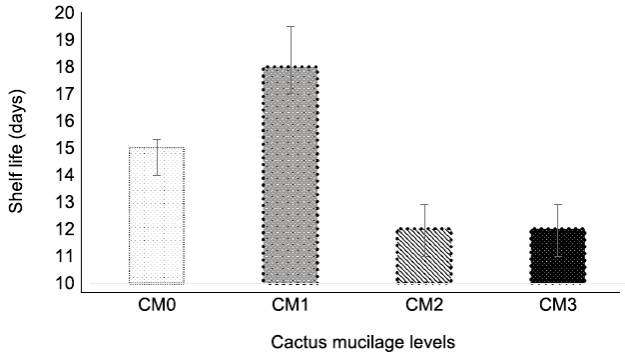

Fig. 2 - Effect of cactus mucilage coating on shelf life of bell pepper. Error bars indicate ±SE of the mean. CM0 is fruit coated with 0% cactus mucilage, CM1 is fruit coated with 1% cactus mucilage, CM2 is fruit coated with 2% cactus mucilage and CM3 is fruit coated with 3% cactus mucilage.

Effect of cactus mucilage coating on total soluble solids of bell pepper fruit

Cactus mucilage coating had a significant effect on total soluble solids (TSS) content of bell pepper fruit from 3 DAH through the end of storage (Fig. 3). In addition, there was an increase in TSS content of bell pepper fruit as storage duration progressed except for fruits coated with 2 and 3% cactus mucilage where a decrease was observed from 9 DAH (Fig. 3). At 3 DAH, fruits coated with 2 and 3% cactus mucilage treatments recorded a significantly lower TSS content, followed by TSS content recorded for fruits coated with 1% cactus mucilage with the highest TSS content recorded for the control treatment (Fig. 3). The trend was the same at 6, 9 and 12 DAH of storage. At 15 DAH, fruit coated with 1% cactus mucilage recorded a significantly lower TSS content as compared to a higher TSS content recorded for the control treatment (Fig. 3).

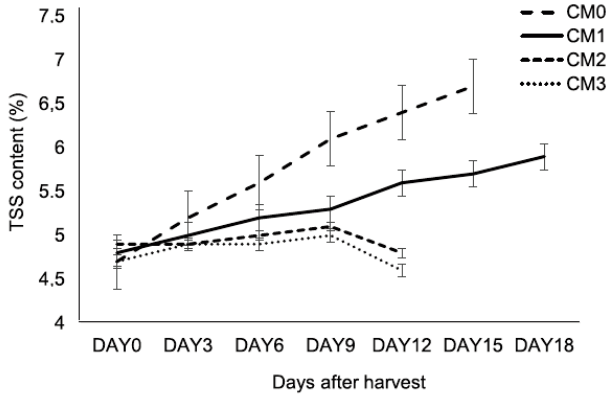

Fig. 3 - Effect of cactus mucilage coating on total soluble solids content of bell pepper. Error bars indicate ±SE of the mean. CM0 is fruit coated with 0% cactus mucilage, CM1 is fruit coated with 1% cactus mucilage, CM2 is fruit coated with 2% cactus mucilage and CM3 is fruit coated with 3% cactus mucilage.

Effect of cactus mucilage coating on iron content of bell pepper fruit

Cactus mucilage coating also had a significant effect on iron content of bell pepper fruit from 4 DAH until the end of storage (Fig. 4). At 4 DAH, fruit coated with 1, 2 and 3% cactus mucilage recorded a significantly higher iron content as compared to a lower iron content recorded for 0% cactus mucilage treatment (Fig. 4). A similar trend was observed at 8 DAH (Fig. 4). At 12 DAH, a significantly higher iron content was recorded for fruits coated with 1% cactus mucilage as compared to a lower content recorded for 0, 2 and 3% cactus mucilage treatments (Fig. 4). Fruits coated with 1% cactus mucilage recorded a significantly higher iron content as compared to a lower content recorded under the control treatment (Fig. 4).

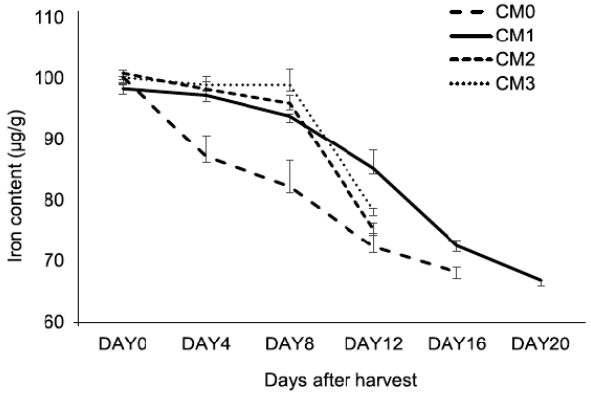

Fig. 4 - Effect of cactus mucilage coating on iron content of bell pepper. Error bars indicate ±SE of the mean. CM0 is fruit coated with 0% cactus mucilage, CM1 is fruit coated with 1% cactus mucilage, CM2 is fruit coated with 2% cactus mucilage and CM3 is fruit coated with 3% cactus mucilage.

Effect of cactus mucilage coating on ascorbic acid content of bell pepper fruit

Ascorbic acid content in bell pepper fruit was influenced by cactus mucilage treatments during storage from 4 DAH through 16 DAH (Fig. 5). At 4 DAH, fruits coated with 1, 2 and 3% cactus mucilage recorded a significantly higher ascorbic acid content as compared to a lower ascorbic acid content recorded for the control treatment (Fig. 5). A similar trend was observed at 8 DAH (Fig. 5). A significantly higher ascorbic acid content was recorded for fruit coated with 1% cactus mucilage as compared to a lower ascorbic acid content recorded for 0 2 and 3% cactus mucilage treatments at 12 DAH (Fig. 5). At 16 DAH, fruit coated with 1% cactus mucilage recorded a higher ascorbic acid content as compared to a lower ascorbic acid content recorded under the control treatment (Fig. 5).

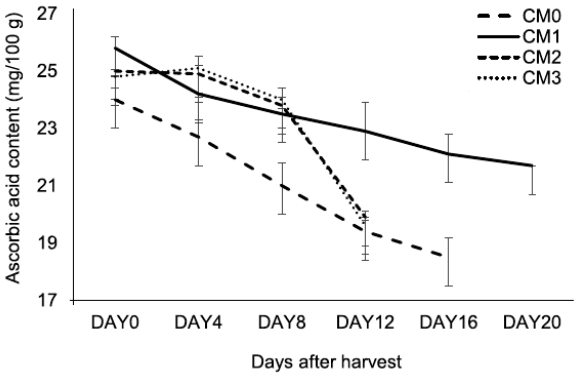

Fig. 5 - Effect of cactus mucilage coating on ascorbic acid content of bell pepper. Error bars indicate ±SE of the mean. CM0 is fruit coated with 0% cactus mucilage, CM1 is fruit coated with 1% cactus mucilage, CM2 is fruit coated with 2% cactus mucilage and CM3 is fruit coated with 3% cactus mucilage.

4. Discussion and Conclusions

Effect of cactus mucilage coating on fresh weight loss and shelf life of bell pepper fruit

Fresh weight loss is an important index in determining postharvest quality and shelf life of pepper. Weight loss in harvested fruit is normally caused by continuous loss of water and stored starch as a result of respiration and evaporation leading to increase in weight loss as storage duration progresses. Cactus mucilage coating forms a film on the fruit's skin/cuticle which acts as a semi-permeable barrier against moisture, oxygen, carbon (IV) oxide, and solute

movement in the produce or between the produce and its environment. This leads to reduced rate of respiration, reduced water loss, starch or sugar loss, weight loss and extended shelf life. This could explain the reduced weight loss and extended shelf life recorded for fruit coated with 1% cactus mucilage compared to control fruit in the current study. Many studies have also reported reduced weight loss and extended shelf life in fruits and vegetables as a result of polysaccharide-based edible coatings (Menezes and Athmaselvi, 2016; Vishwasrao and Ananthanarayan, 2016). A thick layer of fruit coating blocks pores on the fruit's skin, decrease oxygen concentration in the fruit's tissues since oxygen in the fruit's environment cannot get inside and the respiration products cannot also get outside the fruit's tissues. Anoxic conditions initiated leads to ethanol fermentation in which stored carbohydrates and sugars are broken down to lactic acid, ethanol, acetaldehyde and carbon (IV) oxide which explains reduced fresh weight loss observed for fruits coated with 2 and 3% cactus mucilage during storage in the current study. Increased weight loss caused by anaerobic conditions led to a shorter shelf life of fruits. Fermentation bacteria and yeast proliferate the bell pepper tissues and breaks down stored carbohydrate, sugar, water and minerals for their growth and other metabolic activities. This explains the increased rate of fresh weight loss observed after 9 DAH of storage for fruits coated with 2 and 3% cactus mucilage. According to Kareem *et al*. (2017), anaerobic respiration leads to a decrease in stored carbohydrate content in fruits due to the utilization of some of the sugars by the fermenting organisms such as lactic acid bacteria for their growth and other metabolic activities.

Effect of cactus mucilage coating on total soluble solids of bell pepper fruit

Fruit TSS content tends to increase during storage due to biosynthesis of polysaccharides and accumulation of sugars during ripening (Ullah *et al*., 2017) and volatilization of soluble compounds and water. At advanced stages of ripening, disassociation of some molecules and structural enzymes in soluble compounds results in increased levels of TSS. A slower rate of increase in TSS content in cactus mucilage coated fruit observed in the current study could be attributed to the role of fruit coatings acting as a barrier against oxygen, carbon IV oxide and ethylene, slowing down the rate of respiration and ripening leading to reduction in accumulated sugars and polysaccharides. Increased TSS content in control (uncoated) bell pepper fruit could be due to volatility of soluble compounds and water at a faster rate due to lack of a protective barrier on the surface of such fruit. In addition, possible accumulation of sugars and polysaccharides as a result of increased rate of hydrolysis could have led to increased TSS content in control fruits. These results are consistent with that of Menezes and Athmaselvi (2016) in sapota (*Manilkara zapota*). Ethanol fermentation lowers TSS content in fruits due to development of off-flavours as observed for fruits coated with 2 and 3% cactus mucilage. Ethanol fermentation is a two-step process in which pyruvate is first carboxylated to acetaldehyde by Pyruvate Decarboxylase and acetaldehyde is subsequently converted to ethanol by Alcohol Dehydrogenase. This explains the lower TSS content observed for fruits coated with 2% and 3% cactus mucilage as compared to those coated with 1% and dipped in distilled water during storage.

Effect of cactus mucilage coating on iron content of bell pepper fruit

Results of this study indicate that coating bell pepper fruit with cactus mucilage may preserve the fruit's iron content. Iron is stored in fruit's tissues in the chloroplast where 80% of the iron is located as iron-protein complexes known as Fe-phytoferritin, Fe-citrate, Fe-phytosiderophore and Fe-nicotianamine (Maathuis and Diatloff, 2013). Iron is necessary for the synthesis of many proteins (ferredoxin and cytochromes) that carry electrons during respiration in which most iron ions are used to biosynthesize proteins that carry electrons (Bhatla and Lal, 2018). Cactus mucilage coating acts as a barrier against O_2 and CO_2 inside and out of the fruit, thus reducing the rate of respiration and therefore reducing the amount of iron ions used to synthesize proteins that carry electrons during respiration. This could explain the high amount of iron in fruit coated with cactus mucilage during the current study. Rapid decline of iron content in fruit that were dipped in distilled water could possibly have been due to increased respiration rate as a result of increase in O_2 and decreased CO_2 inside and out of the fruit. Results of the current study are consistent with those of Amirthaveni and Daga (2016) who recorded higher iron content in bell pepper coated with *Aloe vera* gel and gum Arabic. Ethanol fermentation causes decline in minerals such as iron in the fruit. The nutrients are utilised by yeasts and lactic acid bacteria as they carry out their metabolism and fermentation activity. In

addition, their growth is supported by the existence of basic compounds such as fermentable minerals. This could offer an explanation for the result observed on fruits coated with 2 and 3% cactus mucilage during storage. These findings are supported by Kareem *et al*. (2017) and Maicas (2020) who reported utilization of minerals by lactic acid bacteria for growth and other metabolic activities during fermentation of fruits.

Effect of cactus mucilage coating on ascorbic acid content of bell pepper fruit

Ascorbic acid is commonly used as a quality indicator of fruits and vegetables since it is very sensitive to degradation due to its oxidation compared to other nutrients during food processing and storage. Plants biosynthesize ascorbic acid mainly through the Smirnoff-Wheeler pathway. In the final step of ascorbic acid synthesis, galactono-1,4-lactone is oxidized by galactono-1,4-lactone dehydrogenase (GLDH) to produce ascorbic acid. Ascorbic acid produced reduces during storage due to degradation mainly through the direct oxidation of dehydroascorbate (DHA) or 4-O-oxalyl-L-threonic acid to produce both oxalic acid and L-threonic acid. In the current study, cactus mucilage coating could have acted as a barrier against oxygen gas that enters the fruit thereby reducing oxidation of dehydroascorbate (DHA) or 4-O-oxalyl-L-threonic acid resulting in higher amounts of ascorbic acid in the fruit during storage. A rapid decrease in ascorbic acid in uncoated fruits could be attributed to increased oxidation of dehydroascorbate (DHA) or 4-O-oxalyl-L-threonic acid due to increased oxygen concentration in the fruit tissues. Results of this study are in agreement with those of a number of scholars who also reported oxidation reactions in the presence of oxygen in uncoated fruits during storage leading to reduction of ascorbic acid (Menezes and Athmaselvi, 2016; Ullah *et al*., 2017). A rapid decrease in ascorbic acid content observed for fruits coated with 2 and 3% cactus mucilage during storage was attributed to ethanol fermentation of fruits as a result of low oxygen concentration in the fruit tissues. During fermentation, microorganisms such as yeasts and lactic acid bacteria uses nutrients such as minerals like ascorbic acid for their growth, reproduction and other metabolic activities leading to a decrease in ascorbic acid content in fermenting fruits.

Based on the objective and findings of this study, it can be concluded that cactus mucilage coating significantly influenced postharvest quality and shelf life of bell pepper. One % cactus mucilage coating was the best treatment in terms of fresh weight loss reduction, maintenance of total soluble solids, iron, ascorbic acid content, and extension of shelf life of bell pepper fruit.

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Organic amendments role in reducing drought stress in *Alcea rosea* L.

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Key words: Antioxidant activity, hollyhock, trace elements, water stress.

Abstract: Water scarcity and dwindling natural resources due to global warming are negatively impacting ornamental plant survival. Soil fertility remains a problem in arid and semiarid regions. In this study, the effects of four media (arable soil, arable soil + cow manure, arable soil + rice hull, arable soil + wheat straw) on macronutrient content and quantitative characteristics of *Alcea rosea* L. under drought stress were investigated. Application of organic amendments mitigated the negative effects of drought in the soil and increased the available organic macronutrients. The application of organic amendments increased the total N, P, and K content in the soil and leaves of hollyhock. Total soluble sugars (by 11.9%), RWC (by 8.75%) and phenolics (by 36.4%) of hollyhock were significantly improved by the application of organic amendments at 80% FC. The amended soil (soil + cow manure) increased the activities of superoxide dismutase and ascorbate peroxidase at 80% FC. Moreover, the soil + cow manure proved to be the best supplement to improve leaf area and dry weight. In conclusion, the application of organic amendments can be successfully used as a cost-effective management method to improve soil fertility and crop production in arid and semi-arid areas.

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1. Introduction

Considering the increasing population of the planet earth, stability in green space has particular importance (Wolch *et al.,* 2014). Exacerbation of environmental stressors lays the groundwork for the loss of ornamental plants. Therefore, one of the critical goals of plant producers and breeders is providing quality and stress-resistant plants for green space (Anguelovski *et al.,* 2020). Among ornamental plants, Alcea, commonly known as Hollyhock, is a perennial plant of the Malvaceae family, with decorative and medicinal importance. People use the flowers of this plant to produce medicinal tea due to pigments (Shehzad *et al.,* 2020). In addition, the antibacterial, anticancer (Lim, 2012), antioxidant (Ahmed *et al.,* 2016), anti-depressant, anti-inflammatory (Ahmadi *et al.,* 2012), anti-fatigue, febrifuge, mouth washing (Burt and Reinders, 2003), and blood circulation enhancer (Lim, 2012) characteristics of this plant have been proven. Regarding the climatic changes and the resistance of this plant to adverse conditions, it can be a desirable plant in the green space (Oraee

et al., 2019).

Among the environmental factors, drought is the most critical factor limiting growth in many areas (Toscano *et al.,* 2019; Bhusal *et al.,* 2020). Based on the scientists' prediction, global temperature could rise by 3 to 9°C with far-reaching effects and significantly increase the dryness of the arable lands (Haile *et al.,* 2020). Regarding the increase in global temperature by 1.5°C, plants selection with high tolerance to drought stress to ensure the survival and stability of green space is an essential strategy (Seleiman *et al.,* 2021). In such conditions, in addition to the skill and accuracy of using and consuming these water resources, recommending tolerant plants, determining the drought tolerance threshold, and increasing soil fertility has become more necessary (Banks *et al.,* 2019; Du *et al.,* 2019).

The elements required in organic matter can increase soil fertility and plant's yield in agriculture (Ukalska-Jaruga *et al.,* 2020). Organic fertilizers provide nutrient balance and increase soil nutrient availability (Verma *et al.,* 2020). These organic substances have benefits, including reducing leaching and wastage of nutrients (Quynh and Kazuto, 2018) and helping the release elements (Mupambwa and Mnkeni, 2018). They enhance root growth due to improved soil structure, increase the amount of organic matter and soil exchange capacity, and ultimately serve as a source for the growth of soil organisms and increase soil yield (Juriga *et al.,* 2018; Chew *et al.,* 2019).

Some studies documented the reduced element transfer in plants under soil drought stress (Liu *et al.,* 2018; Qi *et al.,* 2019). In water shortage conditions, organic matters can conserve water in the soil and prevent the destructive effects of drought stress on plant yield (Kaya *et al.,* 2020). Also, Khosravi Shakib *et al.* (2019) reported that the total dry weight and water use efficiency were about 3-fold higher in marigold (*Calendula officinalis* L.) grown in 30% manure compost substrate compared to the control plants.

Different plant species show a wide range of drought resistance mechanisms, including antioxidant activity and osmoregulatory adaptations (Siddique *et al.,* 2018; Khan *et al.,* 2020). There should be reconsideration of the plants type grown in arid and semi-arid regions. Also, some plants with high water requirements should be replaced with the drought-tolerant plant (De Souza Aguiar *et al.,* 2020). Hollyhock is a low-expectation plant that grows well in natural and marginal areas that can be considered suitable for cultivation in low-input systems. This is a first work useful to develop a suitable methodology for studying the use of soil organic amendments. Also, this study was conducted to identify the drought tolerance threshold of Hollyhock and identify different growth mediums on drought tolerance of Hollyhock.

2. Materials and Methods

Plant material

The experiment was conducted in the research greenhouse of Ferdowsi University in Mashhad, Iran. Seeds were sown in August in the greenhouse at an average temperature of 20±1°C in plug trays containing a mixture of coco peat, perlite and peat (1:1:0.5 v/v/v). Plants grew at 400 µmol m^{-2} s^{-1} combined with a photoperiod of 14 and 10 h d^{-1}. Then, all plants at the 5-6 leaf stage were transferred to pots (18 cm high and 8 cm in diameter) containing arable soil. In May, the plants were transferred to pots of size 20 with a soil mixture of four different substrates (arable soil, arable soil + manure, arable soil + rice hull, and arable soil + wheat straw).

Experimental design and treatments

The experiment consisted of two factors (organic supplements and irrigation regime). The organic supplements and irrigation regimes had four and three levels, respectively. Three sources of organic supplements were used (manure, rice hulls, and wheat straw). The pot experiment (one plant per pot) was conducted factorially in a randomised complete block experiment with three replicates under greenhouse conditions. The mixtures between the soil and cow manure, rice hull and wheat straw were prepared in a 50:50 ratio (50% w/v soil and 50% w/v organic amendments). The soil from the four treatments (control soil and the three additives) was taken to the laboratory for chemical analyses after the mixtures were prepared (as described above). The physicochemical properties of the pot samples are explained in Table 1.

Drought stress treatments

Plants were subjected to drought stress during flowering on June 5. Soil volumetric water content was measured during the flowering period using the

Table 1 - Physicochemical properties of the soils used in the experiment

Growth medium	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	pH	EC (ds/m)
Soil	0.065	23	235	7.15	1.93
Soil + cow manure	0.42	2214	1592	7.76	2.53
Soil + rice hull	0.23	271	343	7.26	1.77
Soil + wheat straw	0.21	117	1572	6.68	3.13

Time Domain Reflectometry (TDR) instrument (TRIKE-FM, England). Plants were exposed to drought stress for one month. Plants were irrigated through a polyethylene piping network with volumetric meters at 80, 60, and 40% FC.

Foliar nutrient analysis

Foliar nitrogen, potassium, and phosphorus were measured at the end of the experiment. Leaf samples (0.2 g) were heated to 400°C with 10 mg sulfuric acid and a catalyst mixture in a digestion apparatus, and then nitrogen content was measured using a Kjeldahl apparatus (Kjeldal, 1998). Leaf potassium was measured using the flame photometer method (Tandon, 1998). Phosphorus content was determined by the colorimetric method (yellow molybdate vanadate) at 470 wavelengths (Rayan *et al*., 2001).

Determination of electrolyte leakage

Leaf electrolyte leakage was measured according to the method of Reddy *et al*. (2004). Tissue samples (0.2 g) from the first true leaf of 9-month-old plants were placed in 50 ml of distilled water. The samples were stored at laboratory temperature for 24 hours and the conductance of the solution was measured using a conductivity meter (Jenway model). All leaves were autoclaved for 20 minutes to measure the final leakage. Electrolyte leakage was calculated as:

$$EL\% = EC1/EC2 \times 100.$$

Total soluble sugars assay

Spectrophotometry at 620 nm was used to analyze total soluble sugars using the Anthron method (McCready *et al*., 1950). Total soluble sugars were extracted by homogenizing the plant material in 80% ethanol. Samples were centrifuged at 3500 rpm for 10 min. Anthrone solution (10 ml 0.15%) was added to 1 ml of the solution. The samples were then heated to 95°C and immediately transferred to an ice bath. The total sugar concentration of the samples was calculated using the standard glucose curve based on mg g^{-1} dry weight (Ebell, 1969).

Determination of proline and relative water content

Proline content and relative water content (RWC) were determined according to the procedure described by Bates *et al*. (1973) and Turner (2018), respectively.

Chlorophyll determination

Chlorophyll was determined using the method of Arnon (1949). For this purpose, 0.2 g of fresh leaves were ground with 80% acetone. The resulting solution was centrifuged at 4000 rpm for 10 minutes. The optical absorbance of the supernatant was measured using a spectrophotometer (Shimadzu UV-160A) to determine the amount of chlorophyll at 645 and 663 nm.

Phenol assay

In this study, total phenol was determined using the Folin-Ciocalteu reagent (FCR) according to the method of Singleton and Rossi (1965). An amount of 4.5 ml of distilled water and 0.1 ml of Folin-Ciocalteu reagent were added to 0.1 ml of the methanolic extract. After 3 minutes, 2% sodium bicarbonate solution was transferred to 0.3 ml solution. The different concentrations of gallic acid were used to construct the standard curve and measure the absorbance using a spectrophotometer (Shimadzu UV-160A) at 760 nm. Total phenol was calculated based on mg GAE g^{-1} dry weight.

Protein and enzyme activity assays

Bradford's method (1976) with slight modification was used to measure total protein . To assay protein, 50 mg of fresh leave was ground in liquid nitrogen. Then 50 mg of polyvinylpolypyrrolidone, 495 µl of extraction buffer solution (including 40 mM hydrogen tris-chloride buffer, 2% sodium dodecyl sulfate, 20% glycerol, and 60 mM dithiothreitol), and 5 µl of phenylmethyl methanol to 200 ml of phenylmethyl methanol were added to each solution. The samples were centrifuged at 8000 rpm at 4°C for 15 minutes. Then 300 µl of the supernatant with 900 µl of acetone containing 10% trichloroacetic acid and 0.07%

dithiothreitol was added and placed at -20°C. 100 µl of adsorption buffer was added to the supernatant.

Nakano and Asada's method (1981) was used to measure the activity of ascorbate peroxidase. The reaction mixture consisted of 20 µl of enzyme extract, 770 µl of 50 mM phosphate buffer, 100 µl of 0.1 mm EDTA, 100 µl of 5 mM ascorbate, and 10 µl of 0.1 mM hydrogen peroxide. The absorption rate of the reaction was read by a spectrophotometer at 290 nm.

Superoxide dismutase activity (SOD) was measured according to the Sairam *et al.* (2002) method with slight changes. The enzymatic reaction mixture consisted of 935 µl of 50 mM phosphate buffer containing 0.1 mM EDTA, 13 mM methionine, and 75 mM nitroblue tetrazolium (NBT), 15 µl of 0.12 mM riboflavin, and 50 µl of enzymatic extract. After preparing the control and blank samples to measure enzymatic activity, the blank sample was placed in the dark for 15 minutes, and the control and enzyme extract samples were shaken for 15 minutes in a shaker at 25°C with two 20 W fluorescent lamps at 100 rpm. The absorbance was read at 560 nm using a spectrophotometer (Shimadzu UV-160A).

Aebi's method (1984) was used to measure the activity of catalase (CAT) enzyme, where 20 µl of enzymatic extract mixed with 50 mM phosphate buffer containing ten mM hydrogen peroxide and their adsorption changes were recorded at 240 nm by spectrophotometer (Shimadzu UV-160A). Glutathione reductase was measured by Sofo *et al*. (2004).

Determination of growth parameters

Leaf area of hollyhocks was determined using a Delta-T Leaf Area Meter (Device Ltd., Cambridge, UK). Plants were dried at 60°C and weighed to calculate their respective dry weights.

Statistical analysis

Statistical analysis was performed using SAS 8.1 software. Data are presented as mean ± SE of three replicates. Means were also compared using the LSD test at a probability level of 5%.

3. Results

Foliar nutrients (N, K, and P) and soil nutrients (N and K) were significantly (P≤0.05) affected by organic amendment and drought. In the desiccated soils, N content increased significantly with the application of manure amended soil. Foliar N content was 50% and 14% higher in the manure-amended and rice hull-amended soils, respectively, than in the control at 80% FC. Finally, N foliar content was higher in soils enriched with manure in all irrigation regimes, followed by soils enriched with rice hulls at 80 and 60%, respectively FC (Fig. 1a). Similar to N content, leaf P content was also higher in soils fertilized with 80% FC. Overall, drought stress (60% FC) had no effect on P content in soils enriched with manure and rice hulls (Fig. 1b). The magnitude of the increase in K content was more significant in plants under 80% FC than in plants under 40% FC, and it was more pronounced in the amended soils than in the control. Drought stress significantly affected K content, decreasing it slightly (by 4.74%) in manure amended soils under 40% FC compared to 80% FC (Fig. 1c).

Leaf nutrient contents analyzed were similar compared with those in soils. In the unamended soils subjected to different irrigation regimes, soil N con-

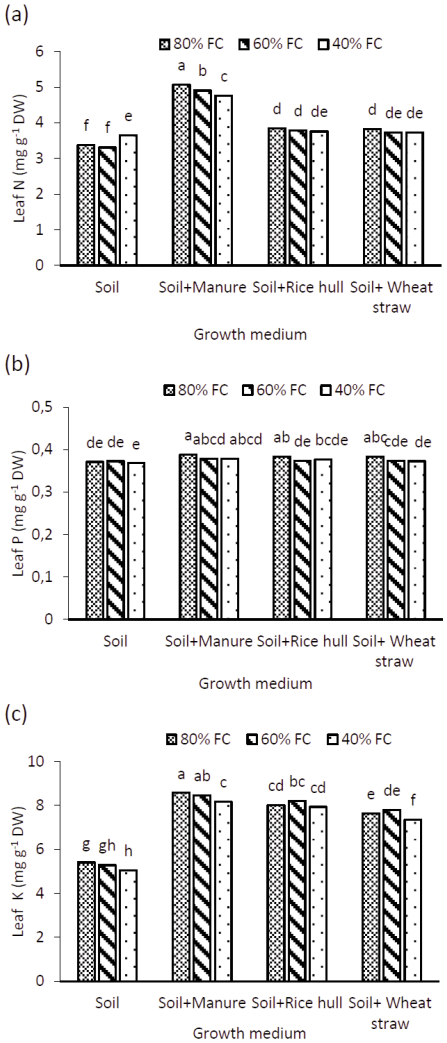

Fig. 1 - Leaf N (a), leaf P (b), and leaf K (c) of hollyhock plants. Values in columns followed by the same letter are not significantly different at the 0.05 level (P≤0.05).

tent decreased significantly. Compared to the control, organic soil amendments caused a less dramatic increase in N content. N content in soils amended with manure was higher in soils amended with 80, 60, and 40% FC than in other treatments (Fig. 2a). P contents in desiccated soils decreased progressively, and these values (P≤0.01) were significantly lower than those determined for well-watered soils. Soil P content was consistently higher in soils amended with manure and rice hulls than in control soils. Application of organic amendments to desiccated soils (40 and 60% FC) also increased K content. K accumulation was lower in soils enriched with wheat straw than in soils enriched with manure (Fig. 2b).

Organic amendment significantly affected pH index (P≤0.01), but EC was affected by organic amendment and drought stress. The pH index in soil amended with manure increased significantly (by 5.57%) compared to the control. Overall, the EC was higher in soils with 40% FC than in the corresponding soils with 80% FC. The EC was 22.9% higher in the

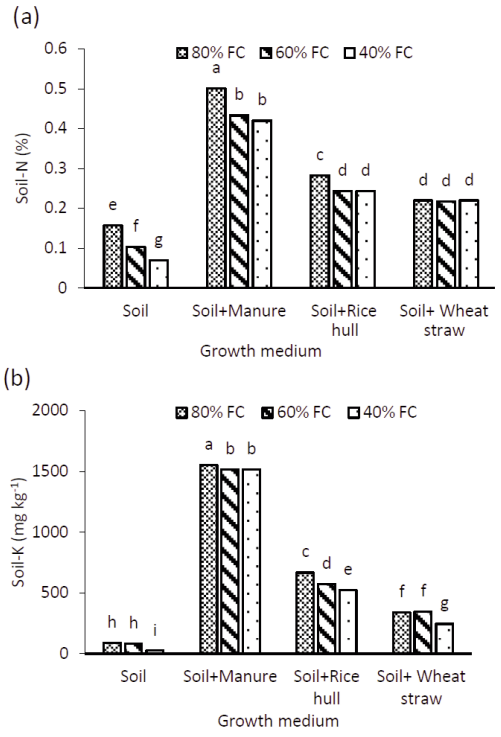

Fig. 2 - The soil-N (a) and soil-K (b). Values in columns followed by the same letter are not significantly different at the 0.05 level (P≤0.05).

manure amended soils than in the control. Organic amendments and drought stress significantly (P≤0.05) affected RWC, EL %, and phenolic content. RWC decreased slightly in amended soils with 40% FC compared to soils with 80% FC. Conversely, 40% FC decreased RWC in unamended soils (Fig. 3a). EL % in unamended soils was higher at 40% FC than in well irrigated soils, while for rice hull and wheat straw there was no difference in this variable at 40% FC (Fig. 3b). Total soluble sugar and proline were significantly (P≤0.01) affected by the interaction between drought stress and organic amendments. In well-watered soils, soluble sugar and proline contents were consistently higher in amended than in control soils. In leaf tissues of plants treated with 40% FC, total soluble sugar content increased by 6.17% in amended soils compared with unamended soils, and this index remained unchanged in leaf tissues of soils amended with rice hull and wheat straw at 60 and 40%, respectively FC (Fig. 3c). Proline tissues were also dependent on drought stress, and a maximum increase of 177% was observed in the soils amended with slurry at 40% FC compared to the control (Fig. 3d).

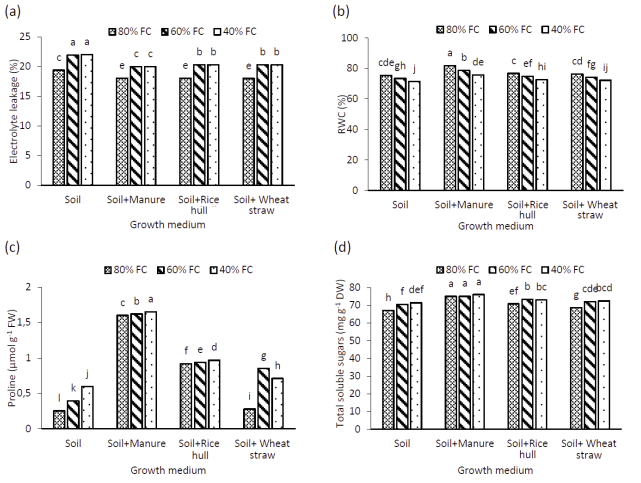

Fig. 3 - Electrolyte leakage (a), RWC (b), proline (c), and total soluble sugar (d) in hollyhock plants under different medium. Values in columns followed by the same letter are not significantly different at the 0.05 level (P≤0.05).

Chlorophyll a, b, and total chlorophyll were significantly (P≤0.05) affected by the interaction between drought stress and organic amendments. It was found that chlorophyll content increased in plants grown with organic amendments under drought stress. The highest chlorophyll a content was found in soils fertilized with 80% cow manure FC (Fig. 4a). Soils fertilized with cow manure showed a significant increase in chlorophyll b under drought stress (Fig. 4b). Between treatments, the highest average total chlorophyll (3.18 mg g^{-1} FW) was measured in soils

amended with cow manure at 80% FC, while the lowest total chlorophyll (1.37 mg g^{-1} FW) was measured in control plants at 40% FC (Fig. 4c). Changes in phenolic content of hollyhocks showed similar trends for both soils (wheat straw enriched soils), such that phenolic content increased under severe water stress, but this index in plant tissues grown in manure and pod enriched soils showed no difference under all irrigation regimes (Fig. 4d).

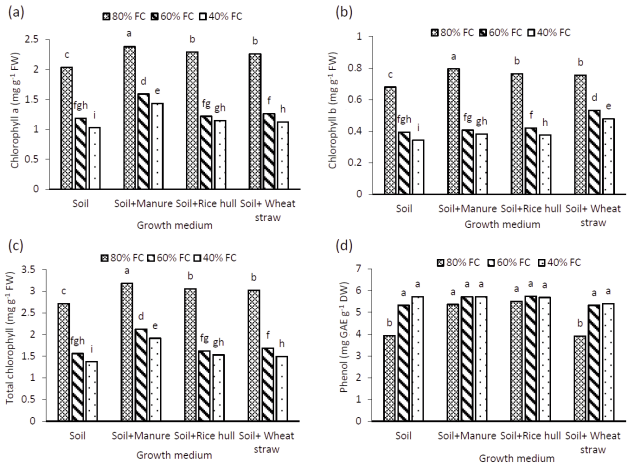

Fig. 4 - Chlorophyll a (a), chlorophyll b (b), total chlorophyll (c), phenol (d) in hollyhock plants under different medium. Values in columns followed by the same letter are not significantly different at the 0.05 level (P≤0.05).

The interaction of drought stress and organic amendments significantly affected antioxidant activity (P≤0.01). Glutathione reductase levels were higher in rice-treated soils than in other treated soils. Drought stress significantly increased this index in amended soils. However, glutathione reductase in leaves of hollyhocks grown in manure amended soils (40% FC) increased significantly by 28% compared to control plants (80% FC). There was no significant difference between glutathione reductase in manure and wheat straw -amended soils at 40% FC (Fig. 5a). In addition, significant improvement of SOD and APX was observed under severe drought stress. The maximum value of SOD was measured in the amended and unamended soils in 40% FC treatments (Fig. 5c). The activity of APX was lower in the unamended soils than in the corresponding soils in amended soils, while there was no significant difference between soil irrigation regimes. Overall, APX activity was higher in manure amended soils at 60 and 40% FC than in the corresponding soils at 80% FC (Fig. 5d). No differences were observed in CAT values between manure-amended and non-manure-amended soils at 40% FC, while a slight increase was measured in manure-amended soils (Fig. 5d).

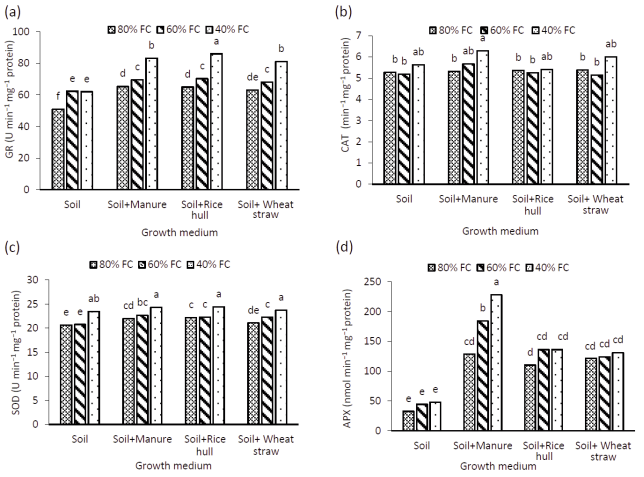

Fig. 5 - GR (a), CAT (b), SOD (c), and APX (d) in hollyhock plants under different medium. Values in columns followed by the same letter are not significantly different at the 0.05 level (P≤0.05).

Leaf area and dry weight of hollyhock (P≤0.05) were significantly affected by drought and amended soils. Leaf area at 40% FC was generally lower than that of the control throughout the evaluation period. Leaf area at 80% FC was higher in manure amended soils than in nonamended soils (Fig. 6a). In well-watered soils, dry weight was consistently higher in amended soils than in control soils. In general, this index was lower in soils at 60 and 40% FC than at 80% FC. Drought (40% FC) had a negative effect on dry weight, especially in the unworked soils. At 80% FC, this index was lower in soils with rice hull admixture than on wheat straw medium (Fig. 6b).

4. Discussion and Conclusions

The results of a recent experiment show that a suitable nutrient medium reduces the negative effects of drought stress. The results show that organic amendments in soils improved macroelements in leaves and soil. Our results are in agreement with those of Banik *et al*. (2006), who showed an increase in nitrogen, phosphorus, and potassium in rice (*Oryza sativa* L.) after the application of various organic matter such as animal and poultry manure and rice. The researchers observed the highest phosphorus and potassium content in rice grain

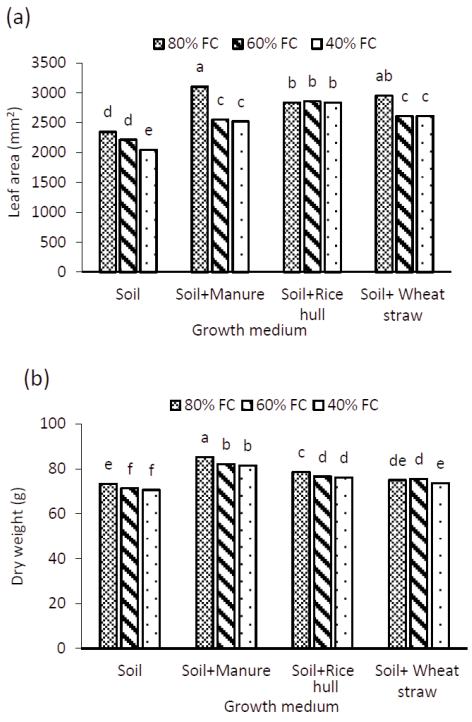

Fig. 6 - Leaf area (a), and dry weight (b) in hollyhock plants under different medium. Values in columns followed by the same letter are not significantly different at the 0.05 level (P≤0.05).

and rice branches, respectively, under cow manure.

He and Dijkstra (2014) found that nitrogen and phosphorus concentrations decreased under drought stress. A significant decrease in nitrogen, phosphorus and potassium uptake in maize (*Zea mays* L.) branches was found to be twofold under drought stress conditions (Alizadeh, 2010). In the studies of Ghazi (2017), increasing drought stress from 50% to 100% FC reduced nitrogen, phosphorus and potassium uptake in maize leaves. Under drought stress, the average potassium concentration in the roots of treated Apocynum plants (*Apocynum venetum* L.) decreased by 40%, but in the leaves of this plant, the amount of potassium remained constant and increased in the stem. The result suggests that the plant stores large amounts of potassium to maintain osmotic adjustments (Cui *et al*., 2018).

Leaf potassium content decreased under severe drought stress because nutrient transport and uptake depend on soil moisture. Decreased element transfer in plants under drought stress conditions has been documented in many studies (Ahangar *et al*., 2016; Li *et al*., 2021). Hollyhock plants were able to maintain potassium content in leaves up to 60% FC, which was consistent with the results of Qi *et al*. (2019), which showed that the accumulation of potassium in the plant was higher under non-stress conditions than under stress conditions, in addition, further reduction of irrigation under severe stress reduced the amount of potassium in the leaves of plants. The reduction in transpiration rates and changes in membrane transporters under drought conditions due to water deficit, which lead to reduced mineral nutrition in plants, showed that phosphorus uptake in rice increases with increasing moisture content (Roy, 2018). The highest phosphorus and potassium uptake occurred due to mineralization of fertilizer elements in the soil. Application of nitrogen and phosphorus fertilizers in tortoiseshell bamboo (*Phyllostachys edulis*) increased soil phosphorus, nitrogen, and leaf phosphorus (Wu *et al*., 2018). There is a positive correlation between soil macroelements and leaf elements (Table 2). Numerous studies have shown that organic fertilizer increases soil pH (Liu *et al*., 2010; Han *et al*., 2016). Organic fertilizers increase calcium carbonate, which is believed to increase buffering and thereby improve soil pH (Whalen, 2000). Electrical conductivity can serve as a critical indicator of nutrient and water uptake (Dhaliwal *et al*., 2019). Application of poultry, cattle, and goat manure significantly increased soil electrical conductivity, and the potential for manure-induced soil salinity was relatively high for poultry and goat manure (Azeez and Van Averbeke, 2012). The increased electrical conductivity of soil is due to the release of salts from fertilizers. As a result of the decomposition of organic matter in the soil, the ions obtained from the decomposition entered the soil solution and consequently increased the salinity of the soil, which was consistent with Roy and Kashem (2014).

An increase in drought stress resulted in an increase in electrolyte loss, such that electrolyte loss increased in leaves of maize (Mozdzen *et al*., 2021) and tomato (Ors *et al*., 2021) compared to the control under drought conditions. In addition, nitrogen uptake occurs by mass uptake and potassium and phosphorus uptake occurs by diffusion (Li Bot *et al*., 2021). In the absence of water, the uptake of nitrogen and phosphorus is reduced. In addition, the presence of potassium under drought stress conditions maintains turgor pressure and osmotic adjustments of cells (Singh *et al*., 2021). In turtle shell bamboo, phosphorus and nitrogen addition to plants under drought stress decreased malondialdehyde and reduced electrolyte loss compared to the control (Wu *et al*., 2018). In the current experiment, due to

Table 2 - Correlation coefficients among different parameters of hollyhocks

	Soil N	Soil P	Soil K	Leaf N	Leaf P	Leaf K	Chloro-phyll	EL	RWC	Proline	Total soluble sugars	GR	CAT	APX	SOD	Leaf area	Dry weight
Soil N		0.82 **	0.90 **	0.92 **	0.55 **	0.74 **	0.47 **	-0.31 **	0.71 **	0.89 **	0.71 **	0.84 **	0.06 NS	0.75 **	0.30*	0.89 **	0.95 **
Soil P			0.65 **	0.97 **	0.52 **	0.66 **	0.46 **	-0.25 *	0.57 **	0.81 **	0.57 **	0.81 **	0.17 NS	0.59 **	0.49 **	0.71 **	0.82 **
Soil K				0.82 **	0.45 **	0.78 **	0.41 **	-0.25 *	0.67 **	0.94 **	0.72 **	0.81 **	0.02 NS	0.72 **	0.28 *	0.79 **	0.56 **
Leaf N					0.47 **	0.67 **	0.47 **	-0.42 **	0.77 **	0.87 **	0.71 **	0.71 **	0.02 NS	0.66 **	0.31 **	0.79 **	0.89 **
Leaf P						0.68 **	0.42 **	-0.70 **	0.64 **	0.42 **	0.36 **	0.49 **	0.19 NS	0.52 **	0.25 *	0.76 **	0.61 **
Leaf K							0.31 **	-0.42 **	0.54 **	0.71 **	0.61 **	0.86 **	0.01 NS	0.87 **	0.37 **	0.91 **	0.82 **
Chlorophyll								-0.52 **	0.81 **	0.19 NS	0.15 NS	0.08 NS	0.36 *	0.06 NS	-0.36 **	0.57 **	0.51 **
EL									-0.73 **	-0.18 NS	0.16 NS	-0.20 NS	-0.24 *	-0.34 **	0.26 *	0.61 **	0.48 **
RWC										0.56 **	0.31 **	0.43 **	0.22 NS	0.37 **	0.05 NS	0.76 **	0.78 **
Proline											0.83 **	0.86 **	-0.06 NS	0.72 **	0.40 **	0.71 **	0.86 **
Total soluble sugars												0.78 **	-0.13 NS	0.70 **	0.58 **	0.55 **	0.65 **
GR													-0.02 NS	0.86 **	0.54**	0.74 **	0.80 **
CAT														-0.02 NS	-0.1 NS	0.20 NS	0.12 NS
APX															0.60 **	0.74 **	0.73 **
SOD																0.26 *	0.31 **
Leaf area																	0.89 **
Dry weight																	

EL= electrolyte leakage, RWC= relative water content, GR= glutathione reductase, CAT= catalase, APX= ascorbate peroxidase, SOD= superoxide dismutase, * P≤0.05; ** P≤0.01; NS= not significant.

the increase of nitrogen, potassium, and phosphorus in the soil and the increase of these elements in the leaves of the irrigated plants by 80% FC, the rate of electrolyte loss in these plants decreased compared to other substrates. Researchers showed that K^+ efflux could play an essential role in anabolic reactions by stimulating catabolic processes and saving "metabolic" energy for adaptation and repair needs in plants (Demidchik *et al*., 2014).

In our study, an increase in drought stress also decreased the amount of chlorophyll in leaves, which is consistent with the opinions of others (Khayatnezhd and Gholami, 2021). In peach plants, the use of phosphorus increased chlorophyll content, but reducing phosphorus decreased protein and chlorophyll content (Dutt *et al*., 2013). The use of nitrogen- and phosphorus-containing fertilizers effectively increased the chlorophyll and carotenoid content of apples because an increase in nitrogen promoted the formation of photosynthetic pigments by thylakoid and stomatal proteins, which also increased the formation of chloroplasts in growing leaves (Jahan *et al*., 2020; Siddiqui *et al*., 2021). The biochemical and biosynthetic properties of photosynthetic pigments require phosphorus as well as nitrogen (da Silva Tavares *et al*., 2020).

In the recent experiment, there was a positive correlation between nitrogen and potassium with chlorophyll (Table 2). The amended soil has increased the leaf elements by increasing the number of elements in the soils under 80% FC. In hyssop (*Hyssopus officinalis*), the effects of drought stress and potassium fertilizer on chlorophyll content were significant, such that the amount of total chlorophyll increased with increasing potassium and irrigation regime (Lopo de Sa *et al*., 2014). In plants under higher drought stress, nutrient deficiencies and reduced energy absorption of sunlight lead to damage to the photosynthesis and chlorophyll systems because nutrients play an essential role in the electron transfer system and carbon metabolism (Xu *et al*., 2020; Ma *et al*., 2021), which is consistent with the study results because the lowest chlorophyll in drought treatment of 40% FC was recorded in the soil substrate.

With increasing drought stress from 80 to 40% FC, the plant's RWC decreased. However, Organic fertilizers reduced the adverse effects of drought stress on the RWC. There have been numerous reports of changes in relative water content and osmotic adjustments in leaves occurring under drought stress, and that these variations were different depending on cultivar, species, duration, and intensity of stress (Kizilgeci *et al*., 2020; Zhu *et al*., 2020). The main rea-

son for the improvement in drought stress relief in the presence of potassium is due to the osmotic adjustments and the preservation of the RWC (Ibrahim *et al*., 2020). Fahri *et al*. (2021) reported an increase in the RWC of two oilseeds with potassium fertilizers under drought stress. Similar responses to potassium fertilizers were reported in corn (Khadem *et al*., 2010) under drought stress. Also, in the study, a positive and significant correlation was recorded between leaf nitrogen and potassium content with the RWC of leaves (Table 2), and the lowest leaves relative water content was recorded in soil-substrate plants with the lowest amount of nutrition.

Drought stress leads to biochemical and physiological changes that lead to osmotic changes and decreased turgor pressure in the cell. These changes degrade nitrate assimilation by reducing nitrate reductase activity (Zhang *et al*., 2009). Plants use osmotic adjustments to reduce the effects of drought stress due to nitrate assimilation, in which proline accumulation plays a vital role in osmotic adjustment (Ozturk *et al*., 2021). Zhang *et al*. (2014) showed that the accumulation of osmotic compounds such as proline, betaine, and potassium ion in two maize cultivars was higher in more resistant cultivars than in more sensitive cultivars under drought stress. Moreover, potassium maintains the turgor pressure by reducing the leaf water potential (Da Silva *et al*., 2021). Potassium application causes proline accumulation by maintaining osmotic adjustments (Aksu and Altay, 2020). In the present experiment, the proline increased with increasing drought stress. Leaf analysis showed that the potassium in leaves of plants in animal manure culture is higher than other substrates, which increases the accumulation of proline in osmotic adjustments. Although its exact mechanism is unknown, it seems to be due to the role of potassium in amino acid metabolism or the effect of potassium on the proline cycle (Zhu *et al*., 2019). Drought stress not only increases the stomatal resistance of plants but also causes the accumulation of osmotic substances (Zahoor *et al*., 2017).

By increasing water uptake and reducing water loss, plants under drought stress prevent further damage (closing stomatal and smaller leaves) (Hill *et al*., 2020). When photosynthesis is not responsive to the plant, carbohydrates break down, maintaining osmotic adjustment in the cell. The sucrose significantly decreased under drought stress (Yang *et al*., 2019). Nutrients play an important role in drought stress on total soluble sugars as an osmotic adjustment and mitigate drought stress. The potassium accumulation in the vacuole and sucrose maintains the turgor and osmotic pressure and effectively increases water uptake in plants (Sardans and Peñuelas, 2021). In addition, drought stress conditions prevent chloroplast damage by maintaining high cellular pH (Cakmak, 2005). Drought stress treatment on apple (*Malus domestica* Borkh.) plants showed that plant sucrose decreased at the beginning of stress but increased over time. Glucose and fructose also had an increasing trend at all times. The process of reducing sucrose reduces vegetative growth in plants under drought stress. The leaf nitrogen decreased with decreasing the amount of applied irrigation water, and with re-irrigation, the nitrogen increased. Phosphorus levels also showed a downward trend in plant parts under drought stress (Jie *et al*., 2010).

In a recent experiment, plants in soil + manure substrates kept the total soluble sugars constant in irrigation at 40% FC compared to 80% FC by absorbing nutrients from the soil. The correlation between the total soluble sugars with nitrogen and potassium proves these results (Table 2). In addition, in the treatment of 40% FC, plants used the total soluble sugars increase for osmotic adjustment and as a system to resist drought stress.

In the present experiment, antioxidant activity was increased under drought conditions. Several studies reported that the antioxidant enzymes activities increased under stress (Hasan *et al*., 2021; Sepahvand *et al*., 2021). Our results were in line with Safari *et al*. (2021) and Babaei *et al*. (2021), who showed that drought stress increased ascorbate peroxidase activity in impatiens and marigold, respectively.

Applying nitrogen and phosphorus fertilizers significantly increased the activity of the peroxidase enzyme under drought-stressed plants when compared to the non-application of these two elements (Wu *et al*., 2018). Using vermicompost organic fertilizer under drought stress conditions in lettuce (*Lactuca sativa* L.) increased the activity of superoxide dismutase and catalase enzymes as an enzyme system, reducing the adverse effects of stress. The correlation between growth traits and the activity of these enzymes under stress showed that the vermicompost application increased plant growth and drought tolerance. The results also showed that the nitrogen and organic matter in vermicompost soil was higher, which led to increased plant growth

(Kiran, 2019).

In plants treated with 80% FC in soil substrate + animal manure with higher leaf elements, enzymatic antioxidant activity increased. Moreover, enzyme activity were positively correlated with elements such as nitrogen and potassium in the leaves (Table 2). The plant needs potassium to maintain enzyme activity and protein synthesis because protein structure need high k^+ in cystocele (Blevins, 1985).

A reduction in leaf area and dry weight under drought stress is attributed to photosynthesis and carbon assimilation. In the present experiment, the plant leaf area decreased with increasing drought stress, and the dry leaf weight decreased with decreasing leaf area, consistent with the results of Khaleghnezhad *et al*. (2021). In the low moisture conditions, the plants reduce the leaf area to decrease the level of respiration. In a recent experiment, plant leaves fell under severe drought stress. To this aim, the leaf area was reduced relative to sufficient moisture conditions, and this may be a positive adaptation to acclimatization to dehydration (Isa *et al*., 2021). Chekanaia *et al*. (2018) showed that the application of nitrogen (ammonium phosphate) and phosphorus (superphosphate) in beans doubled the dry weight and plant yield in both treatments compared to the control. Lubis *et al*. (2021) stated that dry weight of rice and plant height that received elements increased under manure medium. Potassium reduces the negative effect of drought stress on leaf area index and plant's dry weight by increasing the ability of photosynthesis, the rate of CO_2 stabilizing with interference in osmosis, the activity of the enzyme Rubisco, and improving the synthesis and transport of dry matter (Wang *et al*., 2013). The data from the present experiment show that potassium increased plant resistance to drought stress, for which researchers published many reports on the subject (Aksu and Altay, 2020; Aqaei *et al*., 2020).

In the present experiment, the negative correlation between plant dry weight and electrolyte leakage (Table 2) indicates that dry weight plants decreased in severe drought stress with increasing electrolyte leakage, which was consistent with the opinion of Khan *et al*. (2021). They showed that the plants dry weight decreased with increasing electrolyte leakage due to the increase of malondialdehyde in severe drought stress. Also, the results showed a positive correlation between chlorophyll content and plant dry weight (Table 2). Xiao *et al*. (2008) showed that chlorophyll depletion occurred under drought stress due to chlorophyll decomposition.

This is the first work aimed at developing an appropriate methodology to study the use of organic soil amendments for plants such as hollyhock. The application of organic amendments also efficiently improved the uptake of N, P and K from the soil under water stress. Drought stress negatively affected plant traits, while soil amendments in planting beds improved plant performance and promoted plant resistance to drought stress. Based on the results of this study, soil with cow manure is suggested as an efficient soil amendment that is statistically better than rice husks and wheat straw for minimizing water requirements and improving plant tolerance to drought in a potted hollyhock. Due to the availability of cow manure compared to other substrates, the use of this material under potting soil conditions is recommended as these amounts cannot be used in the field. Further studies should be conducted on different methods to optimize the use of organic additives in the field as much as possible.

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Genetic and ampelographic characterization of grapevine accessions maintained in the Lebanese national collection

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Key words: Genetic diversity, ISSR markers, Lebanon, local germplasm, *Vitis vinefera* L.

Abstract: Safeguarding grapevine biodiversity is one of the main concerns in viticulture today. Management of *ex situ* collections requires a comprehensive characterization of the conserved germplasm to provide basic material for selection and breeding programs. In this study, the local grapevine germplasm conserved at the national collection of the Lebanese Agricultural Research Institute and composed of 43 accessions,was submitted to a genetic and ampelographic study. Nine ISSR primers, previously developed in grapevine, were used in this study. These primers generated a total of 51 bands, of which 77.7% were polymorphic allowing the differentiation of 41 genetic profiles *vs.* one case of synonymy that was recorded among three accessions. Ampelographic characterization was conducted using a set of 33 descriptors established by the International Office of Vine and Wine and related to leaf, bunch and berry. Principal component analysis identified 12 descriptors i.e. veraison date, maturity date, berry length, upper and lower vein pigmentation, bunch density, bunch weight, sugar content at harvesting, flesh of juiciness, berry weight, flesh firmness and color skin, as being the most discriminating descriptors. The correlation between the ISSR clustering and the ampelographic one was not significant (r=0.26) because of the divergence of accessions groups, except for the three accessions synonymy case which was confirmed in both dendrograms. Finally, this comprehensive evaluation of the existing local gene pool of grapevine revealed a substantial diversity. It would further allow the promotion of the valuable accessions directly through multiplication scheme, and their sustainable utilization in genetic improvement programs.

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1. Introduction

One of the main concerns for public research in viticulture today is the

need to safeguard grapevine biodiversity by establishing well managed national repositories to ensure germplasm availability to breeders, researchers and farmers (Nass *et al*., 2012). There are presently around 130 grapevine germplasm collections across the word (Dettweiler *et al*., 2000). One of the first grapevine germplasm collections is the one established in Spain in 1950, which contains more than 1066 accessions (Ortiz *et al*., 2004). In France, more than 2,200 varieties originally collected from 35 countries are grouped together at the National Institute of Agronomic Research (Tessier *et al*., 1999). Similar collections of grapevine were also established USA and in Latin America containing grapevine accessions collected all over the world (Martinez *et al.*, 2003). Grapevine is one of the oldest fruit crops growing in the Eastern Mediterranean region including Lebanon (Zohary, 2003). The country was among the first worldwide to have implemented vineyards which gradually became a traditional culture in Lebanon for the production of both table grapes and wine (Chalak *et al*., 2016). Today viticulture occupies the eighth rank in the agricultural sector in the country, with a total production area of 9,240 hectares and an annual production of about 89,000 tons of table grapes (FA0, 2010; FAOSTAT, 2019) *vs*. 3,000 hectares dedicated to wine grape with an annual production of approximately 10,000 tones (Rahal, 2015). In addition, about 800 hectares of vineyards are intended for the production of Lebanese Arak (Roby, 2003). Commercial plantations of table grapes have long been constituted of four local varieties, commonly named 'Tfayfihi', 'Baitamouni', 'Maghdouche' and 'Obeidy'. More recently, the new plantations in the country are mostly constituted of improved varieties imported from Europe and the United States. Around 77 varieties are imported to Lebanon. Nevertheless, the major share went mostly to 'Cabernet Sauvignon Blanc', 'Syrah', 'Viognier', 'Chardonnay' and 'Merlot' which represent 65% of the total saplings quantities imported during 2012-2014 (Tabaja, 2015). The long history of viticulture in Lebanon suggests the existence of a large indigenous germplasm associated to a wide range of traditional varieties that are well adapted to the various agro-climatic conditions of the country (Chalak *et al*., 2016). In Lebanon and despite the increasing interest on the conservation and characterization of plant genetic resources in general, only few studies have addressed the local germplasm of grapevine, although it is threatened by various anthropogenic pressures including in particular the progressive replacement of local varieties by more advantageous improved foreign varieties that are regularly imported into the country (Riachi, 1998; Madi, 2007; Chalak *et al.*, 2016; Rahme, 2016). To face the threat of genetic erosion and avoid the loss of traditional germplasm, the Lebanese Agricultural Research Institute (LARI, Tal Amara station) established in 1998 a national grapevine collection containing numerous local accessions collected from different production areas across the country (Madi, 2007). Within the environmental change and the challenging predictions for the Eastern Mediterranean region including Lebanon (Santillán *et al*., 2019), there is a growing need to address the local genetic resources such as traditional varieties which are recognized to be more adapted to harsh conditions and having better tolerance to various biotic and abiotic stresses (FAO, 2015). Added to this, the wine industry is witnessing a new trend towards the utilization of the local traditional varieties in the production of typical and well prized wines. Therefore clonal selection, with the respect to specific traits, has become the most important way to improve the quality of grape cultivars.

As a consequence, there is a need for reliable and precise methods of clonal characterization for further use by breeders, nurseries and industries (Galet, 1998; Moreno *et al*., 1998). Ampelography is a scientific methodology that has long been the single method used for the characterization of grapevine phenotype, based on the description of different morphological, phenological and pomological characters (Galet, 1998; Sabir *et al*., 2009; Laucou *et al.*, 2011). In addition to the ampelograhic description, in order to discriminate the varieties, synonyms, homonyms and the variation among the accessions, molecular methods has become more frequently used especially markers based on Polymerase chain reaction techniques (Riaz *et al*., 2012; Madhumati, 2014). Among the molecular approaches, the ISSR (Inter Sequence Simple Repeat) has being evaluated for its usefulness in grapevine cultivar identification and in assessing genetic diversity of grapevine germplasms (Moreno *et al*., 1998; Dhanorkar *et al*., 2005; Santiago *et al*., 2005; This *et al*., 2006; Sabir *et al*., 2009; Seyedimoradi *et al*., 2012; Choudhary *et al*., 2014; Castro *et al*., 2016).

This study aimed to characterize the 43 local grape accessions preserved at the Lebanese National Collection of Grapevine at LARI using both ISSR markers and ampelographic descriptors. This work was conducted in an attempt to evaluate the diversity of the local germplasm for further actions of conserva-

tion and sustainable utilization of grapevine genetic resources in diversification strategies.

2. Materials and Methods

Plant material

A total of 43 accessions of traditional grapevine varieties growing in the Lebanese national collection located in Tal Amara, Bekaa (N 33° 49' 59", E 36° 0' 0", 908 m a.s.l.) established in 1998, were considered in this study (Table 1). Accessions initially consisted of farmer's local varieties surveyed and collected across the Bekaa region (Lebanon). The vine cultivars were grafted with four duplications on B41 rootstocks which were planted directly in the collection land a year before the grafting process under the same growing conditions using horizontal trellis training system with the spaces 2.75 x 2.75 m. The site of plantation is characterized by a clay fertile soil and an average of precipitation ranging between 600 to 700 mm/year. For each accession, young leaves were sampled from a single selected plant and stored at -20°C for the DNA extraction and molecular analysis. On the other hand, samples of ten leaves, five clusters and 20 fully mature berries were taken from the same selected plant of each accession between August and October 2020 to undergo the ampelographic characterization.

Molecular characterization

Total genomic DNA was extracted from 300 mg of young leaf tissue after been crushed to a fine powder with liquid nitrogen and stored at -20°C until use. DNA extraction of grapevine landraces was performed according to (Doyle and Doyle, 1990) protocol with minor modifications (Kafkas *et al.,* 2006). DNA concentration was measured by Biospec-nano (Shimadzu corp) and stored at -20 C until further analysis. Nine ISSR primers: UBC807, UBC812, UBC815, UBC816, UBC818, UBC828, UBC864, UBC868 and UBC880 (Table 2) were selected and

Table 1 - Sites of 43 collected samples of traditional cultivars grapevine growing in the national germplasm collection at the Lebanese Agricultural Research Institute (LARI-Tal Amara)

Cultivars growing in the national germplasm collection at LARI	Sites of collected samples	Region
Ainouni	Tamnin	Bekaa Central
Al Mir, Ari, Ashlamish Ahmar, Asouad, Bakhouri, Bourji, Bzaz Al-Anzi, Maksasi, Mariami, Moukh Al-Baghel, Oubaidi, Souri	Nabi Ayla	Bekaa Central
Amarcani Abiad, Amarcani Asouad, Baitamouni, Baitamouni Asouad, Gbaii, Maghdoushi, Nih, Tfaifihi, Zaghzaghani	Niha	Bekaa Central
Amarcani Souri	Ablah	Bekaa Central
Arasani, Souri Zahlaoui	Saghbin	Bekaa Ouest
Ashlamish Abiad, Rins	Rayak	Bekaa Central
Houzairani, Khoudri, Zaitouni	El-Rafid	Bekaa Ouest
Karn Al Ghazal, Maghdoushi Mgaddad, Misky, NABI, Raha, Samaani Abiad, Samani Asouad, Souri Asouad, Souri Mhayyar, Yafaoui, Asali, Boulbouli Asouad, Gnoubi	Unknown Site	Bekaa

Table 2 - The resulting discriminating power, number of polymorphic and monomorphic, the size of bands of ISSR primers, percentage of polymorphism and the polymorphic information content

Primer	Size range (bp)	Number amplified bands	Number of polymorphic bands	Number of monomorphic bands	Polymorphism (%)	PD	PIC
UBC812	350-1000	7	6	1	85.7	0.64	0.33
UBC818	300-700	7	6	1	85.7	0.87	0.59
UBC815	400-650	4	3	1	75	0.62	0.6
UBC828	550-800	3	2	1	66.6	0.44	0.45
UBC864	280-700	7	6	1	85.7	0.9	0.48
UBC868	225-750	4	2	2	50	0.3	0.27
UBC816	300-900	5	4	1	80	0.64	0.64
UBC807	250-800	7	6	1	85.7	0.84	0.47
UBC880	125-600	7	6	1	85.7	0.85	0.58
Total	125-1000	51	41	10	-----	-----	-----
Mean		5.6	4.5	1.1	77.7	0.67	0.49

used in this study based on their good results for amplification and high power of discrimination on grapevine (Seyedimoradi *et al*., 2012).

The ISSR (Inter Simple Sequence Repeat) amplification was carried out as per (Sabir *et al.*, 2009), using 20 µl reaction mixture containing 2 µl of 10 X PCR buffer (750 mM Tris-HCl pH 8.8; 0.1% Tween-20), 0.2 mM dNTP, 2 mM $MgCl_2$; 200 mM Primer; 50 ng genomic DNA and 1 U Taq DNA polymerase (MBI Fermentas Inc, Hanover, MD-21076, USA). The amplification program consisted of 94°C for 4 minutes followed by 35 cycles of 94°C for 1minute, 52°C for 1.05 minutes, 72°C for 2 minutes and a final extension of 8 minutes at 72°C. The amplified DNA was visualized on the 3% agarose gel.

Ampelographic characterization

Thirty-three major morphological descriptors selected from the descriptors list previously developed by the OIV (Organisation International de la Vigne et du Vin) (OIV, 2017) were used in this study (Tables 3, 4, 5). They include 17 qualitative descrip-

Table 3 - Descriptive statistics analysis of quantitative morphological descriptors of 43 grapevine accessions

Descriptors	Mean	Minimum	Maximum	Least significant difference (LSD)
Leaf length (cm)	9.77	7.84	11.26	1.06
Leaf width (cm)	13.29	11.4	15.74	1.35
Petiole (cm)	7.92	4.24	11.12	0.99
Number of lobes	4.89	3.00	5.00	0.09
Bunch weight (g)	576	203	1186	121
Bunch length (cm)	21.76	14.93	30.6	3.60
Bunch width (cm)	13.62	8.15	21.23	2.33
Berry weight (g)	5.7	0.94	9.95	0.47
Berry length (cm)	2.24	1.50	3.40	0.12
Berry width (cm)	1.75	1.16	2.54	0.08
Pedicel length (cm)	1.01	0.49	1.53	0.11
Seed number	2.51	1.00	4.00	0.16
Acidity at veraison (g/L)	2.82	1.35	6.10	0.89
Brix at veraison (◦Brix)	15.67	11.2	18.00	1.77
Acidity at harvesting (g/L)	1.98	1.2	3.10	0.43
Brix at harvesting (◦Brix)	18.38	13.6	23.00	2.13

Table 4 - Leaf descriptors notation and their frequency distribution among the 43 studied grapevine accessions

Descriptors -Leaf	Notation and frequency (Number of accessions)
Shape of blade	Wedge-shaped (31) - pentagonal (3) - circular (9)
Shape of teeth	Both side straight (3) - both side convex (1) - mixture between both side straight and both side convex (39)
Anthocyanin coloration of main veins on upper side	Absent (25) - only on the petiolar point (10) - up to the 1st bifurcation (5) - up to the 2nd bifurcation (2) - beyond the 2nd bifurcation (1)
Anthocyanin coloration of main veins on lower side	Absent (16) - only on the petiolar point (17) - up to the 1st bifurcation (5) - up to the 2nd bifurcation (1) - beyond the 2nd bifurcation (4)
Prostrate hairs on main veins on lower side	None or very low (21) - low (11) - medium (7) - high (4)
Prostrate hairs on main veins on upper side	Absent (40) - present (3)
Density of prostrate hairs on petiole	None or very low (38) - low (1) - medium (2) - high (2)
Opening of petiole sinus	Wide open (13) - open (26) - closed (3) - overlapping (1)
Leaf Length	Short (13) - medium (30)
Leaf width	Narrow (10) - medium (27) - wide (6)
Petiole length	Short (8) - medium (25) - long (10)
Number of lobes	Three lobes (1) - five lobes (42)

tors and 16 quantitative descriptors related to the leaf (12 descriptors), bunch (5 descriptors) and berry (16 descriptors).

Data analysis

To assess the information given by ISSR markers, the following parameters were calculated as follow: number of alleles per locus, power of discrimination (PD= 1 -Σ gi2, where gi is the frequency of the i_{th} genotype), polymorphism information content (PIC= 1-∑ $(Pi)^2$, where Pi is the proportion of samples carrying the i_{th} allele of a particular locus) (Botstein *et al.*, 1980). Genetic distances were calculated according to Jaccard. Trees clustering the data with the unweighted pair-group method (UPGMA) with SAHN-clustering and tree programs of PAST software (Kriege *et al*., 2014).

Qualitative characteristics have been described and scored. For quantitative descriptors, the mean was calculated. To assess the degree of similarity between the units tested and understand the relationships between them, the data were subjected to a principle component analysis (PCA) in order to condense the quantitative and qualitative descriptors in a small number of synthetic components (Saporta, 1990). Thus, the degree of contribution of each of the characters to the total variation was calculated in order to indicate the most relevant characters.

Hierarchical Cluster Analyses was executed using Euclidean distance to classify cultivars into different groups based on morphological evaluation. LSD test (SAS Institute Inc, 1995) was done in purpose to compare means of quantitative characters between different accessions. The correlation between molecular and ampelographic clustering was studied by performing a Mantel test using past program.

3. Results

ISSR markers analysis

The nine ISSR markers (UBC807, UBC812, UBC815,

Table 5 - Bunch and berry descriptors notation and their frequency distribution among the 43 studied grapevine accessions

Descriptors	Notation and frequency (Number of accessions)
Bunch descriptors	
Bunch shape	Cylindrical (21) - conical (15) - funnel shaped (7)
Bunch density	Loose (5) -medium (25) - dense (13)
Bunch weight	Very low (4) - low (13) - medium (13) - high (8) - very high (5)
Bunch length	Short (3) - medium (6) - long (26) - very long (8)
Bunch width	Narrow (13) - medium (21) - wide (8) - very wide (1)
Berry descriptors	
Berry shape	Obloid (2) - globose (9) - broad ellipsoid (11) - narrow ellipsoid (6) - cylindric (1) - obtuse ovoid (7) - ovoid (3) - obovoid (3) - horn shaped (1)
Color of skin	Green yellow (27) - rose (2) - red (1) - dark red violet (8) - bleu black (5)
Thickness of skin	Very thin (2) - thin (17) - medium (17) - thick (5) - very thick (2)
Firmness of flesh	Soft (6) - slightly firm (27) - firm (10)
Juiciness of flesh	Slightly juicy (7) - medium juicy (27) - very juicy (9)
Veraison time	Very early (8) - early (3) - medium (2) - late (24) - very late (6)
Veraison acidity (TA)	Low (5) - medium (32) - high (6)
Veraison brix	Low (6) - medium (27) - high (10)
Harvesting time	Very early (3) - early (4) - medium (7) - late (20) - very late (9)
Harvesting acidity (TA)	Low (6) - medium (30) - high (7)
Harvesting brix	Low (6) - medium (31) - high (6)
Berry weight	Very low (4) - low (6) - medium (26) - high (5) - very high (2)
Berry length	Short (7) - medium (16) - long (15) - very long (5)
Berry width	Very narrow (1) - narrow (21) - medium (20) - wide (1)
Length of pedicel	Very short (1) - short (3) - medium (5) - long (7) - very long (9)
Seed number	One (2) - two (24) - three (11) - four (6)

UBC816, UBC818, UBC828, UBC864, UBC868 and UBC880) showed distinct polymorphism among the 43 different grapevine accessions of this study (Table 2). A total of 41 polymorphic bands were detected. The size of amplified products ranged from 125 bp to 1000 bp. The calculated discriminating power (PD) was between 0.3 (UBC868) and 0.9 (UBC864), indicating a high diversity of the loci and confirming the efficiency of these primers in studying the polymorphism of the Lebanese grapevine germplasm. The polymorphic information content (PIC) value varied between 0.27 (i.e. UBC868) and 0.64 (i.e. UBC816) with an average of 0.49. Number of polymorphic bands varied between two (e.g. UBC868) to six (e.g. UBC812). Eight primers had one monomorphic band, while UBC868 generated two monomorphic bands.

Genetic clustering of the accessions

The allelic diversity data was used to produce a dendrogram by using distance matrix-UPGMA, thus revealing the genetic relationship among grapevine accessions (Fig. 1). The dendogram constructed on the base of the amplification product of ISSR primers of the different accessions showed 41 different molecular patterns. Tfaifihi variety indicated a unique marker with the primer UBC812 (305 bp). One case of synonymy was observed between Gbaii, Arasani and Gnoubi cultivars and they are very close for the most discriminating ampelographic traits. The ISSR analysis showed four distinct groups: G1, G2, G3 and G4 at the distance of 0.67 of similarity including 6, 26, 2, and 9 cultivars respectively.

The first group G1 consists of six accessions 'Baitmouni Asouad', 'Zaitouni, 'Asouad', 'Ainouni', 'Moukh Al-Baghel' and 'Karn Al Ghazal' and have a nine alleles in common with six primers (UBC807, UBC812, UBC816, UBC864, UBC868 and UBC880). The Largest group (G2) gathers 26 grapevine accessions, shared only five alleles in common with UBC807, UBC812 and UBC818. This group can be divided into 4 sub-groups: G2.1 contains six accessions, 'Nabi', 'Misky', 'Asali', 'Bourji', 'Oubaidi' and 'Baitamouni'. G2.2 comprises 11 accessions, with two cases of close similarity detected between 'Souri Zahlaoui' and 'Khoudri', and between 'Yafaoui' and 'Souri Mhayyar', at 0.94 similarity levels. Only one case of synonymy was observed between three different accessions initially collected from the south of Lebanon under different vernacular names and presenting the same genetic profile: 'Gnoubi', 'Gbaii', 'Arasani'. G2.3 consists of four accessions, with a close similarity detected between 'Bzaz Al-Anzi' and 'Amarcani Souri' at a similiraty level of 0.96. G2.4 includes five accessions; 'Bakhouri', 'Amarcani Abiad', 'Maghdoushi Mgaddad', 'Maksasi' and 'Ashalamish Abiad'. The third group (G3) consists only of two accessions: 'Raha' and 'Tfaifihi' had 20 alleles in common with all the nine ISSR markers. The fourth group (G4) consists of nine accessions: 'Souri Asouad', 'Boulbouli Asouad', 'Houzairani', 'Amarcani Asouad', 'Mariami', 'Ari', 'Zaghzaghani', 'Samani Asouad' and 'Samaani Abiad', shared only two alleles in common with UBC815 and UBC864 primers.

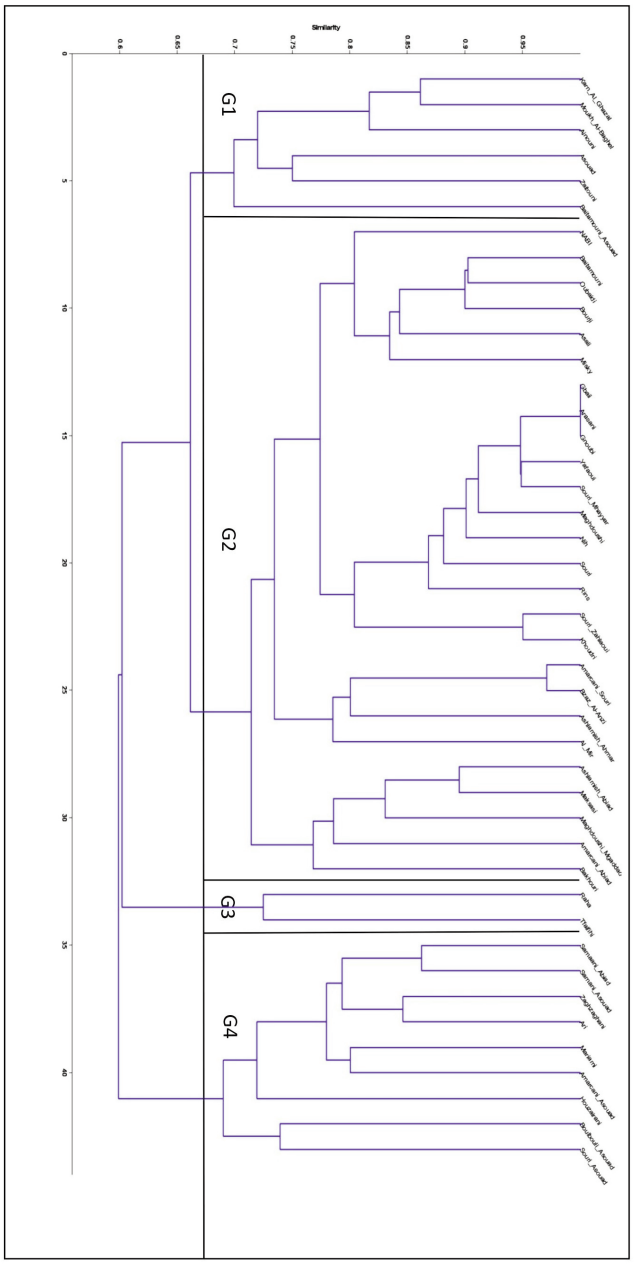

Fig. 1 - Hierarchical clustering analysis of the 43 Lebanese grapevine cultivars, based on 9 ISSR primers using Jaccard distance and UPGMA.

Ampelographic description

A total of 43 cultivars were studied. Locale names were commonly given by farmers or nurseries based on berry colour or on the country of origine (Asouad, Souri Aswad, Amarcani Abiad) and maturity date (Houzairani). For each cultivar, a descriptive list was established with 33 morphological traits related to the leaf, bunch and berry (Tables 3, 4, 5).

Leaf description. The majority of accessions have five lobes except 'Souri Zahlaoui' accession which only have three lobes. Thirty-one accessions presented a wedge-shaped leaf form (e.g. 'Ari', 'Bourji', 'Gbaii'), while only nine accessions had circular leaf (e.g. 'Raha', 'Bakhouri'), and the rest had a pentagonal leaf form (e.g. 'Arasani', 'Asali', 'Souri Mhayyar') (Table 4). Most of the accessions shared same teeth form with both sides straight and both sides convex except for the 'Oubaidi' that possesses a both side convex teeth form, and three accessions ('Boulbouli Asouad', 'Nabi' and 'Samani Asouad') which had a both side straight teeth form (Table 4, Fig. 2).

Anthocyanin coloration of main veins on both the upper and the lower side of blade varied from absent (e.g. 'Al Mir'), limited to the petiolar point (e.g. 'Arasani'), extended to the first bifurcation (e.g. 'Khodre'), and beyond the second burifaction (e.g. 'Zaghzaghani').

The opening of the petiole sinus varied from overlapping sinus (e.g. 'Zaitouni'), closed (e.g. 'Souri Asouad'), open (e.g. 'Misky', 'Rins'), to wide open sinus (e.g. 'Gbaii', 'Souri Zahlaoui').

The density of prostrate hairs between main veins on both sides of the blade and on the petiole varied widely from absent (e.g. Ainouni), low (e.g. 'Bakhouri', 'Zaghzaghani'), medium (e.g. 'Zaitouni', 'Ari'), and high (e.g. 'Tfaifihi', 'Al Mir'). Only three accessions present prostrate hairs on the upper side of the main blade veins ('Rins', 'Souri Mhayyar', and 'Tfaifihi').

Leaves generally presented an average length between 7.84 (i.e. 'Souri Zahlaoui') and 11.26 cm (i.e. Ari*)* with LSD 1.06 and width between 11.4 (i.e. 'Rins') and 15.74 cm (i.e. 'Ashlamish Ahmar'). Petiole length average was between 4.24 (i.e. 'Bzaz Al-Aanzi') and 11.12 cm (i.e. 'Baitamouni Asouad') (Table 3).

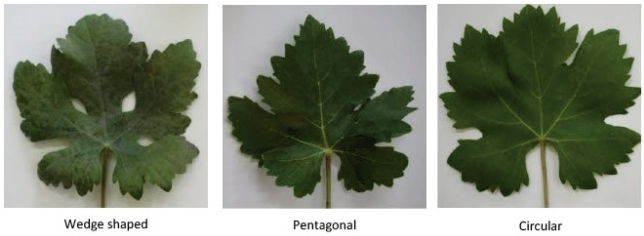

Fig. 2 - Example of variability of leaf shape.

Bunch description. Bunch characteristics investigated showed a great diversity among the accessions studied. Almost 48.8% of the accessions had cylindrical bunch (e.g. 'Ainouni', 'Houzairani'), 34.9% were conical (e.g. 'Boulbouli Asouad', 'Maksasi') and 16.3% of the accessions had a funnel shaped cluster (e.g. 'Arasani, Raha') (Table 5). Both 'Samani Asouad' and 'Samaani Abiad' had the heaviest bunch weight, with an average of 1150 g approximately. For the remaining accessions, bunch weight ranged from 1090 g (i.e. 'Rins') to 203 g (i.e. 'Amarcani Abiad'). Most of the accessions had a bunch length ranging between 14.93 (i.e. 'Maghdoushi Mgaddad') and 30.6 cm (i.e. 'Rins') with 3.6 of LSD. Bunch width ranged between a minimum of 8.15 cm (i.e. 'Bourji') and a maximum of 21.23 cm (i.e. 'Samaani Abiad') (Table 3, Fig. 3).

Fig. 3 - Example of variability of bunch shape.

Berry description. Berry's external appearance presented an important diversity among accessions (Table 5). For the shape, 11 accessions had a broad ellipsoid berry shape (e.g. 'Ainouni', 'Arasani', 'Khoudri'), nine accessions had a globose berry form (e.g. 'Ari', 'Tfaifihi'), and the remaining accessions had obvoid (e.g. 'Al Mir', 'Souri Mhayyar'), narrow ellipsoid (e.g. 'Gbaii', 'Souri Asouad'), obloid (e.g. 'Asali', 'Samani Asouad'), ovoid (e.g. 'Misky', 'Ashlamish Ahmar', 'Yafaoui'), obtuse ovoid (e.g. 'Maghdoushi', 'Asouad', 'Souri Zahlaoui', 'Zaitouni'), cylindical (e.g. 'Bzaz Al-Anzi') and horn berry shaped ('Karn Al-Ghazal'). As to skin color, most of the accessions had green yellow berries (e.g. 'Mekssese', 'Yafawi', 'Karn Al Ghazal'). While 'Houzairani' diverged from others with its red berries, eight accessions had a dark red violet berry color and five other accessions shared the bleu black color. Only the rose color was found in 'Ashlamish Ahmar' and 'Tfaifihi' accessions.

A wide range of variability was also found for the berry quantitative descriptors (Table 3). 'Asouad' and 'Asali' accessions were outstanding with their significantly heavier berries (9.95 g and 9.69 g respectively) while 'Ashlamish Abiad' had only 0.94 g, as berry average weight. For the rest of the accessions, berry weight ranged from 8.3 g in 'Baitamouni Asouad' to 1.71 g in 'Amarcani Abiad'. On the other hand, 'Karn Al Ghazal' accession was distinguished with its long berries (3.4 cm) and its long pedicel (1.53 cm) while 'Asali' accession had the largest berries (2.54 cm).

Principal component analysis

The first three components presented 31.9% of the total variation of the different descriptors (Table 6). The first component consisted of 12.2% of the total variation and included berry skin color, firmness of flesh, bunch weight, harvesting and veraison times, in addition to the brix level at harvesting. Thus, the first component was dominated by the fruit characteristics more than the leaf ones. The second component represented 10.9% of the total variation and included four variables namely berry weight and length, leaf lower vein pigmentation and lower face pilosity. The third component was characterized by a percentage of variation of 8.8% and was dominated by the flesh juiciness and the bunch density. Based on this validation through PCA, this shortlist of discriminating descriptors may be further considered to study the relationships between the grapevine accessions.

Ampelographic accession clustering

The dendogram illustrating the relationship among the 43 studied grapevine accessions was constructed on the 12 most discriminating descriptors as validated by PCA. Four groups were differentiated at the distance -17 of similarity using Euclidean distance (Fig. 4). The first group (G1) consisted of eight accessions of different skin color but sharing same veraison date and the same sugar content at harvesting time. The second and the largest group (G2) consisted of 13 accessions, sharing all the same bunch characteristics in terms of density, juiciness in addition to the common sugar content at harvesting time. Moreover, these accessions started the stage of veraison almost at the same date around the 5th of September. The third group (G3) contained 12 accessions, characterized by medium green berries. A case of close similarity was found between three accessions of different vernacular names 'Gnoubi', 'Gbaii' and 'Arasani'. The fourth group (G4) clustered 10 accessions of which eight accessions with dark red violet (e.g. 'Asali') to bleu black (e.g. 'Mariami') berries, and two accessions with green yellow berries ('Rins' and 'Samaani Abiad'). All these accessions have common leaf characteristics and intermediate sugar content at maturity time.

Table 6 - The first three components of the principal component analysis involving the 33 ampelographic traits and performed for the 43 accessions of the national grapevine germplasm collection

Variable	Factor 1	Factor 2	Factor 3
Leaf form	-0.14042	0.423101	-0.00785
Number of lobes	-0.07649	-0.08669	0.136368
Teeth form	-0.15343	-0.29052	0.301802
Upper vein pigmentation	-0.14288	-0.25638	-0.45432
Lower vein pigmentation	0.069659	**-0.63447**	-0.19775
Lower face pilosity	-0.03692	**0.627619**	-0.00083
Upper face pilosity	0.17382	0.179676	0.009038
Petiole pilosity	0.27927	-0.3332	0.328737
Overlapping of petiole	-0.00076	0.475518	0.226222
Berry shape	-0.0347	-0.00661	0.439309
Color skin	**0.511841**	-0.13872	-0.09665
Skin thickness	0.013082	0.133863	0.46865
Firmness of flesh	**0.519899**	0.152408	0.095108
Juiciness of flesh	-0.27819	0.150394	**-0.55995**
Seeds number	-0.01707	-0.0642	-0.44797
Bunch form	-0.14446	0.507152	-0.03091
Bunch density	0.21315	0.362343	**-0.6217**
Bunch weight	**0.598172**	0.272448	-0.44281
Bunch length	0.344545	0.046891	-0.35337
Bunch width	0.459819	0.390514	-0.32888
Leaf length	0.306676	0.272602	0.398343
Leaf width	0.41474	0.04784	0.18026
Petiole length	0.436157	0.049669	0.154936
Pedicel length	0.269837	-0.46508	0.060371
Berry weight	0.437611	**-0.53381**	-0.29825
Berry length	0.253778	**-0.65709**	0.106556
Berry width	-0.13315	-0.30376	-0.35394
Veraison time	**0.789894**	0.04527	0.01873
Harvesting time	**0.667212**	0.098136	-0.22776
Acidity at veraison	-0.42158	-0.18862	-0.28567
Brix at veraison	-0.49511	0.304928	-0.05688
Brix at harvesting	**-0.57237**	0.156018	-0.1208
Acidity harvesting	-0.44026	-0.21603	-0.20417
Percentage total variation	12.2%	10.9%	8.8%

The characters in bold are discriminant.

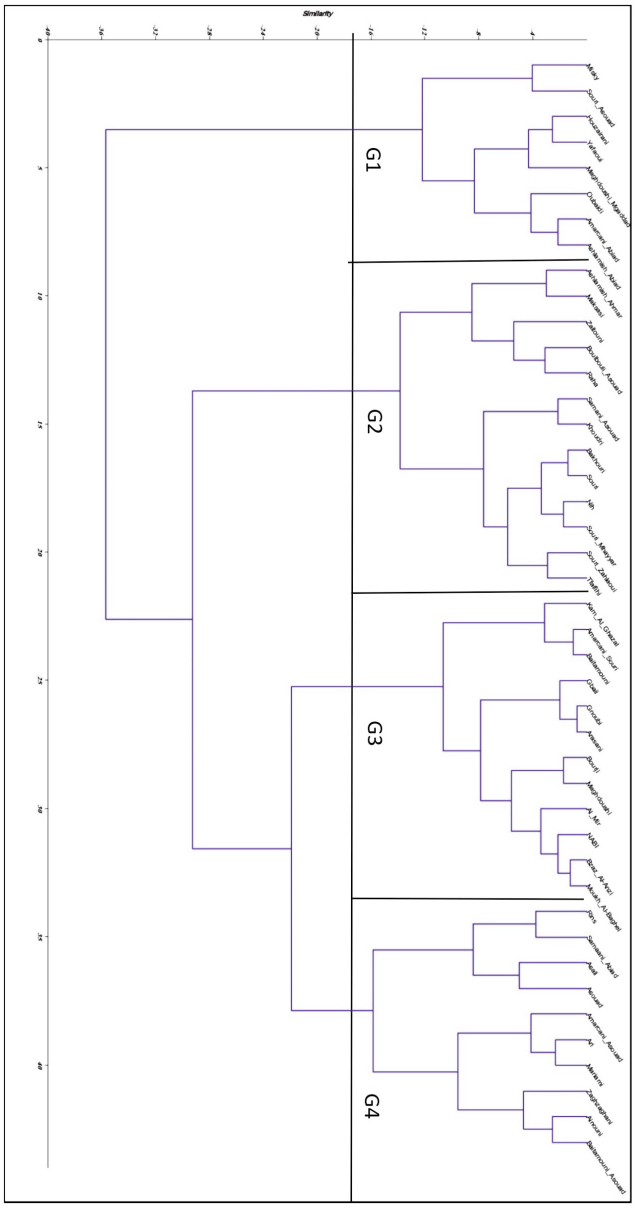

Fig. 4 - Hierarchical clustering analysis of the 43 Lebanese grapevine cultivars, based on the most 12 discriminant morphological traits, using Euclidean distance and Ward method.

4. Discussion and Conclusions

In our study encompassing 43 local accessions, 51 bands were generated from nine primers, out of which 41 were polymorphic. Similarly, 55 polymorphic bands were obtained in Palestine by Basheer-Salimia, (2015) in studying 36 grape accessions using 17 ISSR markers; while 69 polymorphic bands were obtained by Seyedimoradi *et al.* (2012) in studying 21 local Iranian grapevine cultivars using 10 ISSR primers. Our results related to polymorphism rate (50-85%) and size of amplified bands (125-1000bp) are close to the ones previously reported in Portugal (Castro *et al.,* 2016), Turkey (Sabir *et al.,* 2009), Egypt (Hassan *et al.,* 2011), Iran (Seyedimoradi *et al.,* 2012), and Palestine (Basheer-Salimia, 2015). Larger fragments (300-1500 bp) were generated by the same ISSR markers with values up to 1500 bp in India (Dhanorkar *et al.,* 2005) and 2500 bp in Turkey (Sabir *et al.,* 2009). Moreover, our results indicated a discrimination power value ranging from 0.3 (UBC868) and 0.9 (UBC864) and PIC value varying between 0.27 (i.e.UBC868) and 0.64 (i.e.UBC816) with an average of 0.49, which was similar to the results obtained in Iran by Seyedimoradi *et al.* (2012).

Our ISSR results revealed an important genetic diversity within the national collection of grapevine germplasm. About 41 different molecular profiles were clearly differentiated within the 43 accessions, with only one case of synonymy found between three accessions ('Arasani', 'Jbai' and 'Jnoubi'). These findings certainly confirm one more time the efficiency of these ISSR markers in investigating the genetic variability of grapevine. It also indicates the efficiency of the initial survey and collection of local accessions in establishing this national collection of grapevine germplasm with only 5% synonymy. In Spain, the molecular characterization of grapevine accessions of the national gene bank at Alcalá de Henares allowed the differentiation of 177 accessions (30%) over 621 initially collected (Ortiz *et al.,* 2004). Furthermore, the SSR analysis of the Eastern European cultivars led to the differentiation of only 659 unique profiles over 997 accessions studied (Maul *et al.,* 2015). In Italy, Cipriani *et al.* (2010) reported 200 groups of synonyms vs. 774 unique genotypes out of 1005 grapevine accessions studied. Also, Lopes *et al.* (1999), studying 49 supposed different cultivars from the Portuguese grapevine national collection of Terciera (Azores Island) through microsatellite markers, detected only 36 different profiles after determining the synonym cases.

Along with the genetic differentiation of the grapevine accessions, an ampelographic description was carried out for the 43 Lebanese accessions using 33 descriptors recommended by the OIV, and mostly related to various descriptors including yield components like bunch and berries dimensions and weight (Kara, 1990; Ortiz *et al.,* 2004; Santiago *et al.,* 2005; OIV, 2007; Akram *et al.,* 2019). Mature leaf descriptors did not vary significantly among the Lebanese accessions. Most of these accessions had leaves with

five lobes similarly to the results reported in other studies (Ecevit and Kelen, 1999; Chalak *et al.,* 2016). On the other hand, our results revealed a large variability of bunch shape, weight, density, as well as for the berry shape, color and size, similarly to the results reported in previous works in the Eastern Mediterranean (Sabir *et al.,* 2009; Biniari and Stavrakaki, 2016). On the other hand, the multivariate analysis was efficient to analyze large data generated in our study by qualitative and quantitative descriptors to further identify patterns and relationship among powerful statistical techniques.

PCA allowed to extract the most discriminating descriptors, e.g. veraison date, maturity date, berry length, upper vein pigmentation, lower vein pigmentation, bunch density, bunch weight, brix at harvesting, juiciness of flesh, berry weight and firmness of flesh and skin color. Our results were in accordance with those previously obtained by Riachi (1998), Madi (2007) and Chalak *et al*. (2016) confirming one more the stability of the discriminating descriptors over years. This also in accordance with Leao *et al.* (2010, 2011) who reported the stability of the discriminating characters for two consecutive years. Phenotypic clustering allowed differentiate the 43 accessions studied in four main groups. The synonymy case first revealed between the three accessions 'Gbaii', 'Gnoubi', and 'Arasani' by the ISSR clustering was also confirmed by using ampelographic. This is the minimum rate for verifying synonyms and for clone selection processes (Cervera *et al.,* 2002).

Furthermore, many cases of close phenotypic similarity, first revealed by Madi (2007), were also recorded in this study. It is worthy to note that some of our accessions were also described in other countries. This is the case of 'Bzaz Al Anzi' reported in Egypt by Hassan *et al.* (2011) and presenting almost similar ampelographic characteristics of the bunch and the berries.

When comparing the two dendograms generated apart by the molecular and ameplographic descriptors, the Mantel test indicated a weak correlation (r = 0.26, data not shown) between them. This reflects different structure for the accessions clustering whether it is generated by molecular or ampelographic descriptors. Exception is made to the case of synonymy between the three accessions 'Gnoubi', 'Gbaii', and 'Arasani', which was confirmed by both, ampelographic and molecular clustering. This discrepancy between the two clustering types was also reported in other studies in grapevine collections (Knezović *et al.,* 2017) and other fruit crops (Talebi *et al.,* 2008; Zdunic *et al.,* 2008).

On the other hand, considering some key ameplographic descriptors, it was possible to categorize the existing diversity of the collected Lebanese germplasm according to the phenological stage, and according to berry skin color. In five groups of maturity time; with 27 accessions had green yellow berries, only one accession with red berries, two accessions with rose berries, five accessions with bleu black berries and eight accessions with dark red violet berries. Surprisingly, similar ratios between green and red accessions were also reported in Canary Islands and Madeira by Marsal *et al.* (2019).

Additionally, most of the accessions studied had crunchy berries with thick skin and very low juice content. This indicates the potentiality of using these accessions for table grapes rather than wine grapes. Only three accessions had thin skin, juicy flesh and high sugar content and, therefore, may be tested for fermentation. Such descriptive ampelographic characterization of the berries is hopefully necessary for evaluating grapevine accessions conserved in field genebanks with respect to their usage as table grape or wine grape (Sabir *et al.,* 2009; Ates *et al.,* 2011; Basheer-Salimia, 2015).

This diversity upon the 43 grapevine accessions conserved at the Lebanese national collection of grapevine as revealed by ISSR markers and ampelographic descriptors indicate the efficiency of sampling/collecting strategy conducted in 1998 in the Bekaa and Chouf areas.

Nevertheless, additional grapevine varieties growing locally under different vernacular names, which do not appear in the national grapevine germplasm collection, were recently assessed in their growing site by (Chalak *et al.,* 2016). Therefore, it is strongly recommended to further extend the survey and mission collection to cover multiple grapevine production areas, particularly the North, the South and of Mount of Lebanon in order to enrich the national collection. Combining both molecular makers and ampelographic descriptors as it was shown in this study, would be very useful in optimizing the collection of accessions by clarifying the mislabeling cases, understanding the genetic distances among accessions, and setting up a descriptive assessment for these accessions. The comprehensive evaluation of the existing local gene pool of grapevine would further allow the sustainable utilization of the valuable accessions directly for multiplication in certified nurs-

eries and also in further breeding programs.

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Economic viability and development of radish (*Raphanus sativus* L.) under different soil water tensions and mulching types

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Abstract: There is a lack of information on the production of irrigated radish associated with the use of mulching and on the economic viability of these production technologies. The objective of this study was to evaluate the growth, yield, and economic viability of the radish crop under different soil water tensions (SWT) and mulching types. The experiment was conducted in a greenhouse. During the experiment, the following variables were evaluated: growth parameters, yield and economic viability. SWT at 7 kpa in the treatments without mulching and at 12 kPa in the treatments with black plastic and black nonwoven resulted in higher growth parameters and yield. The leaf area index and the root diameter were the parameters that had a high and positive correlation with yield. Expenses with variable resources represent on average 75% of the total production cost. Therefore, the investment pays all resources applied in the activity and provides an economic profit. In this context, the higher radish yield with 37.5 t ha^{-1} provided the highest profitability of the evaluated treatments, thus, for radish production, the recommendation is to use 12 kPa as an indicator of the moment for irrigation, associated with the use of black plastic.

1. Introduction

Radish crop (*Raphanus sativus L.)* is a vegetable belonging to the Brassicaceae family grown worldwide, consumed mainly in salads, cooked, and even pickled (Chihoub *et al.,* 2019). The radish has been gaining prominence among vegetables due to its rusticity and its short cycle (Kim *et al.,* 2014; Zhang *et al.,* 2021), ideal for small and medium producers. However, in Brazil, its production and consumption are still small, and it can be better exploited by horticulturists, creating a market niche. In Brazil, according to the agricultural census (2006), around 10,500 tons of

radishes were produced, generating revenue of R$ 9 million (CONAB, 2010). The South and Southeast regions are the regions with more radish production, with 4,587 and 4,456 tons, respectively. In general, radish productivity in Brazil is between 11 and 30 t ha^{-1}.

Despite its rusticity, the high yield and profitability of the radish crop will only be achieved under optimal conditions of soil moisture, air temperature, and fertilization (Gruver *et al.,* 2016). Therefore, adequate management techniques are necessary to achieve this optimal cultivation condition. Proper irrigation management is one of the ways to achieve maximum yield. The timing and amount of water applied are critical to the irrigation effectiveness. Excessive irrigation increases pumping costs, water waste, and crop disease susceptibly; nevertheless, deficit irrigation generally causes losses and reduces production quality (Contreras *et al.,* 2017). Soil water tension (SWT) is one of the ways to determine the timing of irrigation and the volume of water to be applied. SWT is a fundamental variable to describe the water availability in the soil and the capacity for that water to be used by plants (Meyer and Green, 1981). According to Masseroni *et al.* (2016), the local measurement of SWT is one of the most effective options for irrigation management.

In addition to adequate irrigation, mulching is also a technique used in the search for high yield. Soil mulch can decrease the evaporation rate, maintain the soil moisture, moderate the temperature, and form a barrier to weed growth, which can significantly affect yield and water consumption (An *et al.,* 2015; Gao *et al.,* 2019). According to Carmichael *et al.* (2012), mulching significantly increased the radish yield. However, the acquisition, installation, and maintenance of the irrigation and mulching require a high investment, which represent important additional costs to production.

Based on agricultural production costs, it is possible to evaluate the profitability and efficiency of the production system adopted by the rural producer and makes it possible to obtain information for decision-making on agricultural activities (Artuzo *et al.,* 2018). The production cost is grouped into fixed costs, variable costs, operating costs, and total cost. Economic analysis compares the production cost with the gross revenue obtained from the sale of the product produced. Therefore, the success of the enterprise is related not only to the production cost, but also to the final product price and, mainly, to crop yield (Schwerz *et al.,* 2017).

Radish yield can be influenced by numerous factors, including the plant's response to the production environment. Therefore, it is necessary to understand the crop traits that contribute to high yield and their interrelationships. Knowledge of the relationship between yield and crop growth variables obtained through correlation analysis helps in plant selection and develop high yielding varieties (Carmichael *et al*., 2012; Schwerz *et al.,* 2017). Correlation coefficient (r) measures the degree (intensity) and nature (direction) of association between characters (Abd El-Mohsen *et al.,* 2013).

Given the above, the present study suggests that the combined use of water tension in the ideal soil with mulching can increase development, yield, and profitability. Besides, the gain in profitability with the use of these techniques can exceed the costs of implementation and generate high profits. Therefore, the aims of this study were to evaluate the growth variables and economic viability of radish production under different water tensions in the soil associated with types of mulching, under unheated plastic greenhouse. Also, to determine the relationship between morphological variables and the radish yield to help radish producers how to determine what growth parameters could be efficiently used to raises yield.

2. Materials and Methods

Experimental site characteristics and cultural practices

The experiment was conducted during September and October of 2018, in greenhouses covered by low-density polyethylene, at the experiment area of the Department of Water Resources and Sanitation of the Federal University of Lavras (UFLA). The experiment area is located in the southern region of the state of Minas Gerais (21°14' S, 45°00'W and 918,84m altitude). The climate in the region is classified as Cwa, according to the methodology proposed by Köppen (Dantas *et al.,* 2007).

Soil characteristics of the study area

The soil used in this study was classified as a distroferric red latosol (oxisol) with a clayey texture according (Santos *et al.,* 2006). The chemical characteristics of the soil were: pH = 7.0; K = 106.7 mg dm^{-3}; P = 0.7 mg dm^{-3}; Ca = 3.53 $cmol_c$ dm^{-3}; Mg = 0.39

$cmol_c\ dm^{-3}$; Al = 0.04 $cmol_c\ dm^{-3}$; H+Al = 1.54 cmol dm^{-3}; M.O.= 2.11 dag/kg e V = 73.12. It was applied in the planting fertilization 1500 kg ha^{-1} de P_2O_5 in the form of single superphosphate, 145 kg ha^{-1} of urea (N), 320 kg ha^{-1} of potassium chloride (K), 620 kg ha^{-1} of limestone and 12,5 kg ha^{-1} of commercial product Solubor (17.5% boron - B).

Irrigation management and experimental design

The drip irrigation system was used, with self-compensating emitters, spaced 0.3 m apart, and operating with a discharge of 4.3 L h^{-1}. The SWT was determined utilizing tensiometers at two depths (0.15m and 0.25m) in each treatment. The soil water-retention curve was adjusted according to the model proposed by Van Genuchten (1980). The soil moisture of 0.453 $L^3\ L^{-3}$ (5 kPa) was the corresponding to the field capacity (θcc), according to the model proposed by Mello *et al.* (2002).

$$\Theta = 0.235 + \frac{(0.614 - 0.235)}{[1+(0.269 \times \frac{1}{2}\Psi m\frac{1}{2}^{2.064}]^{0.515}} \qquad R^2 = 0.93$$

Where: θ is the soil moisture content ($cm^3\ cm^{-3}$) and Ψm is the soil water tension (kPa). The functioning of the irrigation system was calculated based on the gross water depth, according to (Pizarro Cabello, 1996), considering a 0.2 m effective root. A 90% water-application efficiency of the irrigation system was adopted and a water distribution-uniformity coefficient (DUC) of 98% was obtained.

The experimental design was randomized complete in a 4 x 3 factorial, replicated four times, totaling 12 treatments. The four SWT used were 7, 12, 20, and 50 kPa. The mulching materials used were black polyethylene film (black plastic); black polypropylene (black non-woven film); and no mulch (control) (Table 1).

Table 1 - Treatments of soil water tension and mulching in the radish crop

Treatment code	SWT (kPa)	Mulch material
NM7	7	Control (no mulch)
BP7	7	Black Plastic
BNW7	7	Black non-woven film
NM12	12	Control (no mulch)
BP12	12	Black Plastic
BNW12	12	Black non-woven film
NM20	20	Control (no mulch)
BP20	20	Black Plastic
BNW20	20	Black non-woven film
NM50	50	Control (no mulch)
BP50	50	Black Plastic
BNW50	50	Black non-woven film

Experimental plots, data collection and analysis

The transplant of Comet Radish seedlings occurred 6 days after the planting of the seeds in the experimental plots prepared, fertilized, and with mulching fixed. The plots were kept with moisture close to the field capacity (θcc) for 3 days, after that period, the irrigation differentiation level started. The plot received 24 plants with 0.2m between rows and 0.05m between plants.

At harvest time, eight central plants were used for the analysis of leaf weight (LW), leaf number (LN), plant height (PH), root diameter (RD), root length (RL) and root weight (RW). The total yield (TY) was estimated considering the total weight of the roots within the useful area. Commercial Yield (CY) was estimated by subtracting the percentage of cracked and defective roots from TY. Also, the soil cover fraction (SCF) and leaf area index (LAI) were measured. The SCF was estimated using the following equation:

$$SCF = PPA/UA$$

Where: SCF is the soil cover fraction, PPA is the plant projection area (PPA), and UA is the useful area of the plot. For the leaf area index the following equation was used:

$$LAI = TLA/UA$$

Where: LAI is the leaf area index, TLA is total leaf area of the plants, and UA is the useful area of the plants. Moments before harvest, photos of the plots were taken using a camera 1m away to obtain the PPA (Fig. 1B). After harvesting, the plant leaves were placed on a platform and were photographed at 0.6m away to obtain the TLA (Fig. 1D). PPA and LA were calculated using ImageJ Software (Fig. 1C, 1E), which is free to use.

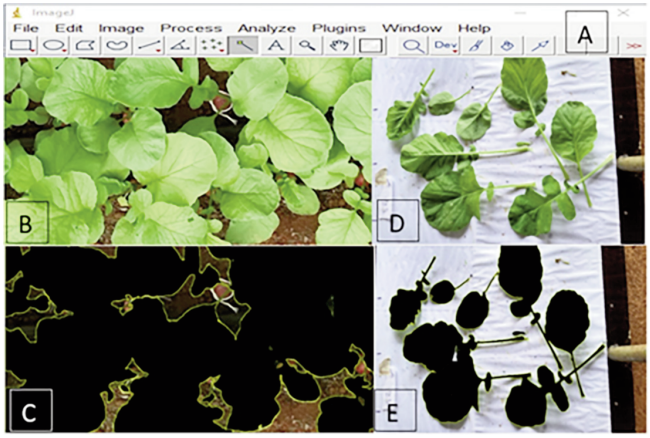

Fig. 1 - (A) ImageJ Software Interface; (B) Image of the useful area of the experimental plot; (C) Projection area calculation; (D) images of plant leaves and (E) Calculation of leaf area.

Fixed and variable cost

The radish yield (t ha^{-1}) and the costs per cultivated hectare were used for the cost analysis, in approximate values of Brazilian Real (R$). In the estimate of commercial yield in the greenhouse, the useful planting area index of 57% was used (Araújo Neto *et al.,* 2012) in addition to subtracting the percentage of defective roots.

In this study, the methodology proposed by Reis (2002) was used to estimate production costs. The production cost is the integration of all inputs, labor, depreciation, and operational values in the production process, including the alternative cost. Production cost were grouped into: fixed costs, variable costs, and total cost. Fixed cost refers to depreciation and alternative costs. The variable costs are those related to the crop costs during the cycle of the production process. The sum of fixed and variable costs represents the total cost.

Depreciation is defined as the cost necessary to replace capital goods when rendered useless by physical or economic wear and tear. The linear method was used, considering 6 cycles per year, which corresponds to the average cultivation cycle of the radish cultivar used in addition to the rest and soil preparation period. The Depreciation (D) was calculated by the following equation:

$$D = (Vp - Vr)/Lu \times P$$

Where: D is depreciation (R$), Vp is the present value of the asset (R$), Vr is the residual or resale value (R$), Lu is the useful life or period of activity of the asset (years), and P is the period of analysis or productive cycle (years).

The interest rate of 7% per annum (p.a) was considered for the analysis of the alternative cost of fixed and variable resources allocated to production, Above the recommended by the Companhia Nacional de Abastecimento - CONAB (2010). The alternative fixed cost allocated to the radish cultivation was calculated using the following equation:

$$AC_{fixed} = [(Lu - A) /Lu] \times Vp \times Ir \times P$$

Where: AC_{fixed} is the alternative fixed cost (R$); A is the average duration of the asset use (years), considered 50% of Lu, and Ir is the interest rate (decimal).

The alternative cost of the variable assets (AC_{var}) allocated to the radish cultivation was calculated according to equation:

$$AC_{var} = V_{exp}/2 \times Ir$$

Where: AC_{var} is the alternative variable cost (R$), and Vexp is the financial investment for the acquisition of inputs and services for the crop production (R$).

The fixed cost corresponding to the sum of the contributions of fixed factors in total product in each production cycle. The alternative cost of the production factor was added to the depreciation in calculating the fixed cost. The following items were considered in this calculation.

Land and Rural Land Tax: The value of the Rural Land Tax (RLT) was not considered, due to the exemption for properties below 30 ha. The land is not depreciated when proper soil management is adopted, and all chemical elements extracted by the plant are replaced through the practice of soil fertilization. The value considered was the alternative cost, based on the land rental value. The rental value was R$ 131.35 per hectare and per month, as mentioned in the on the agricultural price indexes of the Department of Business Administration and Economics of UFLA (DAE/UFLA) and corrected by the General Price Index - Internal Availability (GPI - IA), for November 2018 amounts (R$).

Mulching: The value of black polyethylene film (BP) and black non-woven film (BNW) were R$ 0.41 m^{-2} and R$ 0.67 m^{-2}. Based on the area of mulching required in the experiment, approximately 72% of the greenhouse area. Expenditures on BP and BNW were R$ 3,028.1 ha^{-1} and R$ 4,834.2 ha^{-1}, respectively. A useful life of 1 years was considered.

Seedling tray and greenhouse: The expenses with 2860 seedling tray were R$ 6,292.0 ha^{-1} (20% more to guarantee the quantity of seedlings necessary for transplantation) and the useful life of 3 years. Expenses for greenhouse structure and low-density polyethylene film coverage were R$ 380,000.00 ha^{-1} and R$ 25,525 ha^{-1}, respectively, considering the useful life of 20 years and the change of greenhouse cover every 2 years.

Irrigation system: The quantities of material and the irrigation system cost is influenced by the unevenness degree of land, the water collection distance, and the equipment used. A project with the following characteristics was considered: 5 hp motor-pump set, system automation set with starter switch, contactor and relay, programmable irrigation controller with nine outlets, relief valve, air valves and vacuum, electric control valves (solenoids), underground irrigation pipes DN 150 for main irrigation system, PVC piping (50 mm in diameter) connecting the main system to sectors, DN 16 mm low density polyethylene (LDPE) tube, tube connection fittings DN 16 mm, self-compensating dripper with a nominal

flow of 4.3 L h^{-1} and 2 disc filters with automatic backwash. The useful life considered was 20 years, except for LDPE pipes and connection fittings, which useful life considered was 3 years. The residual value was estimated at 20% of the acquisition value. The maintenance and operation of the system is equivalent to 2% of the acquisition value.

The expenses for the services and products acquisition in each crop cycle, added to the alternative cost, was used in the variable cost calculation. The following items were considered in this calculation.

Inputs: related to investment in the acquisition of substrate, seeds, chemical fertilizers, and pesticides. The value of each input was based on the report on agricultural inputs (CONAB, 2018) and the values provided by companies producing seeds and substrates. The amount of inputs needed were based on the quantity used in the experiment, according to the soil analysis and the recommendations for the crop.

Labor: Expenses with labor refer to the implementation, conduction and harvesting of the crop, operation of machines and irrigation system, and post-harvest processing (cleaning, bagging, and transportation within the property). The unit value practiced was R$ 954.00 (minimum wage practiced in 2018) plus 51.56% as social charges, according to the methodology proposed by CONAB (2010).

Energy: The energy cost was calculated according to the following equation:

$$EC = V_{kWh} \times T \times (736 \times Pwr)/1000 \times h$$

Where: EC is the energy cost (R$); T is the total operating time of the irrigation system in each treatment (h); V_{kWh} is the kWh price (R$); Pwr is the motor pump power (hp), and h is the motor pump efficiency (decimal). R$ 0.49 is the price per kWh charged by Minas Gerais Electric Power Company (MINISTÉRIO DE MINAS E ENERGIA, 2018). The cost for the volume of water used was not considered, the collection being considered public or for use by the producer.

Administration and Post-harvest cost: Expenses with administrative labor and technical assistance were 6% of the variable costs (CONAB, 2010). Post-harvest cost refers to expenses with product improvement, wooden boxes for packaging and transportation to the destination. The quantities used changed depending on the average yield of each treatment.

Machines and implements: Referring to the investment in renting machines and implements in the preparation of the soil. The unit values considered were those mentioned in the Department of Business Administration and Economics of UFLA (DAE/UFLA) agricultural price indices. The quantities used for each resource were estimated according to the quantity used in conducting the experiment.

Alternative cost: The real interest rate of 7% p.a. was considered for calculating the alternative cost of each item of the fixed and variable cost.

Economic analysis

The radish price adopted was R$ 1.20 kg^{-1}, which is equivalent to the average price paid by the Food Acquisition Program (Programa de Aquisição de Alimentos - PAA), operated by the National Supply Company, and the prices practiced in the Supply Centers of Minas Gerais (Centrais de Abastecimento de Minas Gerais S.A. - CEASAMINAS) in October 2018.

The operating cost considers the depreciation and inputs used, equivalent to the analysis period, without the alternative cost. Average total operating cost (TOC) and average total cost (ATC) were calculated in unit terms in the economic analysis. The TOC, in R$ kg^{-1} of radish, is divided into average fixed operating cost (FOC), which is composed of the depreciation, and the average variable operating cost (VOC), which is composed of the disbursements during the analysis period (Reis, 2002).

The economic analysis evaluates the TOC and the ATC in relation to the practiced price. This analysis can result in different conditions, and each result suggests an interpretation. To carry out this interpretation of the economic analysis, the situations of economic and operational analysis of the productive activity, described by Reis (2002), were considered. Thus, this study presents a diagnosis of the economic-financial behavior of irrigated radish cultivation, with information about the remuneration obtained and the allocated resources in comparison with the remuneration provided by investment alternatives (alternative cost).

Statistical analysis

Data were statistically analyzed using the Statistical Analysis System Learning Edition 8.0 (SAS, 2003) computer program. Data were initially examined for homogeneity of variance and then subjected to analysis of variance. Tukey test ($p>0.05$) was used to compare the difference between the treatments for the growth variables, root variables and radish yield. In order to analyze the correlation of plant growth, root growth, and yield variables, Pearson

correlation was conducted, which was qualitatively evaluated for intensity using the following criteria, proposed by Callegari-Jacques (2003): null (0), low (0 to 0.3), regular (0.3 to 0.6), strong (0.6 to 0.9), very strong (0.9 to 1.0) and full (1.0) .

3. Results and Discussion

Growth and yield of radish

The results related to radish growth and yield can be seen in Table 2. For the variables leaf weight and plant height was possible to observe a similar behavior, were mulched system resulted in higher values of leaf weight and plant height than those without mulch (Table 2). The variables leaf weight and plant height were significantly higher with black plastic mulching at 12 kPa, and treatment without mulching at 50 kPa resulted in the lowest growth of these parameters. The water quantity applied had a direct influence on leaf weight and plant height and increase in the water quantity resulted in plants with heavier leaves and taller plants. The BP7 and BP12 resulted in a higher leaf number, however there was no significant difference between treatments.

The leaf area index and soil coverage fraction grew with the increase in the water quantity used and mulching treatments resulted in higher values (Table 2). The highest leaf area index and soil coverage fraction values were observed with BP7 and BP12, respectively. The lowest leaf area index and soil coverage fraction observed were with the NM50 treatment, and the NM50 differed significantly from the BP7 treatment. These results agree with previous findings by Carmichael *et al.* (2012), who reported an increase in radish growth parameters with increasing irrigation depth. These authors also demonstrated that the use of mulching can significantly influence the growth of the radish. Other authors have also observed similar results. Kang and Wan (2005) reported a maximum leaf area index at 15 kPa in the radish crop in 2002. Yaghi *et al.* (2013) reported that different types of mulching created a positive effect on the growth of cucumber plants.

There was a significant difference between the treatments applied in the root diameter and root length parameters, however, in the root weight parameter, there was no significant difference between treatments. The highest values of root diameter, root length, and root weight were obtained with treatments BP12, NM7 and BNW12, respectively (Table 3). The lowest values of root diameter, root length, and root weight were obtained from the NM20, NM50, and NM50 treatments in decreasing order. The increase in SWT resulted in a decrease in root growth parameters in treatments without mulching; however, a higher growth was observed at 12 kPa in treatments with black plastic and black non-woven.

For the total yield and commercial yield (Table 3) was possible to observe that treatments without mulching, maximum total yield and commercial yield were obtained at 7 kPa and the increase in SWT

Table 2 - Effect of irrigation and mulching on growth variables of radish

Treatment	Growth variables				
	Leaf weight (g)	Leaf number	Plant height (cm)	Leaf area index	Soil coverage fraction
NM7	25.3 abc	6.6 a	32.7 abc	5.78 ab	2.32 abc
BP7	30.3 ab	7.2 a	35.5 ab	6.46 a	2.73 a
BNW7	22.8 bc	7.0 a	33.2 ab	5.02 abc	2.40 abc
NM12	22.4 bc	7.0 a	30.7 bc	4.49 abc	2.32 abc
BP12	34.1 a	7.2 a	37.3 a	6.30 ab	2.55 ab
BNW12	24.8 abc	6.8 a	33.5 ab	5.15 abc	2.19 abc
NM20	22.4 bc	6.6 a	30.1 bc	4.11 bc	1.86 bc
BP20	25.8 abc	6.8 a	34.4 ab	4.81 abc	2.34 abc
BNW20	23.2 abc	6.4 a	32.9 ab	4.46 abc	2.28 abc
NM50	14.9 c	6.1 a	27.0 c	3.13 c	1.74 c
BP50	21.8 bc	6.8 a	32.0 abc	4.45 abc	2.12 abc
BNW50	23.5 abc	6.4 a	33.9 ab	4.48 abc	1.93 bc
CV	15.52	6.62	5.97	15.51	10.63

* Different letters within columns indicate significant difference by Tukey test at 5% probability level.

decreased these variables. However, the behavior of total yield and commercial yield was different between treatments with black plastic and black non-woven. The maximum total yield and commercial yield were reached at 12 kPa in these treatments. Therefore, keeping soil moisture close to the field capacity associated with mulching resulted in water stress suffered by the plant, due to the mulch's characteristics of maintaining high soil moisture. However, with the proper SWT, the use of mulching increased yield when compared to treatments without mulching. Gao *et al.* (2019) and Li *et al.* (2018) also reported a significantly increase of yield of different crops with mulching.

The BP12 treatment resulted in the highest total yield and commercial yield, 41.8 t ha^{-1}, and 37.5 t ha^{-1}, respectively (Table 3). This result highlight the importance of the use of irrigation and water management in radish crop. Kang and Wan (2005) reported the total yield of 47.4 t ha^{-1} under irrigation management system. These yield differences observed in the literature can be explained by the radish cultivars used, and the difference in cultivation techniques adopted. Yield values above those obtained in the present study indicate a potential for yield growth that can be achieved by new techniques.

Correlation and multiple linear regression analysis

The yield of the radish root is usually determined by some characters of a morphological nature. Adequate knowledge of the relationship between yield and morphological characters is essential for the identification of selection criteria to be used to improve the yield of radish. Significant association was found between all the contributory characters studied and total and commercial yield (Table 4). The results of the person correlation presented in Table 4 showed that the leaf area index (plant growth parameter) and the root diameter (root growth parameter) that had a very strong and positive correlation with total and commercial yield. The other characters also showed a positive correlation, however with lower r values. Therefore, the increase in leaf area index and root diameter implies a greater increase in yield when compared to the other characters. These results indicate that concentrating efforts on the selection of plants with high leaf area index and higher root diameter would be accompanied by high yield capacity in these conditions.

The findings in this paper confirm previous studies by Ullah *et al.* (2010), which found a positive and significant correlation between radish yield with plant height, root length, and root diameter. Khatri *et al.* (2019) found a positive relationship between yield and plant height, root length and root diameter, however leaf number showed highest significant positive correlation with total yield of radish. However, these papers showed that different morphological characters can have a greater relationship with yield under different cultivation conditions.

Table 3 - Effect of irrigation and mulching on root growth variables and radish yield

Treatment	Root variables			Yield	
	Rooot diameter (cm)	Root length (cm)	Root weight (g)	Total yield (t ha^{-1})	Commercial yield (t ha^{-1})
NM7	42.9 ab	60.6 a	53.3 ab	37.6 ab	32.0 ab
BP7	41.0 abc	60.1 a	46.8 abcde	33.0 abcde	30.4 abcd
BNW7	39.0 abcde	63.6 a	47.7 abcde	33.7 abcde	30.8 abc
NM12	37.8 bcde	59.2 a	40.4 bcde	28.5 bcde	25.9 bcd
BP12	43.4 a	67.6 a	59.2 a	41.8 a	37.5 a
BNW12	42.9 ab	60.4 a	49.1 abc	34.6 abc	31.9 ab
NM20	35.1 de	56.5 a	31.2 de	22.0 de	20.0 cd
BP20	40.9 abc	68.4 a	48.4 abcd	34.2 abcd	31.4 ab
BNW20	40.3 abcd	64.8 a	40.3 bcde	28.5 bcde	25.6 bcd
NM50	33.7 e	55.1 a	30.4 e	21.4 e	19.3 d
BP50	37.1 cde	64.6 a	39.9 bcde	28.2 bcde	25.6 bcd
BNW50	36.7 cde	61.9 a	35.9 cde	25.3 cde	22.8 bcd
CV (%)	4.76	7.78	13.53	13.54	13.52

* Different letters within columns indicate significant difference by Tukey test at 5% probability level.

Table 4 - Pearson correlation coefficients among yield and its related characters in radish

	Plant growth					Root growth		Yield	
	LW	LN	PH	LAI	SCF	RD	RL	TY	CY
LW	1								
LN	0.69 **	1							
PH	0.87 **	0.63 **	1						
LAI	0.91 **	0.72 **	0.83 **	1					
SCF	0.61 **	0.48 **	0.66 **	0.67 **	1				
RD	0.70 **	0.44 *	0.66 **	0.77 **	0.56 **	1			
RL	0.56 **	0.51 **	0.69 **	0.55 **	0.35 *	0.51 **	1		
TY	0.74 **	0.56 **	0.71 **	0.83 **	0.62 **	0.90 **	0.66 **	1	
CY	0.75 **	0.59 **	0.73 **	0.82 **	0.64 **	0.89 **	0.68 **	0.99 **	1

LW= Leaf weight; LN= Leaf number; PH= Plant height; LAI= Leaf area index; SCF= Soil coverage fraction; RD= Root diameter; RL= Root length; TY= Total yield; CY= Commercial yield.
**, *= Significant at 1 and 5 % probability levels, respectively.

Production cost economic analysis

The percentage contribution of the fixed and variable cost items that make up the total cost are shown in Table 5. The total fixed cost and the total variable cost represent, on average, 25% and 75% of the total cost, respectively. Greenhouse (structure and cover) has the largest percentage contribution in total fixed cost, and input followed by labor has the largest percentage contribution in total variable cost. A similar result was observed by Silva *et al.* (2007) in the cultivation of sunflower, however Boas *et al.* (2011) and Lima Junior *et al.* (2014) in the onion and carrot crop, respectively, observed a percentage contribution of the total fixed cost in the total cost of less than 25%. These results can be explained by the non-use of greenhouse and mulching in the cultivation of these vegetables, reducing fixed costs. These authors also observed a greater percentage contribution of input and labor in total variable cost.

Despite the depreciation of the greenhouse constituting a high percentage in the total fixed cost (Table 5), its use decreases the susceptibility to weather and allows the planting of the crop throughout the year. According to Araújo Neto *et al.* (2012), despite the high cost of greenhouses, its use generates higher yield, reduces the average total cost, and increases profitability. Inputs and Labor represent, on average, 47% of total production costs. Despite the inputs cost being difficult to reduce, family labor is recommended in the quest to reduce labor costs and

Table 5 - Percentages of fixed and variable costs of radish production in different mulching types and soil water tension

	% Total cost											
	NM7	BP7	NW7	NM12	BP12	NW12	NM20	BP20	NW20	NM50	BP50	NW50
Land	1.09	1.07	1.06	1.13	1.03	1.05	1.18	1.07	1.09	1.19	1.11	1.12
RLT	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mulching	0.00	2.15	3.38	0.00	2.07	3.36	0.00	2.14	3.49	0.00	2.22	3.56
Seedling tray	1.62	1.60	1.58	1.69	1.54	1.57	1.75	1.60	1.63	1.76	1.66	1.66
Greenhouse structure	15.77	15.58	15.34	16.36	14.95	15.25	17.00	15.51	15.84	17.10	16.07	16.13
Greenhouse cover	4.77	4.71	4.64	4.95	4.52	4.61	5.14	4.69	4.79	5.17	4.86	4.88
Irrigation System	0.24	0.24	0.24	0.25	0.23	0.24	0.26	0.24	0.25	0.27	0.25	0.25
Dripper and LDPE pipes	1.19	1.18	1.16	1.23	1.13	1.15	1.28	1.17	1.20	1.29	1.21	1.22
TFC	24.61	26.47	27.32	25.53	25.40	27.17	26.54	26.35	28.22	26.69	27.29	28.73
Inputs	23.65	23.42	23.06	24.68	22.33	22.91	25.82	23.29	23.94	25.99	24.26	24.44
Labor	23.10	22.87	22.52	24.11	21.81	22.37	25.22	22.75	23.38	25.38	23.70	23.87
Energy	0.54	0.53	0.53	0.53	0.48	0.49	0.42	0.38	0.39	0.33	0.31	0.31
Post-harvest expenses	20.02	18.83	18.83	16.97	22.20	19.33	13.71	19.37	16.25	13.29	16.48	14.76
Administration Costs	4.15	4.05	4.01	4.10	4.12	4.02	4.04	4.06	3.95	4.03	4.00	3.92

production costs.

The NM7 treatment had the lowest percentage contribution of the total fixed cost and the highest percentage contribution of the total variable cost in the total cost, among the treatments applied (Table 5). The lack of mulching in this treatment contributed to the decrease in total fixed cost, also, NM7 resulted in high yield, increasing expenses with post-harvest and administrative costs. The BNW50 treatment showed the highest percentage contribution of the total fixed cost due to expenses with non-woven film and low yield, which reduces the total variable cost. Post-harvest expenses and administrative costs were high in BP12 treatment, due to high yield, resulting in a low percentage participation of total fixed cost in the total cost, despite the use of plastic mulching.

In the simplified economic study, R$ 1.20 kg^{-1} was considered as the average price practiced in the period of October 2018, and the average total cost and total operating cost for radish crop varied according to the treatment applied (Table 6). The BP12 treatment resulted in the lowest average total cost (R$ 0.71 kg^{-1}) and total operating cost (R$ 0.60 kg^{-1}). Although black plastic increased production costs, the mulching characteristics helped to increase yield and profitability, offsetting the expenses with the mulching. The BNW12 treatment resulted in the lowest average total cost (R$ 0.81) and total operating cost (R$ 0.68) among black non-woven film treatments, however they were lower than the values observed with the BP12 and NM7 treatment. Although the black non-woven film increased the yield, the expenses with mulching did not compensate for the application. Therefore, that its use is viable, the prices practiced in the commercialization of non-woven film must be below the prices presented in the present study. Between the treatments without mulching, keeping the humidity close to the field capacity (7 kPa) provided the lowest production cost. The NM50 and NM20 treatments resulted in the highest average total cost and total operating cost, due to low yield, therefore they are not recommended.

Although the application of plastic mulching results in an increase in yield and financial return, its use must be done in a correct and controlled manner, to decrease the negative impact on the environment of plastic film pollution. In addition, accumulation of plastic residue in soil over time may produce negative effects on crop production (Gao *et al.,* 2019). Therefore, an effective cleaning is necessary after the useful life of mulching or the acquisition of biodegradable mulching film in order to develop sustainable agriculture.

All treatments applied exhibited average returns higher than average total cost. Therefore, the investment pays all resources applied in the activity and provides an economic profit, even in treatments with low yield. In this situation, investment is higher than market alternatives, and the trend in the medium and long term is for expansion and entry of new companies into the activity, attracting competitive invest-

Table 6 - Average economic and operating costs of radish production, in R$ kg^{-1}, at different types of mulching and soil water tension

	Average fixed cost	Average variable cost	Average total cost	Average fix operating cost	Average viarable operating cost	Average total operating cost
NM7	0.19	0.60	0.78	0.08	0.57	0.66
BP7	0.21	0.62	0.83	0.11	0.60	0.70
BNW7	0.22	0.61	0.83	0.11	0.59	0.70
NM12	0.23	0.69	0.92	0.10	0.67	0.77
BP12	0.17	0.53	0.71	0.09	0.51	0.60
BNW12	0.21	0.60	0.81	0.11	0.57	0.68
NM20	0.30	0.85	1.14	0.13	0.81	0.95
BP20	0.21	0.60	0.81	0.10	0.58	0.68
BNW20	0.26	0.70	0.96	0.14	0.67	0.81
NM50	0.31	0.87	1.18	0.14	0.84	0.98
BP50	0.25	0.70	0.95	0.13	0.67	0.80
BNW50	0.30	0.76	1.06	0.15	0.73	0.89

ments.

The analysis was carried out with the product price at R$ 1.20 kg^{-1}, however the price may vary according to the market at the time of harvest. If the price is lower than practiced, a new analysis must be made. However, there are alternatives to increase sales such as the sale of deformed and cracked roots (difference between total yield and commercial yield), for companies specialized in purchasing these products and increasing yield with new techniques and with more productive cultivars.

4. Conclusions

The different SWT levels and the use of mulching resulted in different growth and yield responses. The BP7 treatment resulted in the highest leaf area index and soil cover fraction. The BP12 treatment resulted in the highest leaf weight, plant height, leaf number, root diameter, root length, total yield and commercial yield. In this context, this treatment was recommended for the radish producers. The plant variables leaf area index and root diameter were the parameters that presented the highest and positive correlation with the radish yield.

Expenses with variable resources increased the final cost in all treatments studied. In conditions similar to those observed experimentally, it is recommended to adopt a black plastic cover keeping the SWT close to 12 kPa, to obtain the highest profitability in the productive activity. However, if the practice of mulching is not feasible, the SWT should be kept close to 7 kPa.

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Short note

Contrasting aspects of the physical and physiological dormancy by seeds from four peach rootstocks

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Abstract: Considering that cultivars of peach rootstocks selected to be propagated by seeds show variation in the degree of physiological dormancy and peculiarities regarding the limitation imposed by the endocarp, it is essential to define the cold stratification period and the ideal temperature to be used during the pre-germination treatment. Isolated for each cultivar, knowledge of these variables which will provide viability in the production of peach rootstocks via seeds, in the presence of the endocarp, at a larger scale. Two germination tests were carried out, in a completely randomized experimental design and 4 x 3 factorial scheme, with four replications, each one consisting of 25 endocarps. The first experiment was performed with four cultivars (Aldrighi, Capdebosq, Okinawa Roxo and Tsukuba 1) and three stratification temperatures (1°C, 4°C and 7°C) for a period of 90 days. For the second experiment, the same cultivars were used, two temperatures (7°C for 'Aldrighi' and 'Capdebosq' and 4°C for 'Okinawa Roxo' and 'Tsukuba') and three stratification periods (40, 80 and 120 days), followed by sowing in substrate under greenhouse conditions for a period of 45 days, with subsequent endocarp breaking and re-sowing. The pre-germination treatment at 7°C for 90 days is sufficient to obtain high germination percentage of 'Aldrighi' and 'Capdeboscq' seeds in the presence of the endocarp. Under the stratification conditions tested, the seeds of 'Okinawa Roxo' and 'Tsukuba 1' require rupture of the endocarp to reactivate the germinative embryonic process.

1. Introduction

The production of peach rootstocks [*Prunus persica* (L.) Batsch] is traditionally done by seed, being the simplest and the most inexpensive method when compared to clonal propagation (Martins *et al.,* 2014; Fischer *et al.,* 2016). Despite the fact that stone fruit production is very important in Brazil, the country still presents relatively low orchard average productivity, mainly in Rio Grande do Sul state (Fischer *et al.,* 2016). Factors associated with this low productivity include pest incidence, dis-

eases, climate and seedling quality, mainly in the form of rootstock production; in general, they are obtained from seeds discarded by the canning industry and, therefore, with no genetic identity (Souza *et al.,* 2018).

Currently, at Federal University of Pelotas and Embrapa Temperate Climate, a wide range of rootstock cultivars for peach seedling production are available to growers (Souza *et al.,* 2018; Menegatti *et al.,* 2019 a; Souza *et al.,* 2019 a; Menegatti *et al.,* 2020). However, peach seeds of different genotypes exhibit considerable divergence in germination rate as a result of the variability in the degree of physical and physiological dormancy of the seeds, constituting a limitation for commercial seedling production of the specie (Souza *et al.,* 2017; Menegatti *et al.,* 2019 b). Souza *et al.* (2017) and Souza *et al.* (2019 b) suggest that the combination of physical and physiological dormancy presented by seeds of temperate climate species, such as peach, may be one of the main factors responsible for the variation in the germination percentage of rootstocks seeds. This variation requires the exposure of seeds to different combinations of time and low temperatures to overcome dormancy and, consequently, to the maximizing and homogeneity of germination. According to Souza *et al.* (2016) the overcoming of this dormancy can be achieved through techniques that promote the rupture of the endocarp, popularly known as the stone. Endocarp removal, suggested as a method for obtaining high germination percentages in peach seeds (Souza *et al.,* 2017), is an operation that results in an additional cost for the nurseryman, mainly due to the high cost of the procedure, which must be performed manually and individually in order not to compromise the physical integrity of seed structures (Wagner Júnior *et al.,* 2013).

Considering that cultivars of peach rootstocks selected to be propagated by seeds show variations in the degree of physiological dormancy and peculiarities regarding the limitation imposed by the endocarp, it is essential to define the cold stratification period and the ideal temperature to be used during the pre-germination treatment, isolated for each cultivar (cv.), which will provide viability in the production of peach rootstocks via seeds, in the presence of the endocarp, at a large scale. Therefore, this study aimed to evaluate contrasting aspects of physical and physiological dormancy shown by seeds of different peach rootstock cultivars.

2. Materials and Methods

The present study was carried out with plant material obtained from ripe fruits harvested from clonal matrix plants of peach cvs. Aldrighi, Capdeboscq, Okinawa Roxo and Tsukuba 1, from Federal University of Pelotas Peach rootstock Germplasm Collection. Two different experiments were performed, both referring to germination tests. In the first one, the endocarps were packed in tulle bags and placed in plastic boxes (40 x 27 x 10 cm) and covered with fine vermiculite, previously and periodically moistened with distilled water and fungicide solution (Benlate 500 - 2 g L^{-1}). Then the plastic boxes were placed in Biochemical Oxygen Demand (BOD), in different temperatures, where they remained for 90 days in cold weather for stratification, with four cvs. (Aldrighi, Capdebosq, Okinawa Roxo and Tsukuba 1) and three stratification temperatures (1°C, 4°C and 7°C), with four repetitions, each one consisting of 25 endocarps.

At the end of the germination test (at 90 days), the percentage of seed germination was determined, with germinated seeds considered as those that presented radicle protrusion of at least two millimeters (MAPA, 2009). With these data, it was possible to determine the ideal temperature to be used during the pre-germinative treatment of the seeds of each peach rootstock cv. in the presence of the endocarp.

From these results, the second experiment was performed in which the endocarps were conditioned and kept under similar conditions to the previous experiment but were subjected to different stratification periods using the temperatures predetermined in experiment I, specifically 7°C for cvs. Aldrighi and Capdebosq and 4°C for cvs. Okinawa Roxo and Tsukuba 1, and three stratification periods (40, 80 and 120 days), with four repetitions, each one consisting of 25 endocarps. At 40, 80 and 120 days, the germination percentage was determined according to MAPA (2009), and the endocarps in which the seeds did not germinate during the cold stratification process were sown, at 1.0 cm depth, in polystyrene trays of 72 cells (114 cm^3 per cell) containing as substrate composed of 25% orchard soil + 25% vermiculite + 25% medium sand + 25% commercial. The trays were kept under controlled greenhouse conditions, with irrigation as needed, for a period of 45 days.

After this period, the percentage of seedlings

emerged under greenhouse conditions was determined (MAPA, 2009), and the endocarps in which the seeds did not germinate were removed from the trays and broken to extract the seeds using a manual lathe. The seeds were then re-sown in the polystyrene trays, under the same conditions mentioned above, and kept under greenhouse conditions to evaluate seedling emergence.

The percentage of seedling emergence was daily monitored, and the experiment was finished at 15 days after the direct re-seeding during which, in three consecutive evaluations, no new seedlings emerged in any of the treatments.

All analyses was performed with the program SigmaPlot version 10.0 (Systat, 2006).

3. Results and Discussion

The seeds of cvs. Aldrighi and Capdeboscq presented superior results regarding to germination percentage in the presence of endocarp when stratified at 7°C for 90 days (Table 1), suggesting that such conditions are adequate to overcoming seed dormancy and appropriate for inducing germination. A temperature of 7°C employed in cold stratification, for a period of 90 days the cvs. Aldrighi and Capdeboscq provided average values of germination percentage of 82 and 80% respectively, results considered satisfactory according to MAPA (2009). Souza *et al.* (2017) evaluated the stratification of the seeds of these same cultivars, under identical conditions, but for a period of 60 days, registered lower values, which were: 56.5 and 21%, respectively, indicating that the period of 60 days is not sufficient to overcome the dormancy process and, consequently, to obtain satisfactory germination results.

These results also suggest that the 30-day increase, from the 60 days suggested by Souza *et al.* (2017), to 90 days proposed in the present study, for the pre-germinative treatment of seeds from these cultivars. in the presence of endocarp, might have promoted significant changes in the hormonal balance that controls the process of physiological dormancy, thereby making the seed metabolism more active and the embryo able to resume development. Additionally, it was observed that when the stratification temperature was reduced to 4°C and 1°C, the germination percentages decreased significantly for 'Capdeboscq' and 'Aldrighi', being about 54% and 15%, respectively (Table 1).

Table 1 - Mean germination values (%) of four peach rootstocks, in accordance of the temperature used in the pre-germination cold stratification treatment for 90 days

Temperature (°C)	Germination (%)			
	Aldrighi	Capdeboscq	Okinawa	Tsukuba 1
1°C	15	12.5	2	4
4°C	54	52.5	10	11
7°C	82	80	2	0

The seeds of cvs. Okinawa Roxo and Tsukuba 1 showed low average germination percentages at the end of the stratification period, regardless of the temperature tested. The best results were obtained in the treatment in which the endocarps remained in stratification at 4°C for 90 days, which were: 10% and 11%, respectively (Table 1). These results are superior to those obtained by Souza *et al.* (2017), who reported zero germination rate for these two cvs., when the seeds in the presence of the endocarp were stratified at 7°C, for a period of 60 days.

Although these results suggest that the prolongation of seed stratification period in the presence of the endocarp and the reduction in temperature from 7°C to 4°C, allow expressive increases in the germination percentage of seeds of these cultivars, these values are still considered unsatisfactory according to MAPA (2009). Based on the best results obtained in the first experiment, seeds from the four rootstocks were subjected to stratification temperatures that were considered most appropriate, with reductions and extensions of the stratification period. Thus, 'Aldrighi' and 'Capdeboscq' endocarps were placed to germinate at 7°C and 'Tsukuba 1' and 'Okinawa Roxo' were stratified at 4°C.

In figure 1 A and B, the effects of the prolongation of the cold stratification period (in days) on the seed germination percentage in the presence of the endocarp are shown under the ideal temperatures of 7°C for 'Aldrighi' and 'Capdeboscq', and 4°C for 'Okinawa Roxo' and 'Tsukuba 1', that were previously described as ideal for pre-germinative treatment. The prolongation of the cold stratification period induced an increase in the germination percentage, with the maximum obtained at 120 days independent of cv. evaluated, however, the most significant increase in germination rate was registered for 'Aldrighi' (Fig. 1). These results corroborate with the research done by Wagner Júnior *et al.* (2013), in which they observed superiority in the effect of cold stratification on seeds

of some *Prunus* cvs. according to the number of hours of accumulated cold increases.

It is important to highlight that at 120 days of stratification at 7°C, 'Aldrighi' and 'Capdeboscq' presented an average germination percentage of 82% (Fig. 1A), similar to that obtained before 90 days of cold stratification at this same temperature (Table 1). These results indicate, with greater reliability, the use of pre-germinative stratification treatment at 7°C for 90 days to overcome seed dormancy of these cultivars, in the presence of the endocarp, and may have greater impact on the large-scale production of rootstocks.

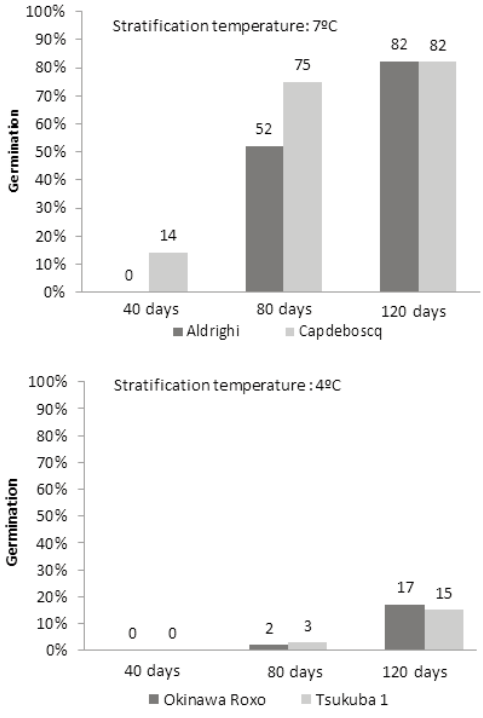

Fig. 1 - Mean values of seed germination (%) in the presence of the endocarp, of the peach rootstocks cultivars, in Biochemical Oxygen Demand (BOD), in accordance of the period used in the pre-germination treatment.

Besides ensuring germination efficiency, the use of this pre-germination treatment to overcome seed dormancy of these cutlivars excludes mechanical procedures such as endocarps rupture, a method commonly indicated for many of the seeds of the *Prunus* genus of commercial interest in which the endocarp acts as a physical limitation to the initiation of the germination process (Souza *et al*., 2019 c).

The superior seed germination rate and the lower degree of physical dormancy has promoted the use of 'Capdeboscq' as a rootstock in the production of peach seedlings in southern Brazil (Souza *et al.,* 2018) for a long time. However, the use of this genotype is not currently recommended due to attributes such as the high susceptibility to different species of the genus *Meloidogyne* (Almeida *et al*., 2015, Souza *et al.,* 2019 c), a parasitic nematode, and the adherence of the fruit pulp in the endocarp. Removal of the pulp necessitates the cleaning of the endocarps, facilitating the potential development of pathogens that may compromise the phytosanitary quality of the postharvest seeds. These characteristics are also exhibited by 'Aldrighi', discouraging the use of these particular cultivars, and promoting of the use of cutlivars with opposite characteristics.

The increase in germination percentage with an extension of the stratification period was also observed in 'Okinawa Roxo' and 'Tsukuba 1' seeds kept at 4°C (Fig. 1B). However, at 120 days of stratification, satisfactory germination rates were not reached (MAPA, 2009), indicating that the increase in the number of cold hours beyond this period is not the main factor determining the low germination rate of these cultivars.

Thus, it is suggested that physical limitations imposed by the presence of the endocarp may contribute decisively to the low seed germination rate of these cvs., as already evidenced by Wagner Júnior *et al.* (2013) and Fischer *et al.* (2016), which suggests that the endocarp is a highly lignified structure, that confers high resistance to water absorption. This factor could compromise seed germination due to the difficulty of the embryo to overcome such a barrier during initial stages of the germination process when imbibition of water is critical.

For these same four cultivars, Souza *et al.* (2017) obtained germination rate of 100% when seeds were stratified without endocarp at 7°C for 25 days. On the other hand, when the seeds were stratified at 7°C for 60 days, without endocarp removal, there were 0 and 0,7% of germination rates for 'Tsukuba 1' and 'Okinawa Roxo', respectively, suggesting that in addition to the presence of the endocarp, the early maturation of the fruits of these cvs., in relation to the other genotypes, directly influence into the physiological quality of the seeds, especially in relation to the embryo maturation. According to Pérez-Jiménez *et al.* (2012) when an embryo is not fully developed, it requires maintenance under specific conditions for a period of time to induce full development in order to ensure the establishment of a new seedling.

The differences in germination percentage of seeds previously treated with cold stratification by different temperatures and periods, followed by sowing in the presence of the endocarp in trays containing commercial substrate, and kept for 45 days under greenhouse conditions are shown in figures 2 and 3. Seeds of 'Aldrighi' stratified previously at 7°C during the periods of 40 and 80 days (Fig. 2), and the seeds of 'Capdeboscq' stratified for 40 days (Fig. 2B), followed by sowing in the greenhouse did not demonstrate a continuity in seed germination. For these treatments, a higher percentage of germination was expected during maintenance in the greenhouse given that during the period they were kept in BOD the values were considered low. Different behaviour was exhibited by seeds submitted to stratification treatment at 7°C for 120 days, for these two cultivars (Fig. 2A and B), since they had already shown high germination percentage in BOD, not showing significant increases in the percentage of germination in the greenhouse.

On the other hand, the seeds of 'Okinawa Roxo' and 'Tsukuba 1' did not show high germination percentage either under BOD conditions or when submitted to different stratification periods at 4°C followed by a period under greenhouse conditions (Fig. 3). This suggests once more the necessity of rupturing of the endocarps for the continuity of the germinative process by the embryo. Souza *et al.* (2019 c) and Menegatti *et al.* (2019 a) point out that in some peach rootstock cvs. the physical barrier imposed by the endocarp may be more determinant in germination than the low temperature and the cold stratification period. The results show that physiological seed dormancy is always present and it needs to be overcome with cold stratification so that the germination occurs, but besides physiological dormancy, the presence of the endocarp may also limit or even prevent

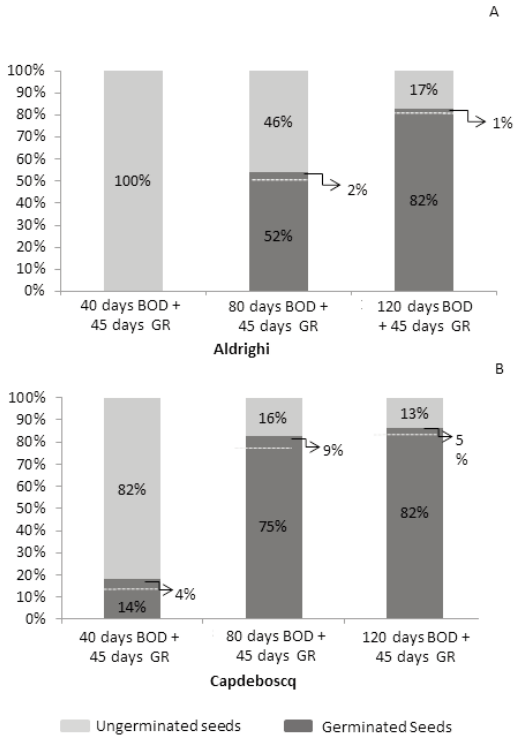

Fig. 2 - Mean values of increase in percentage (%) of germinated seeds after pre-germination stratification treatment in accordance with the period, (A) 'Aldrighi' and (B) 'Capdeboscq', at 7°C, followed by sowing of the seeds in the presence of the endocarp in trays containing commercial substrate and kept for 45 days in greenhouse (GR). Dotted lines on darker bars indicate the limit of the average germination percentage obtained during stratification in Biochemical Oxygen Demand (BOD), and the additional germination obtained after 45 days in greenhouse.

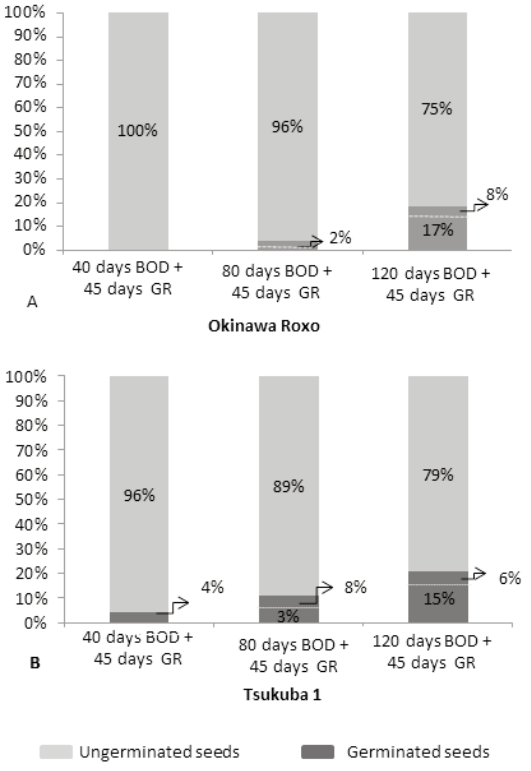

Fig. 3 - Mean values of percentage increase (%) of germinated seeds after pre-germination stratification treatment in accordance of the period, (A) 'Okinawa Roxo' and (B) 'Tsukuba 1', at 4°C, followed by seed sowing in the presence of the endocarp, in trays containing commercial substrate and kept for 45 days in greenhouse (GR). Dotted lines on darker bars indicate the limit of the average germination percentage obtained during stratification in Biochemical Oxygen Demand (BOD), and the additional germination obtained after 45 days in greenhouse.

seed germination of some cultivars resulting in non-uniform seedlings, as demonstrated by Souza *et al.* (2019 c), Souza *et al.* (2017), and the present study.

Among non-germinated seeds of 'Aldrighi' and 'Capdeboscq' after pre-germination treatment, followed by sowing and maintenance in trays for 45 days in the greenhouse, when the endocarp was ruptured, a high percentage of nonviable seeds was found (mostly rotten), This percentage was highest with the cold stratification treatment for 40 days (Table 2). Moreover, the results showed that the increase of the cold stratification period induced a reduction in the number of dead seeds of these same cultivars. These results show that the presence of the endocarp, involving the seeds of these cultivars., doesn't totally restrict the entry of water and cold to overcome dormancy, but probably the accumulation of 40 and 80 days of cold was not enough to promote effective changes in hormonal balance.

This response was not observed in the great majority of 'Okinawa Roxo' and 'Tsukuba 1' seeds (Table 2), which when extracted from the endocarp were intact, with no turgidity, i.e., no evidence of water entering, which allowed the re-seeding and maintenance in the greenhouse again, for a period of 15 days. After this period, it was possible to observe a significant increase in the germination rate directly proportional with the extension of the stratification period employed, reaching a maximum value of 60 and 58%, respectively, after the stratification period of 120 days.

The results obtained in this study reinforce the results published by Souza *et al.* (2017), which compared the seed germination rate of 'Tsukuba 1' after cold stratification in the presence and absence of the endocarp and found that the germination rate was zero in the presence of the endocarp. In contrast, the authors obtained approximately 95% germination when the physical barrier (endocarp) was eliminated. Similar response was also obtained by Souza *et al*. (2017), having found that seeds without endocarp of 'Okinawa Roxo' and 'Tsukuba 1' achieved germination percentages greater than 90%.

In 'Okinawa Roxo' and 'Tsukuba 1', the presence of the endocarp, besides preventing the entry and absorption of water by the embryo, evidenced by the absence of turgidity in the seeds present inside the endocarp even after stratification for 120 days, may also have prevented the leaching of the inhibitor substances present in the seeds. This factor would make the germination process in the presence of the endocarp more demanding regarding the stratification period. This behavior occurs especially in cultivars with greater resistance to rupture of the endocarp, as is the case with these two previously mentioned cultivars (Souza *et al.,* 2017) and 'Okinawa Roxo' according to work by Fischer *et al.* (2016).

4. Conclusions

Pre-germinative treatment at 7°C for 90 days is efficient to obtain high germination percentages of seeds of 'Aldrighi' and 'Capdeboscq' in the presence of the endocarp. Total removal of the endocarp from the seeds of cultivars 'Okinawa Roxo' and 'Tsukuba 1' enables a more efficient overcoming of physical and physiological dormancy and allows for a greater optimization germination rate these cultivars that could benefit large-scale peach rootstock production.

Pre-germination treatment at 4°C for 80 days is not efficient to obtain high germination percentages of seeds of 'Okinawa Roxo' and 'Tsukuba 1' in the

Table 2 - Mean values of the percentage of non-germinated seeds after pre-germination stratification treatment, 'Aldrighi' and 'Capdeboscq', at 7°C (B) 'Okinawa Roxo' and 'Tsukuba 1' at 4°C, for different periods, and kept for 45 days in greenhouse, percentage of dead seeds after this period, percentage of seeds germinated after the whole process followed by endocarp rupture and re-sowing and percentage of lost seeds

	days in BOD at 7°C + 45 days in GR						days in BOD at 4°C + 45 days in GR					
	Aldrighi			Capdeboscq			Okinawa Roxo			Tsukuba 1		
	40	80	120	40	80	120	40	80	120	40	80	120
% S Non-germinated	100	46	17	82	16	13	100	96	75	96	89	79
% S dead	94	12	11	78	12	8	0	2	1	0	3	2
% S germinated after rupture-endocarp	0	17	2	0	2	3	32	44	60	20	40	58
% S lost	6	17	4	4	2	2	68	5	14	76	46	19

%S= percentage of seeds; GR= greenhouse; BOD= Biochemical oxygen demand.

presence of the endocarp.

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Short note

AHS
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Evaluation of *Hemerocallis* germplasm using single nucleotide polymorphisms of nrITS and chloroplast interspacer region

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The authors declare no competing interests.

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Abstract: This study was initiated to distinguish nocturnal (night) flowering *Hemerocallis* species from day flowering species based on the single nucleotide polymorphisms (SNPs) of nuclear internal transcribed spacers 1, 2 in a ribosomal RNA gene (nrITS) and a chloroplast interspacer region (cpIS). Four nocturnal flowering species, *H. citrina*, *H. thunbergii*, *H. minor*, and *H. lilioasphodelus* were collected including Korea, and compared with day flowering species that included *H. vespertina* and *H. hongdoensis.* Based on the haplotypes of nrITS and cpIS, nocturnal species cannot be distinguished from day flowering species. Discrepancies in flowering time and haplotypes among *H. minor* accessions suggest that more germplasm with diverse geographic origins should be evaluated and identification of other genes is required to effectively distinguish nocturnal species from day flowering species.

1. Introduction

There are about 15-26 species/varieties in the genus *Hemerocallis* (USDA, ARS, National Genetic Resources Program, 2015). Using amplified fragment length polymorphisms (AFLP) markers, *H. fulva* L. were grouped separately from the nocturnal species *H. thunbergii* Baker and *H. lilioasphodelus* L., while nocturnal *H. minor* Mill. and *H. citrina* Baroni, were grouped together in a different sub-cluster (Tomkins *et al*., 2001). The flowers of nocturnal *Hemerocallis* open late in the afternoon and wither the next morning (Fig. 1) (Chen and Noguchi, 2000). However, Gulia *et al*. (2009) classified *H. minor* as a diurnal species rather than a nocturnal species, confirming the study by Krestova and Nesterova (2003) that *H. minor* flowered in the morning under sunny weather at >16°C and with-

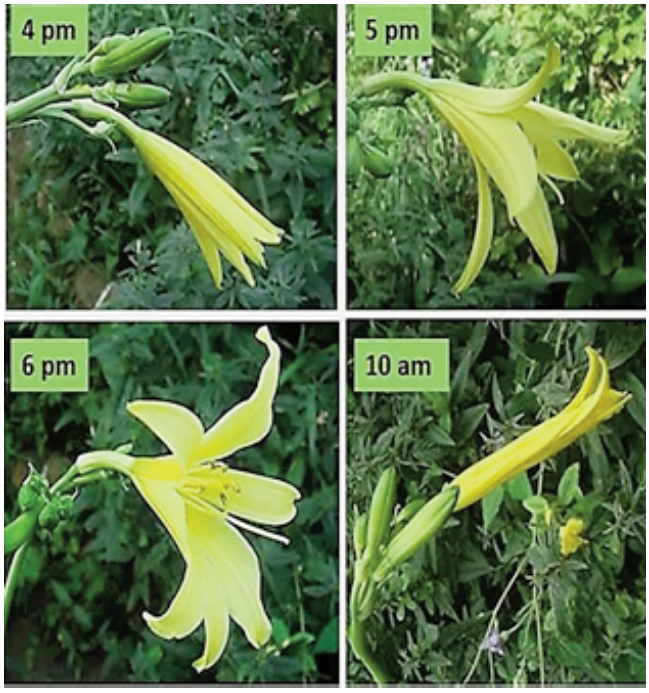

Fig. 1 - Nocturnal *H. thunbergii* accession 7 collected from Korea (K7) showing flower opening at 4, 5, and 6 pm and closed by 10 AM following day.

ered in the afternoon.

Molecular markers have not previously been tested to determine timing of flowering among *Hemerocallis* species. Therefore, this study was conducted to investigate the use of SNPs of nuclear internal transcribed spacers 1, 2 in a ribosomal RNA gene (nrITS) and a chloroplast interspacer region (cpIS) to evaluate the genetic relationships between nocturnal and day flowering species of *Hemerocallis*.

2. Materials and Methods

The nocturnal flowering species *Hemerocallis thunbergii, H. minor, H. lilioasphodelus,* and *H. citrina,* and the day flowering species *H. hongdoensis* M.G. Chung & S.S. Kang*, H. vespertina* H. Hara*, H. dumortierii* C. Morren and hybrid 'Stella de Oro' were collected from Korea (K), China (C), United Kingdom (UK), United States of America (UA) and Germany (GE) (Table 1). Samples were designated as, for example, K1 (mother plant)/1-3 (seedling 1, 2, 3 from mother plant K1).

Genomic DNA was isolated from young leaves using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Polymerase chain reaction (PCR) for nrITS was performed with a 18S rRNA gene specific forward primer ITS1 (5'-TAG AGG AAG GAG AAG TCG TAA CAA GG-3') and primer ITS2 (5'- GATTTTCAGTCCTCTGCTC-TAC-3'). The reaction mix consisted of 12.5 µl of 2X F-star *Taq* Smartmix (SolGent Co., Daejeon, Korea), 2 µl of each primer (0.4 µM final concentration), and 2 µl of genomic DNA. For cpIS, forward primer (5' -TCGT-GAGGGTTCAAGTCCCTCT-3') and reverse primer (5'-GATTTTCAGTCCTCTGCTCTAC-3') were used.

The reaction mix consisted of 12.5 µl of 2X F-star *Taq* Smartmix (SolGent Co., Korea), 2 µl of each primer (0.4 µM final concentration), and 2 µl of genomic DNA (50 ng) and 5 µl of 5× Band Doctor buffer (SolGent), and made up to the 25 µl final volume with PCR ultrapure water. The PCR conditions were: 2 min at 95°C, followed by 35 cycles of 20 sec at 95°C, 40 sec at 65°C, and 1 min at 72°C, followed by 5 min of 72°C using an ABI Veriti Dx Thermal Cycler (Life Technologies, Grand Island, NY, USA). PCR products were direct sequenced as described by Park *et al*. (2014). Sequences were registered in the National Center for Biotechnology Information GenBank (NCBI, http://www.ncbi.nlm.nih.gov/).

3. Results and Discussion

Based on the sequences of nrITS, three haplotypes were identified: T I-1, T I-2, and T II (Table 2) and based on the sequences of cpIS, 7 haplotypes were identified: T I, TI-1, TII, TIII, TIV, TV, and TVI (Table 3). Sequences of cpIS were more informative to separate accessions than those of nrITS, suggesting that the ITS region in *Hemerocallis* may not be useful as a potential source for species identification, although accessions of cultivated origin *Kolkwitzia amabilis* (Graebn.) Christenh were derived from the accession of known wild origin (AA816-84A) (Park *et al*., 2014).

Nocturnal flowering *H. citrina*, *H. thunbergii* and *H. lilioasphodelus* flower in the afternoon and wither the following morning (Table 1, Fig. 1). The difference between two types of floral morphology of *H. thunbergii,* based on the presence or absence of bracts subtending the flower buds; K7 lacked bracts while K8-9 had bracts was detected in the haplotype of cpIS, with K7 belonging to type I-1 and K8-9 belonging to type I that may result from that the sequence of the ITS 2 region is more variable than that of the ITS 1 region (Ma *et al*., 2014). Nocturnal flowering *H. thunbergii* accessions collected from the same location in Korea (K8-9, and K 8-13) should be evaluated

further since these accessions were grouped together with other *H. thunbergii* accessions (K1-3).

There is no correlation between these nrITS haplotypes and timing of flowering observed in *H. citrina*; collected from China (C2) and the United Kingdom (UK5), both belonging to type I, from Germany (GE4), belonging to type II, and from the United Kingdom (UK1), belonging to type III. This requires further examination collecting more accessions from different collection sites. Further, in cpIS, day flowering *H. hongdoensis* and *H. vespertina* belonged to type I and II, respectively.

When T was assigned for the ambiguous code C or T at the positions 78, 86, 99, 113, and 120 of nrITS sequence for type I-1, and A was assigned for A or G at 231, types I-1 and I-2 can be combined and all accessions can be assigned as type I, separated from *H. minor* (UK6 and GE3) as type II (Tables 1 and 2). They may be derived from different geographic origins of K14 or UA5 which were collected from Korea, belonging to type I-2, and exhibit some degree of genetic variation revealed in this study using universal primers for nrITS. However, different strains or populations of *H. minor* may exist since *H. minor*

Table 1 - Accession information on *Hemerocallis taxa* (mother plants and their seedlings) with flowering characteristics. Haplotype based on the sequence analysis of nrITS and cpIS are indicated

Scientific name	Mother plant (leaf)	Seedlings [a]	Flowering time [b]	Source, Country	Haplotype (T) [c]	
					nrITS	cpIS
H. thunbergii	K1 [d]	K1/1-3	N		I-2	I
H. thunbergii	K2	K2/1-3	N	Jungseon-gun, Korea	I-2	I
H. thunbergii	K3	K3/1-3	N		I-2	I
H. thunbergii	K7		N	J.W. Chang, Gomyeong-dong, Jecheon-si, Chungcheongbuk-do, Korea	I-2	I-1
H. thunbergii	K8,9 [e]		N	E.J. Kim, Gomsi-gil, Ungdam-ri, Paju-gun, Kyunggi-do, Korea	I-2	I
	Ka8-13 [d]		N	E.J. Kim, M.S. Roh, Gomsi-gil, Ungdam-ri, Paju-gun, Kyunggi-do, Korea	I-2	IV
H. thunbergii	K11		N	Hantaek Botanical Garden, Korea	I-2	I
H. thunbergii	UK3, 4		N	Royal Botanical Garden Edinburgh, UK (19300128A [f])	I-2	I
H. thunbergii	UA7	-	N	United States National Arboretum, Washington, DC, USA (USNA; NA54757.3 collected from Korea)	I-2	I
H. minor	-	K14/1-3	D	Hantaek Botanical Garden, Korea	I-2	I-1
H. minor	UK6	UK6/1-3	N?	Royal Botanical Garden Edinburgh, UK	II	V
H. minor		GE3/1-3	N?	Botanischer Garten Leipzig (XX-O-LZ-AD439/2006)	II	V
H. minor	UA5	-	D	USNA; NA31800.1	I-2	I
H. lilioasphodelus	-	C1/1	N	X.W. Wu, China	I-2	I
		C1/2-3			I-1	I
H. liliasphodelus	UA6	-	N	USNA; NA54879.3	I-2	I
H. citrina	-	C2/1-3	N	X.W. Wu, China	I-2	I
H. citrina	UK1		N	Royal Botanical Garden Edinburgh, UK (19685548A [u])	I-2	III
H. citrina	UK5		N	Royal Botanical Garden Edinburgh, UK	I-2	I
H. citrina		GE4/1-3	N	Botanischer Garten Leipzig (XX-O-LZ-AW78/1998, 2000)	I-2	II
H. citrina 'April Flower'	-	C3/1-3		X.W. Wu, China	I-2	I
H. vespertina	K12	K12/1-3	D	Hantaek Botanical Garden, Korea	I-2	II
H. dumortierii C. Morren	K13		D	Hantaek Botanical Garden, Korea	I-2	VI
H. hongdoensis	-	K15/1-3	D	Hantaek Botanical Garden, Korea	I-2	I
'Stella de Oro'	UA2	UA2/1-3	D	M.S. Roh, Ann Arbor, MI., USA	I-1	I

[a] Seedlings 1, 2, 3 from mother plant K1. Designations of accession of mother plant and three seedlings are indicated as K1 and K1/1-3, respectively.

[b] Flower opens in the morning and withers in the late afternoon (day, D) or opens in the late afternoon and withers early on the next morning (night, N). Flowering characteristics were not evaluated in this study for *H. minor* collected from Royal Botanical Garden Edinburgh, UK and Botanischer Garten Leipzig (XX-O-LZ-AD439/2006).

[c] Refer to Table 2 for nrITS and Table 3 for cpIS haplotypes and single nucleotide polymorphisms.

[d] Collected or received from Korea (K, Ka), United States of America (UA), United Kingdom (UK), Germany (GE), and China (C).

[e] Samples of K8 and 9 and of Ka 8-13 were collected from the same location in 2011 and 2014, respectively.

[f] Samples were of garden origin from Dendrologische Gartenerei in Pruhonice, Czech Republic via Peter Brownless.

flowers in the morning under sunny weather at >16°C and withers in the afternoon, as reported at the Botanical Garden Institute, Far East Branch, Russian Academy of Sciences (Krestova and Nesterova, 2003). The *H. minor*, received from the US National Arboretum (USNA; NA31800.1) originally collected from Korea, flowers in the morning in Ann Arbor, MI, USA. Therefore, further investigation of *Hemerocallis minor* is needed to determine whether accessions collected from China, Korea and the far eastern part of Russia are of two different flowering types.

Hemerocallis hybrid 'Stella de Oro' (UA2), day flowering landscape plant of unknown parentage, was grouped with *H. minor* collected from Korea (K14), but not with *H. minor* (UK6 and GE3) accessions and their seedlings (K14/1-3) and UK6/1-3, based on the haplotypes of SNPs with a nrITS region (Table 2) and with cpIS (Table 3). Grouping of *H. dumortieri* (K13) with *H. minor* (UK6 and GE3) with nrITS region was also different from that with cpIS (Table 3). McGarty (2006) used AFLP markers from *Hemerocallis* species, and placed *H. lilioasphodelus* with *H. thunbergii* in one sub-group and *H. citrina*, *H. minor*, and *H. dumortieri* in another. This suggests that day and night flowering species cannot be separated either by AFLP markers, or by SNPs from a nrITS region or cpIS, as attempted in this study. Difficulties with identifying species of *Hemerocallis* native to Korea may not be easy to resolve and flowering time in F_1 hybrids between *H. fulva* (day flowering) and *H. citrina* (nocturnal flowering) showed discontinuous bimodal distribution (Hasegawa *et al*., 2006).

Beyond the identification issue for the accessions *H. thunbergii* Ka8-13, grouping of species investigated in this study differs significantly between nrITS and cpIS (Tables 2 and 3). Difficulties with identifying species of *Hemerocallis* native to Korea may not be easy to resolve and flowering time in F1 hybrids between *H. fulva* (day flowering) and *H. citrina* (night flowering) showed discontinuous bimodal distribution (Hasegawa *et al*., 2006).

Distinguishing nocturnal flowering forms of *H. minor* from day flowering forms is not possible due to the existence of two different genotypes collected from China, Korea and far eastern Russia and by testing the universal primers by SNPs of nrITS region and cpIS. However, these primers were used successfully to identify mother plants and seedlings of *Ligustrum quihoui* Carrière (Ma *et al*., 2014).

Table 2 - Haplotype based on single nucleotide polymorphisms (SNPs) and insertion and deletion (IN/Del) of a nuclear internal transcribed spacer 1, 2 in a ribosomal RNA gene of Hemerocallis accessions

Haplotype	NCBI Registration	Positions and codes of nucleotide												
		Single nucleotide polymorphisms												In/Del
		78	86	99	113	120	170	206	210	231	254	432	568	597	435-441
I-1	KT189161	C/T	C/T	C	C/T	C/T	A	G	A	A/G	A	A	G	G	C7
I-2	KT189162	C	C	C	C	C	A	G	A	A	A	A	G	G	C6-7
II	KT189163	C	C	T	C	C	G	C	C	C	C	G	A	A	C7

Table 3 - Haplotypes based on single nucleotide polymorphisms (SNPs) and insertion and deletion (In/Del) of a chloroplast interspacer region of *Hemerocallis* accessions

Haplotype	NCBI registration	Positions and codes of nucleotide							
		Single nucleotide polymorphisms						In/Del	
		26	216	243	246	291	315	37	302
I	KT189164	T	C	C	C	A	A	T9	T7
I-1	KT189165	T	C	C	C	A	A	T9	T8
II	KT189166	T	C	C	C	C	A	T9	T8
III	KT189167	T	C	C	A	A	A	T9	T8
IV	KT189168	C	C	A	A	A	A	T8	T8
V	KT189169	C	C	C	A	A	A	T8	T8
VI	KT189170	C	A	C	A	A	A	T10	T8

Seedlings grouped in the haplotype with their mother plants, except the mother plant (C1) and seedlings 2-3 of *H. liloasphodelus* (C1/2-3), which belonged to type I, type I-2 and I-1, respectively, in nr-ITS region (Tables 1 and 2). The current primers for nrITS region and cpIS cannot be used to differentiate nocturnal flowering species from day flowering species. Lee and Maki (2015) reported that cpDNA in the majority of cultivars were inherited from *H. albomarginata*, although the leaf morphology was similar to *H. sieboldiana*, indicating that nrITS should further be investigated.

4. Conclusions

The sequence variations of nrITS region and cpIS cannot be used to distinguish nocturnal flowering species from day flowering *Hemerocallis* species. Markers other than those evaluated in this study should be evaluated. Genetic variations among seedlings or between mother plant and their seedlings were observed in *H. lilioasphodelus* (C1/1 vs. C1/2-3), requiring seedlings of *H. lilioasphodelus* to investigate to confirm the results of this study. Discrepancies in flowering time among *H. minor* accessions also suggest that more germplasm with diverse geographic origins should be evaluated.

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