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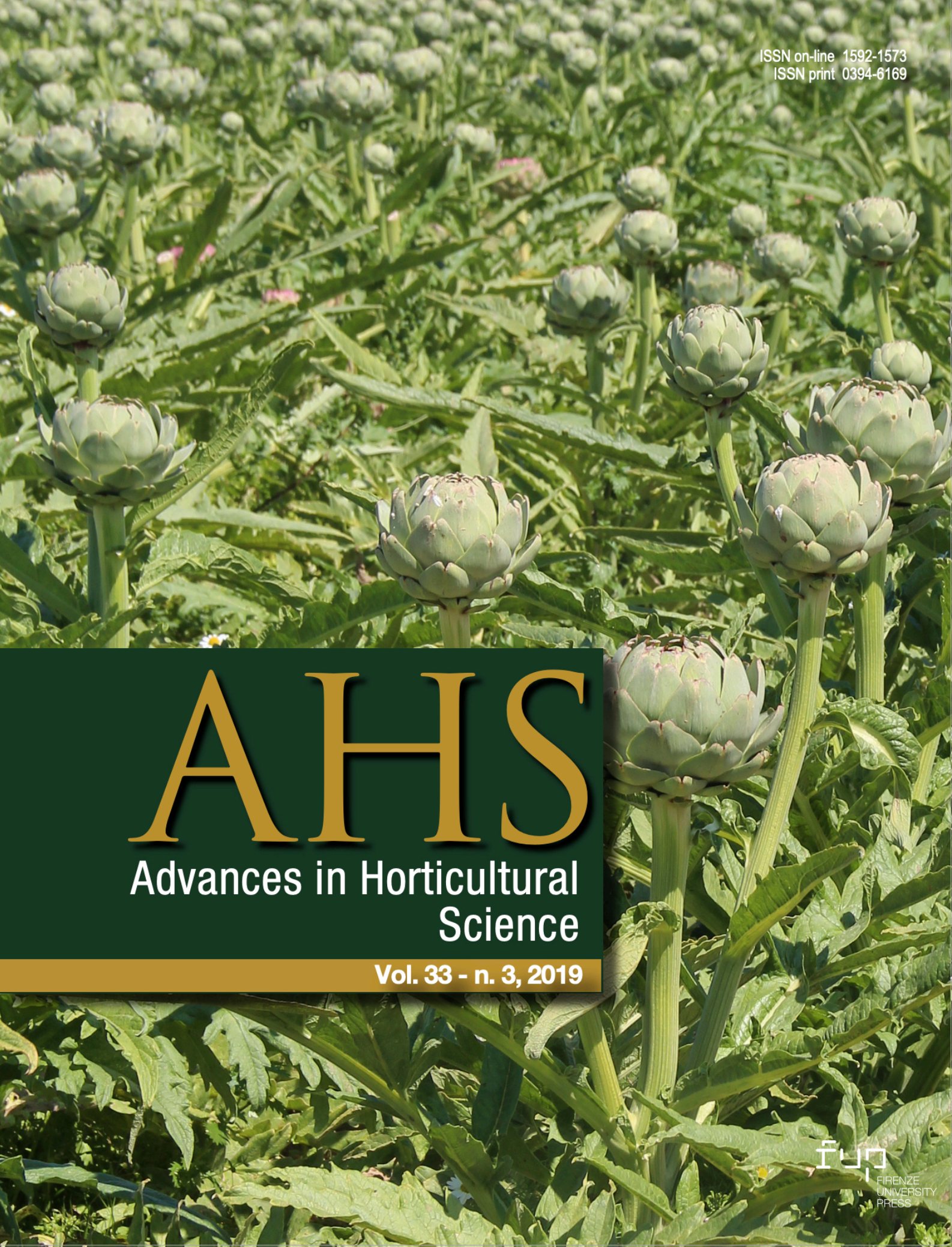

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Short Note

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Descriptions of okra seed longevity loss behavior using nonlinear regression models

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Abstract: Seeds longevity loss is an inevitable problem of seed storage. Applications of nonlinear regression models to describe and predict the aging damages to seed germination would be reliable and helpful for understanding the relationship between seed quality and storage condition. In this study, various nonlinear models, including logistic, Hill, Weibull, Sigmoid, Gompertz and Probit, were applied on seed germination data, obtained from the accelerated aging test of three Iranian okra landraces. Results revealed that the Weibull 4 parameter and Probit 4 parameter functions failed to describe cumulative germination of Ahwaz ecotype in contrast to sigmoid models. The best three parameters sigmoid model to describe germination data of Isfahan ecotype was Hill 3p (AICc=26.89) while there was a failure to fit germination data using Weibull 4p and Probit 4p. Mashhad germination and vigor was well described using Hill 3p (AICc=33.72 and 32.22). It is suggested that the use of the Hill, Gompertz and Weibull parameters provided more information of viability and vigor loss of okra seeds during deterioration conditions.

1. Introduction

Okra is a crop belonging to Malvaceae family, largely cultivated in Africa and Asia (Düzyaman, 2005; Sorapong, 2012). It is mainly planted for its fresh, tender and tasty pods as food purposes alongside high generic medical or industrial potential applications (Dhankhar and Singh, 2009). Recent researches revealed that okra seed oil has a great potential use as industrial products and liquid biofuels (Anwar *et al.,* 2010; Moosavi *et al.,* 2018).

Good seed quality assures rapid, uniform seed germination and healthy seedling establishment.

Seed germination as the complex physiologically active process is initiated with water uptake by dry seeds and completed by radicle protrusion from the seed coat (Bewley and Black, 1994; McDonald and Kwong, 2005).

Germination characteristics of okra seeds depend at least partly on the

duration, temperature and relative humidity of storage condition. Seeds from different geographical locations commonly react differently to germination conditions (Campbell and Sorensen, 1979). Okra is a crop of warm and humid climate and when it comes to seed production, one of the main concerns would be storage damages to see due to storage conditions (George, 2009). Understanding the pattern of seed quality loss under various storage conditions would be very useful for okra seed producers to estimate their seed loss and find solutions for better seed storage condition.

The conventional method of seed germination data analysis depends on the single-value indices or descriptive statistics such as mean, variance, final germination, mean germination time to represent the results of experimental treatment (Shafii *et al.,* 1991). However, such indices retain no information on either the initiation or rate of germination (Brown and Mayer, 1988; McNair *et al.,* 2012). Moreover, there is no room to put information of lag, speed, and extent phase of germination into the single value data. Another concern of statistics in seed germination is the skewness of germination frequency (Soltani *et al.,* 2016). Therefore, cumulative germination will not only provide ambiguous information about seed germination but also it will make much easier to understand and amenable to statistical analysis (Brown and Mayer, 1988).

Regression analysis studies the relationship between dependent variable y and one or more explanatory or independent variables x_i. Basic model is:

$$Y_i = h\,(x_i\,(1), x_i\,(2), \ldots\ldots, x_i(n); \theta_1, \theta_2, \ldots, \theta_{pi}) + E_i\,.$$

Where h is an appropriate function that depends on the explanatory variables and parameters.

Application of nonlinear model in cumulative data analysis is a powerful tool to predict and understand the changes in cumulative germination data (Yin *et al.,* 1995; Aparecida Guedes *et al.,* 2014). The logistic model is a nonlinear regression method, which developed for the sigmoid models. It is symmetric about the inflection point but its application to fit germination data needs special care due to the skewed nature of such data (Shafii *et al.,* 1991). In contrast to logistic, Gompertz is asymmetric sigmoidal function. The Gompertz model was first introduced to fit the relationship between increasing death rate and age by the Mr. Benjamin Gompertz and now it is one of the most useful sigmoid models fitted to different data types including growth data (Gompertz, 1825; Tjørve and Tjørve, 2017). The Weibull function has widely been used by seed researchers to explain germination data (Scott *et al.,* 1984; Dumur *et al.,* 1990; Akbari *et al.,* 2016). This regression model works well with small sample data and provides reasonable, simple and useful failure graph and analysis (Abernethy, 2006). The four-parameter Hill function was useful to explain seed germination variations among different seed lots of coniferous by incorporating germination rate, germination onset and cumulative germination data (El-Kassaby *et al.,* 2008). Probit model analysis has been widely applied to seed longevity experiments to estimate the probabilities of germination at each level of treatment (Probert *et al.,* 2009; Gazola *et al.,* 2015).

Large amounts of naturally existing plants produce seed populations with a different degree of germinability (Meyer and Kitchen, 1994; Gesch *et al.,* 2016). Prediction of germination behavior of such germplasm will help us to provide a better genetic pool to breed better crops.

The aim is also to compare different methods of nonlinear regression models for the description of okra seed germination behavior after exposure to the accelerated aging condition.

2. Materials and Methods

Seed materials

The okra seeds were purchased in dry state form local seed stores of Ahwaz, Isfahan and Mashhad. Seeds were then cleaned from the white pappus and sent immediately to seed technology laboratory of the department of plat production and genetics. All seed germination measurements were performed based on the ISTA rules for seed testing (ISTA, 2013).

Accelerated aging test

For the accelerated aging test, 200 mL of distilled water were added to each plastic box (20*15*10 cm) and 200 seeds were placed on a wired mesh tray (19*14*10 cm) inside the box. To avoid the direct content of seed with water, they were placed in the middle part of the tray. Seeds were aged at 40°C and 99% humidity for 24, 48, 72 and 96 h using one box for each aging/time combination (Demir *et al.*, 2004; Souza *et al.,* 2017). Seeds were disinfected before standard germination test using 3% solution of NaOCl (Sauer and Burroughs, 1986).

Germination test

To test the germination of Okra seeds, 50 seeds

were disinfected with 3% sodium hypochlorite for 5 minutes, then cultured by sandwiching between two layered filter papers. The counting of germinated seeds was done regularly after every 24 h and the appearance of 2 mm or more of radicle was considered as germination. Germination test was ended after 14 days when the number of germinated seeds was equal in two sequential counting. The seedling size was measured on the last day of the germination test. Final germination percentage (FGP) was calculated using the following formula (Sumithra *et al.,* 2006):

$$FGP\ (\%) = \frac{\text{Number of total geminated seeds}}{\text{Total number of seeds tested}} \times 100$$

Seedling vigor

Seedlings vigor was determined when the number of germinated seeds at the two subsequent counts remained constant (day 14th). All seedlings that had completed morphological parts without lesions or defects, were selected and computed as vigorous seedlings and average seedling length and weight of 10 seedlings were measured for calculating seedling vigor index (SVI) by a modified formula of (Abdul-Baki and Anderson, 1973; Williamson and Richardson, 1988):

$$SVI = \frac{FGP\ (\%) \times \text{means of seedling length (cm)}}{100}$$

Statistics

The experimental design was a factorial experiment fitted into the randomized complete design with three replications. Treatments were three Okra ecotypes and five durations (0, 24, 48, 72 and 96 h) of accelerated aging conditions arranged as the first and second factor, respectively. All acquired experimental data were subjected to fit with non-linear regression models to evaluate model parameters and their performance of data explanations. Models applied were:

i) Sigmoid 3 parameter: $Y= \frac{a}{1+e^{-\left(\frac{x-x0}{b}\right)}}$ and Sigmomoid 4 parameter: $Y=y_0\frac{a}{1+e^{-\left(\frac{x-x0}{b}\right)}}$

ii) Standard Logistic 3 parameter: $Y= \frac{a}{1+\left(\frac{x}{x0}\right)^b}$ and Logistic 4 parameter: $Y=y_0\frac{a}{1+\left(\frac{x}{x0}\right)^b}$

iii) Gompertz 3 parameter: $Y =ae^{-e^{-\left(\frac{x-x0}{b}\right)}}$ and Gompertz 4 parameter: $Y =y_0+ae^{-e^{-\left(\frac{x-x0}{b}\right)}}$

iv) Hill 3 parameter: $Y= \frac{ax^b}{c^b+x^b}$ and Hill 4 parameter: $Y=y_0+\frac{ax^b}{c^b+x^b}$

Where *a* is Y_{Max} or *upper asymptote*, *b* was a *slope*, x_0 *Critical point* or the *x* that reached 50% of Y_{Max} and y_0 is a lower *asymptote.*

Variable "y" corresponds to the germination percentage and "x" to the time of accelerated aging, respectively.

Also, other non-linear regression models included:

vi) Weibull 4 parameter:

$$Y= a\left[1-e^{-\left[\frac{x-x0+bln\,2^{\frac{1}{c}}}{b}\right]^c}\right]$$

vii) Probit 4 parameter:

$$Y= y_0+ (a-y_0) \times \text{normal distribution}\left(\frac{(x-x0)}{b}\right)$$

Where *a* is Y_{Max} or *upper asymptote*, *b* is the *slope*, x_0 is the c*ritical point* or *the x* that reached 50% of Y_{Max} and y_0 is a *lower asymptote.*

Sigma plot v. 11 was used for calculating the type of regression equation. R^2, AICc, and RMSE was applied to determine the best estimates of the parameters. R^2 was calculated using the following formula:

$$R^2= SSR/SST$$

Where SSR denotes the sum of squares (SS) for regression ($\sum^n_{i=1}$ *L*-$\bar{L}$) and SST the total SS ($\sum^n_{i=1}$ *Li*-$\bar{L}$). *Li* is the *observed value* and $\bar{L}$ is the *corresponding estimated value*. In addition, root mean square error (RMSE) calculated using following the formulae:

$$RMSE =\sqrt{(1/n)} \sum (Y_{obs}-Y_{pred})^2$$

Where Y_{obs} denotes *observed* value, Y_{pred} *predicted* value, and *n* is the *number of samples*.

To identify the best model for estimating, the Akaike Information Criterion corrected (AICc) was used. This statistic incorporates the amount of reduction of RSS and the model complexity (Butler and King, 2004; Kamkar *et al.,* 2012).

$$AICc=n \ln + (RSS/N) + 2K\,[2K\,(K-1)]/(N-K-1)$$

Where *n* is *number of data points*, and *K* is the *number of parameters* in the model.

3. Results and Discussion

The data subjected to model data analysis were from an experiment to study seed longevity loss during accelerated aging conditions. Main investigated traits were cumulative seed germination and seedling vigor. Results of fitted parameters with both three and four parameters nonlinear regression models are presented in Table 1. Among investigated ecotypes,

Table 1 - Comparative indices of models performance to describe cumulative germination data of three okra ecotypes (Ahwaz, Isfahan and Mashhad) under deterioration conditions

Model		Normal			24 h			48 h			96 h			192 h		
		Ahwaz	Isfahan	Mashhad	Ahwaz	Isfahan	Mashhad	Ahwaz	Isfahan	Mashhad	Ahwaz	Isfahan	Mashhad	Ahwaz	Isfahan	Mashhad
Sigmoid 3P	R^2	0.998	0.996	0.994	1.00	0.997	0.998	0.999	0.992	0.961	0.989	0.978	0.968	1.00	0.983	0.982
	RMSE	1.74	2.70	3.31	0.31	1.98	1.38	0.49	3.25	7.03	4.74	6.45	6.90	0.14	4.01	1.42
	AICc	25.29	29.08	30.85	10.55	26.39	23.28	14.41	30.70	37.40	33.97	36.64	37.23	3.38	32.52	23.52
Logistic 3P	R^2	0.996	0.999	0.997	0.999	0.992	0.999	0.997	0.994	0.994	0.988	0.972	0.964	0.999	0.993	0.983
	RMSE	2.93	1.54	2.20	0.43	3.61	0.52	2.11	2.76	2.76	4.79	6.55	7.31	0.23	2.46	1.41
	AICc	29.80	24.24	27.30	13.20	31.60	14.78	26.96	29.26	29.29	34.07	36.77	37.73	8.00	28.29	23.48
Gompertz 3P	R^2	0.993	0.999	0.998	0.999	0.996	1.00	0.996	0.996	0.978	0.988	0.978	0.966	1.00	0.992	0.984
	RMSE	3.75	0.97	1.94	0.50	2.63	0.12	2.48	2.06	5.20	4.81	6.57	7.12	0.21	2.70	1.40
	AICc	31.94	20.20	26.22	14.53	28.58	2.53	28.34	26.73	34.7	34.09	36.81	37.50	7.31	29.10	23.37
Hill 3P	R^2	0.996	0.990	0.997	0.999	0.992	0.999	0.997	0.994	0.994	0.988	0.978	0.964	0.999	0.993	0.983
	RMSE	2.93	1.54	2.20	0.43	3.61	0.52	2.11	2.76	2.46	4.79	6.55	7.31	0.23	2.46	1.41
	AICc	29.80	24.24	27.32	13.20	31.60	14.78	26.96	29.26	29.29	34.07	36.77	37.73	8.00	28.29	23.48
Sigmoid 4P	R^2	0.998	0.997	0.996	1.00	0.998	0.999	0.999	0.992	0.961	0.993	0.987	0.968	1.00	0.987	0.982
	RMSE	1.83	2.65	3.10	0.27	1.81	1.35	0.53	3.51	7.60	3.90	5.44	7.42	0.126	3.66	1.53
	AICc	30.54	33.76	35.12	14.20	30.45	27.93	19.86	36.20	42.90	37.12	40.0	42.69	7.32	80.49	29.02
Logistic 4P	R^2	0.997	0.999	0.997	1.00	0.992	0.999	0.998	0.994	0.994	0.993	0.987	0.969	0.999	0.994	0.983
	RMSE	2.84	1.60	2.35	0.35	3.90	0.56	1.69	2.98	2.98	3.88	5.41	7.34	0.25	2.52	1.52
	AICc	34.35	29.39	32.72	16.23	37.10	20.28	29.85	34.76	34.79	37.07	39.95	42.60	13.50	33.32	28.97
Gompertz 4P	R^2	0.996	0.999	0.998	0.999	0.996	1.00	0.998	0.997	0.978	0.993	0.987	0.968	1.00	0.992	0.983
	RMSE	3.11	1.04	2.089	0.38	2.83	0.136	1.91	2.14	5.61	3.88	5.41	7.48	0.235	2.80	1.511
	AICc	35.18	25.66	31.68	17.06	34.34	7.99	30.91	31.9	40.27	37.07	39.95	42.76	12.73	34.24	28.86
Hill 4P	R^2	0.997	0.999	0.997	1.00	0.992	0.999	0.998	0.994	0.994	0.993	0.987	0.969	0.999	0.994	0.983
	RMSE	2.84	1.60	2.35	0.35	3.90	0.56	1.69	2.98	2.98	3.88	5.41	7.34	0.25	2.52	1.52
	AICc	34.35	29.39	32.72	16.23	37.10	20.28	29.85	53.3	34.79	37.07	39.95	42.60	13.50	33.32	28.97
Weibull 4P	R_2	0.999	0.999	0.998	0.999	0.999	1.00	0.999	0.998	0.995	0.989	0.980	0.966	1.00	0.996	0.985
	RMSE	0.40	0.67	1.77	0.54	1.20	0.133	0.47	1.67	2.60	4.93	6.76	7.70	0.002	2.002	1.53
	AICc	17.48	21.92	30.25	20.02	26.89	7.77	18.87	29.78	33.6	39.14	41.89	43.01	0.001	31.31	28.19
Probit 4P	R_2	0.998	0.997	0.996	0.999	0.999	0.998	0.999	0.998	0.994	0.993	0.986	0.967	1.00	0.985	0.982
	RMSE	1.71	2.51	3.03	0.44	1.23	1.83	0.77	1.38	2.77	3.94	5.49	7.57	0.017	3.85	1.53
	AICc	59.03	66.74	70.49	32.08	52.53	60.3	43.04	54.77	68.69	75.70	82.35	88.75	0.00	75.27	56.9

Mashhad ecotype was more susceptible to longevity loss, especially at higher aging stress durations (Fig. 1, A_1-A_5). Among four parameter models, Weibull and Probit functions were produced the best curve fit as illustrated in figure 2 (E_1-E_5; F_1-F_5).

Our results revealed that at most aging treatments, the best-fitted data obtained from three parameter nonlinear regression models (Fig. 3).

The differences in parameter estimates among aging treatments had biological significance. The maximum germination, estimated by the parameter *a* (asymptotic part of the model), decreased as aging duration increased meaning that aging damages decrease the proportion of seeds which are able to survive and complete germination by radicle protrusion. The estimates of parameter *b*, which is the rate of seed germination were also reduced due to the increase in aging durations (Fig. 3). Deterioration damages due to oxidative stress caused by aging resulted in a reduction of seed germination properties and aged seeds germinated slower than no aged seeds (Anderson and Baker, 1983; Goel *et al.,* 2003; Balešević-Tubić *et al.,* 2007; Parmoon *et al.,* 2015).

Under no aging conditions, there was a significant difference in the estimated parameter for all three okra ecotypes. Using sigmoid 3p, Weibull 4p models parameter a, was well predicted and as shown in figure 3 it was very close to actual observed value (dash red line). Our results revealed that only Gompertz model did not provide precise estimation for Parameter x_0 (the time required for 50% of viable seeds to germinate) while other 3 parameter models performed well. Parameter X_0 in Ahwaz and Isfahan ecotypes well fitted using 4p Weibull and Hill while in Mashhad ecotype probit model was closer to the observed value.

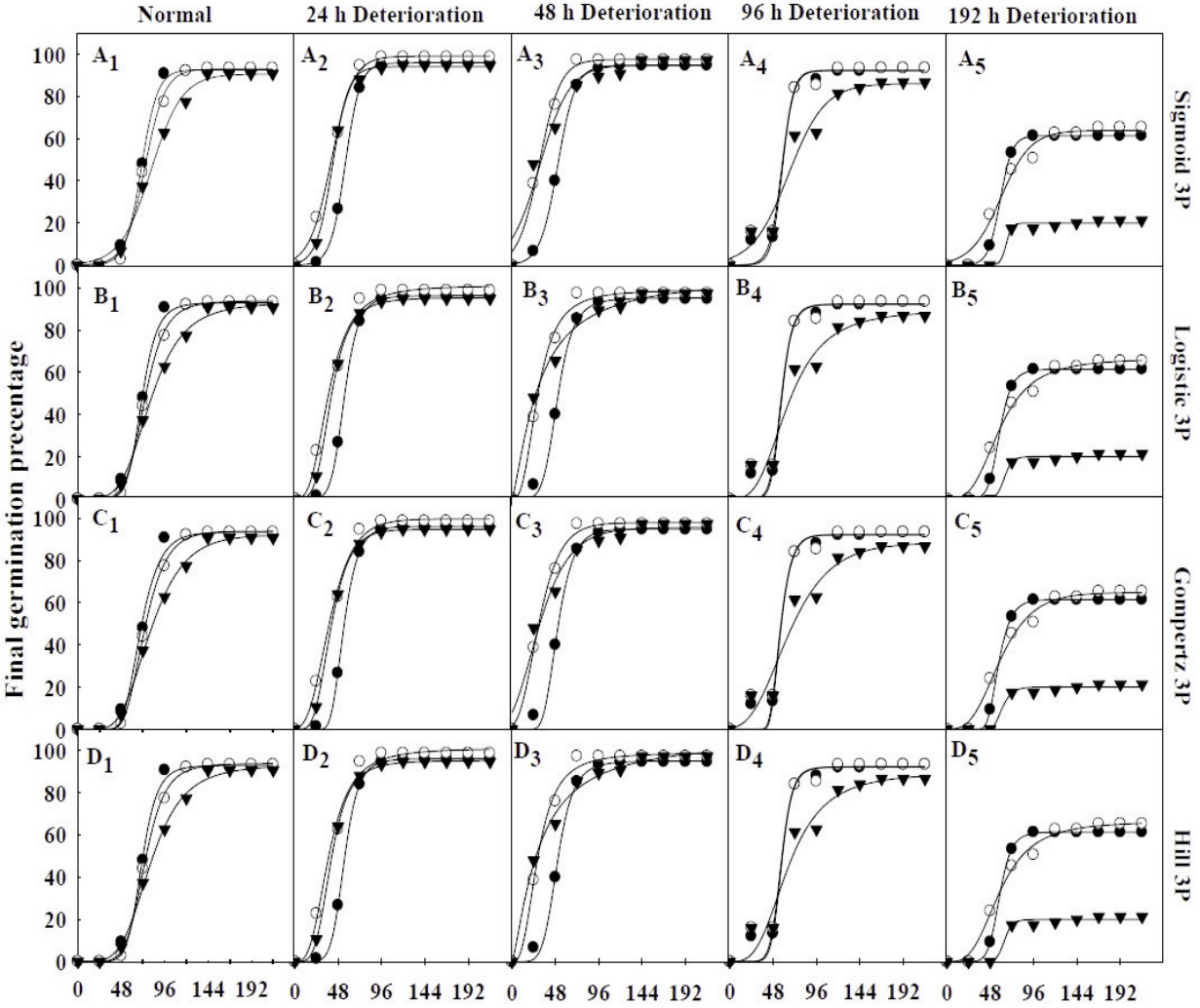

Fig. 1 - Model fit function 3 parameter of germination ecotypes okra (*Abelmoschus esculentus* L.) under deterioration condition. ● Ahwaz, ○ Isfahan and ▲ Mashhad ecotypes.

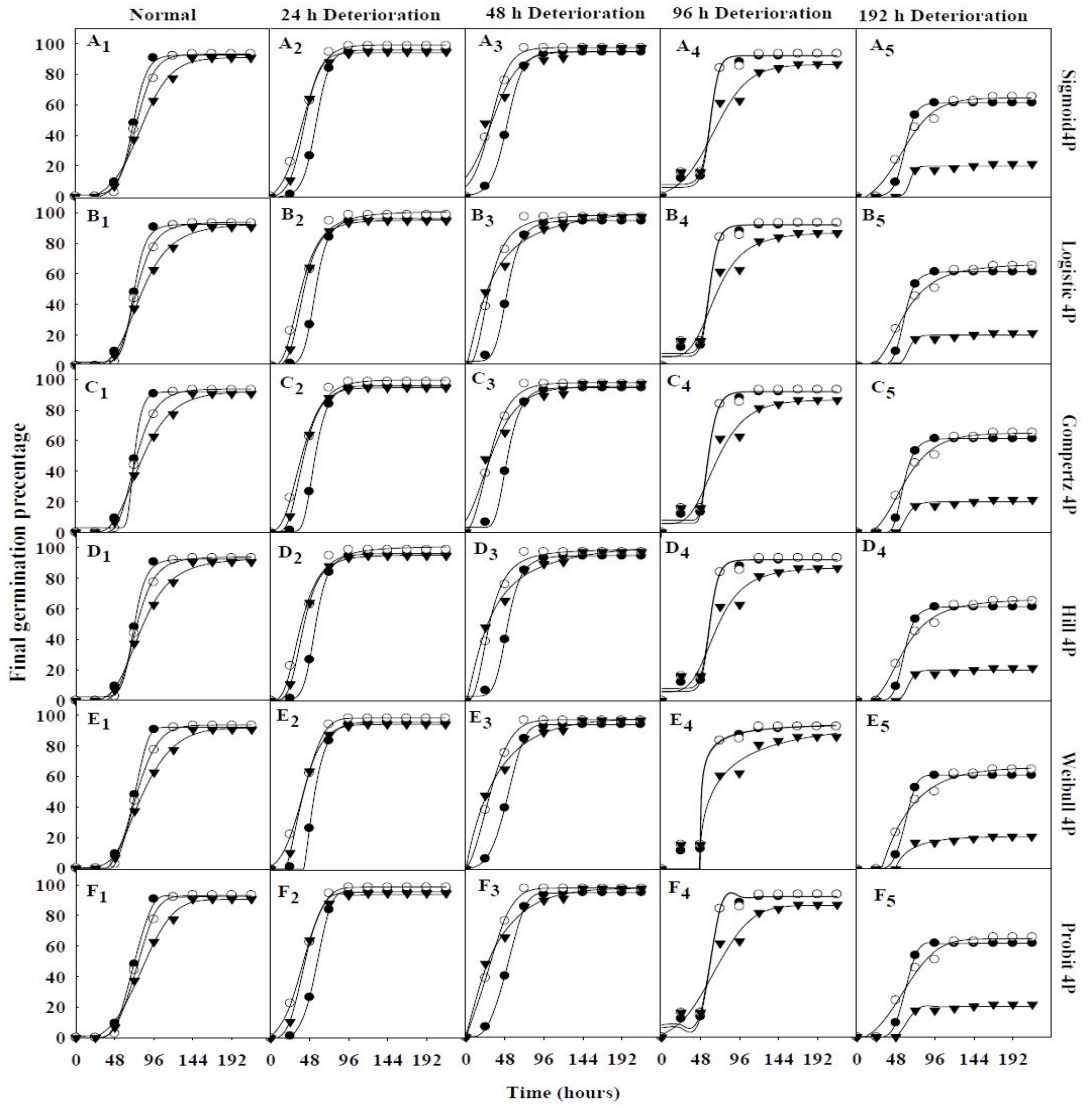

Fig. 2 - Model fit function 4 parameter of germination ecotypes okra (*Abelmoschus esculentus* L.) under deterioration condition. ● Ahwaz, ○ Isfahan and ▲ Mashhad ecotypes.

Accelerated aging (deterioration) after 24h, resulted in a reduction of X_0 value compared to no aged seeds (normal condition). Results revealed that an increase in deterioration treatments led to a decrease in observed maximum germination and predicted parameter.

Model output for parameter a (the maximum germination) of Ahwaz ecotype using sigmoid 3p model was less than the observed value. The result of the Weibull 4p function showed that the predicted parameter a, was the same as the maximum value of observed germination. There was no difference in estimated parameter a (asymptote), for Isfahan ecotype using Gompertz 3p and Weibull 4p. Model performance to predict germination of okra ecotypes under deterioration conditions varied among regression models (Table 1). For instance, at no aged treatment (normal condition), the best model to predict cumulative germination of Ahwaz ecotype were Weibull 4p and Sigmoid 3p with AICc values of 17.48 and 25.29 respectively. Results showed that, at the 96 h of accelerated aging conditions, the best fit and the lowest AICc values were obtained from three parameter growth models. In the case of Mashhad ecotype, the Gompertz 3p provided the best model fit to estimate germination loss at both no aged and severe aging treatments with the AICc values of 23.37 and 26.22, respectively. Application of regression models will result in various measures of the asymptotic point of Cumulative seed germination curves (Aparecida Guedes *et al.,* 2014). To compare the model performance of parameter estimation, AICc was a well discriminant index (Hurvich and Tsai, 1989; Spiess and Neumeyer, 2010). The lower AICc,

the better parameter estimation of the model is expected (Sandvik, 2008).

Under 48 and 96 hours of deterioration, the estimated parameter a using Sigmoid 4p, Logistic 4p and Hill 4p, was less than the maximum observed germination. Our results showed that the sigmoid 3p model predicts lower values for actual maximum germination, while Sigmoid 4p and Logistic 4p estimated higher values especially in Isfahan ecotype seeds.

Weibull was the best model to show the alteration of the parameter *b* (rate of increase in seed germination), at different deterioration durations. Aging reduced the value of parameter b, which mean that deterioration because the reduction of seed germination rate of seed lot compared to no aged (normal condition) seeds (Fig. 3).

Results of experiments suggested that the Weibull model was the best among all of four parameter models for cumulative germinating data of Ahwaz and Mashhad ecotypes while Gompertz 4 parameter performed well for the fitting of Isfahan data.

The Study of the germination data of deterioration seeds after 24 hours showed that the best three parameter model to fitted data of Ahwaz and Isfahan ecotypes was Sigmoid 3p (AICc= 10.55 and 26.39) while Gompertz exhibited the best fit for Mashhad ecotype (AICc= 2.53) (Table 1). Germination data of deterioration seeds for 48 hours were best-fitted using Sigmoid 3p, Gompertz 3p and Hill 3p in Ahwaz, Isfahan and Mashhad ecotypes respectively. Among four parameter models, the best fit data was Weibull 4p for all ecotypes. Results of fitting cumulative ger-

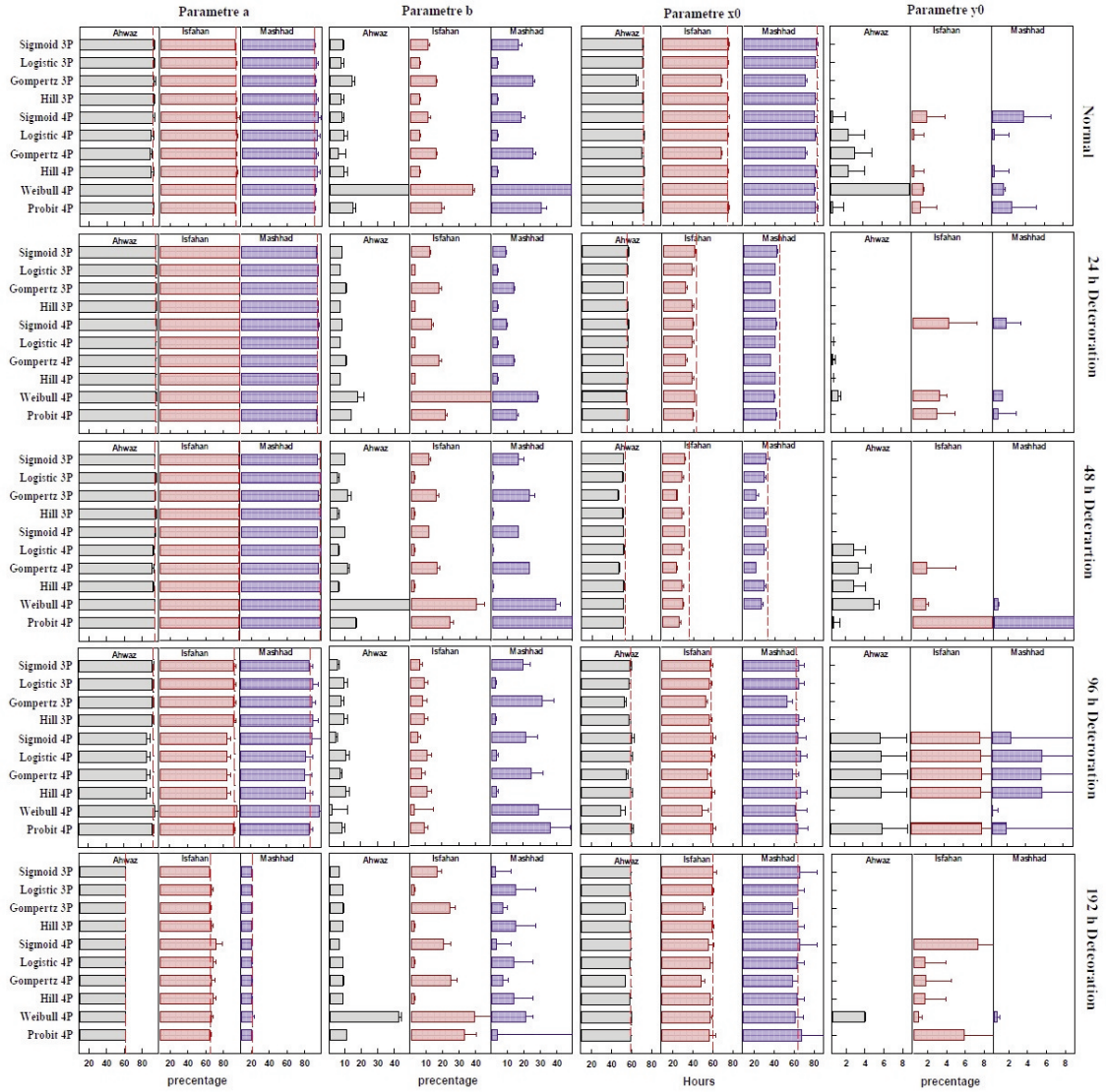

Fig. 3 - Estimate parameter of fit models germination ecotypes okra (*Abelmoschus esculentus* L.) under deterioration condition. Dash red line indicated observed parameter value. Parameter a= Asymptote (theoretical maximum for y); parameter b=rate of increase; X0= time required to completion of germination in 50% of seed population or D50; Y0= initial seed germination.

mination data of deterioration seeds for 96 hours showed that the sigmoid 3p model was the best model to describe data. Among four parameter models, Gompertz and Hill both performed well to describe germination data of Ahwaz and Isfahan ecotypes having similar AICc, while Mashhad ecotype was well-fitted using Logistic 4p and Hill 4p.

The study performed at the 192 hours of deterioration showed that the sigmoid 3p was the best-fit model for Ahwaz, Isfahan while for the Mashhad ecotype Gompertz was the best model (Table 1). It is concluded that the Weibull 4p was the best-fit model to describe germination data of okra seeds.

Seed vigor is an important index of seed quality and any change in this trait resulted in a reduction of seedling emergence. Among all three parameter models, Hill 3p well-illustrated changes of seed germination and vigor loss for Mashhad ecotype (Fig. 4). Model comparisons to describe germination and vigor of okra ecotypes during deterioration conditions revealed that Weibull 4p and Probit 4p functions failed to describe cumulative germination of Ahwaz ecotype in contrast to sigmoid models. The best 3p model to describe germination data of Isfahan ecotype was Hill 3p (AICc=26.89) while there was a failure to fit germination data using Weibull 4p and Probit 4p. Mashhad germination and vigor was well described using Hill 3p (AICc=33.72 and 32.22) (Table 2). Non-linear model fit to germination of vigor data of okra ecotypes showed that in almost all treatments decline of seed vigor initiated earlier than germination loss. However, predictions of vigor loss with an increase in deterioration treatments was not well described by four parameter models (Fig. 5). Application of logistic and Weibull functions to describe the germination and vigor of alfalfa seeds

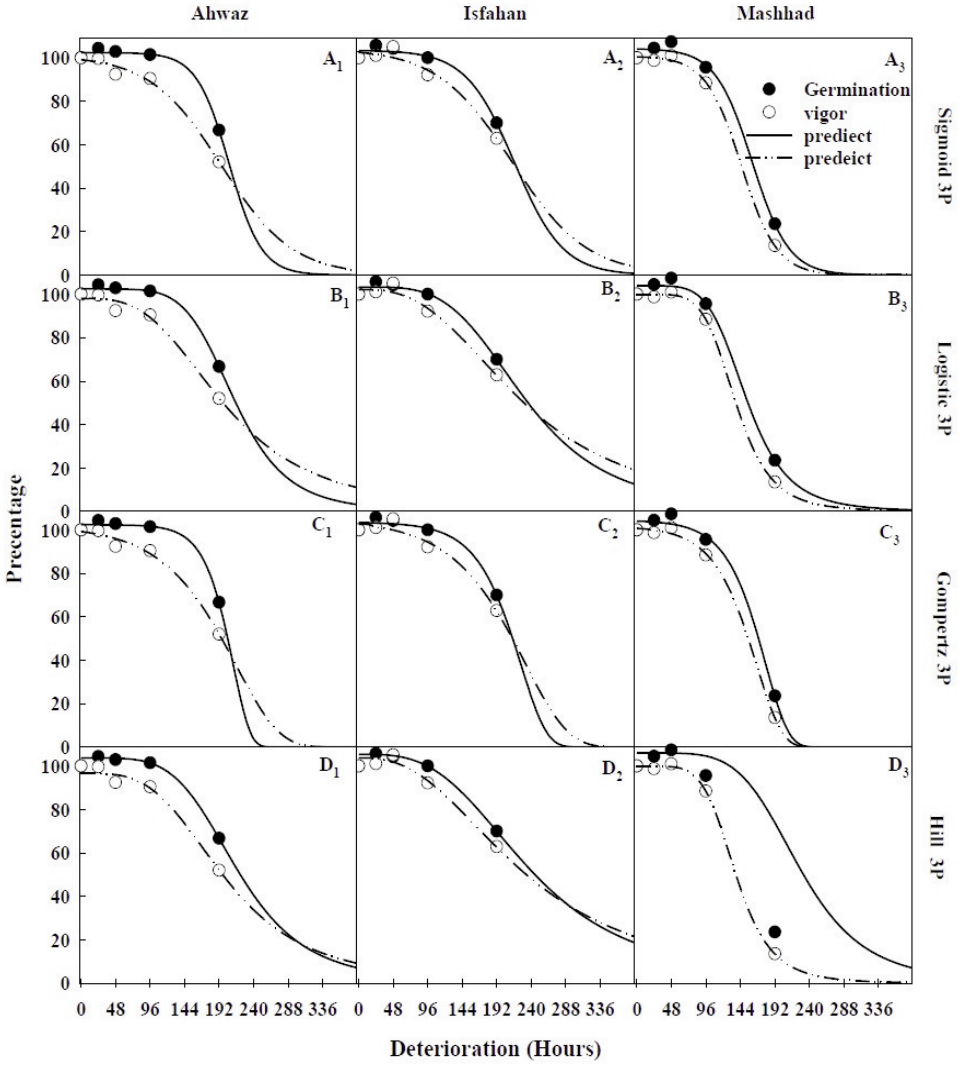

Fig. 4 - Model fit function 3 parameter of process deterioration germination and vigor ecotypes okra (*Abelmoschus esculentus* L.). Points are the percentage of improvements in seed properties compared with control treatment (no aged seeds) (● germination percentage, ○ seedling vigor) and lines are predicted values.

Table 2 - Comparative indices of models performance to describe seed germination and vigor of okra ecotypes

Model		Ahwaz		Isfahan		Mashhad	
		Germination	Vigor	Germination	Vigor	Germination	Vigor
Sigmoid 3P	R^2	0.990	0.987	0.976	0.968	0.992	0.998
	RMSE	2.23	3.11	3.18	4.29	4.28	1.79
	AICc	35.33	37.05	37.17	38.72	38.72	34.17
Logistic 3P	R^2	0.990	0.981	0.979	0.977	0.9942	0.999
	RMSE	2.21	3.80	3.02	3.59	3.80	1.23
	AICc	35.28	38.10	36.89	37.80	38.10	32.22
Gompertz 3P	R^2	0.990	0.988	0.976	0.967	0.991	0.998
	RMSE	2.23	30.046	3.20	4.37	4.49	2.18
	AICc	35.33	36.87	37.20	38.82	38.96	35.20
Hill 3P	R^2	0.991	0.984	0.999	0.984	0.998	0.999
	RMSE	0.68	3.34	0.44	2.97	1.64	1.23
	AICc	29.12	37.34	26.89	36.81	33.72	32.22
Sigmoid 4P	R^2	0.990	0.989	0.979	0.987	0.994	0.999
	RMSE	3.13	4.11	4.20	3.79	5.23	1.56
	AICc	25.74	30.17	30.36	28.77	33.78	14.92
Logistic 4P	R^2	0.990	0.985	0.979	0.987	0.994	0.999
	RMSE	3.13	4.87	4.20	3.79	5.23	1.56
	AICc	25.74	32.68	30.36	28.77	33.78	14.92
Gompertz 4P	R^2	0.990	0.977	0.979	0.987	0.994	0.999
	RMSE	3.13	6.04	4.20	3.70	5.23	1.56
	AICc	25.74	36.04	30.36	28.77	33.73	14.92
Hill 4P	R^2	0.998	0.981	0.999	0.992	0.999	0.999
	RMSE	1.02	5.09	0.49	2.84	2.07	1.55
	AICc	8.28	33.35	0.00	24.25	19.35	14.85
Weibull 4P	R^2	-	-	0.00	0.00	0.00	-
	RMSE	31.9	191.41	29.51	34.16	70.6	75.3
	AICc	62.05	90.05	60.82	63.11	74.46	75.48
Probit 4P	R^2	0.731	0.938	-	0.987	0.993	0.963
	RMSE	16.53	9.86	29.51	3.79	5.65	14.49
	AICc	51.76	43.68	60.82	27.77	35.02	49.7

showed that the Weibull parameter was more informative than the logistic (Bahler *et al.,* 1989). Among ecotypes, Mashhad ecotype vigor and germination data were fitted more uniformly to four parameter models. The Hill 4p was not capable of fitting germination data of the Isfahan ecotype. We found that none of the four-parameter models is suitable to describe seedling vigor loss of okra seeds due to accelerated aging condition. At high levels of aging treatments, seed germination was declined to zero and so there is no lower limit to produce Y_0 parameter, and like the results, model equations could not predict satisfied result (Fig. 6). Interestingly, results showed that aging condition did not exceed to deteriorative level before 24 hours and instead it might improve seed germination by providing after-ripening requirements.

4. Conclusions

In this study, we test the moments and indices of seed germination using growth models with three to four parameter. This method of data description provides sufficient information about the dispersion of germination rate in time and extent of germination due to aging conditions. The Hill and Weibull models were capable of good predictions across cumulative germination and seedling vigor data of three okra ecotypes. We recommend non-linear regression

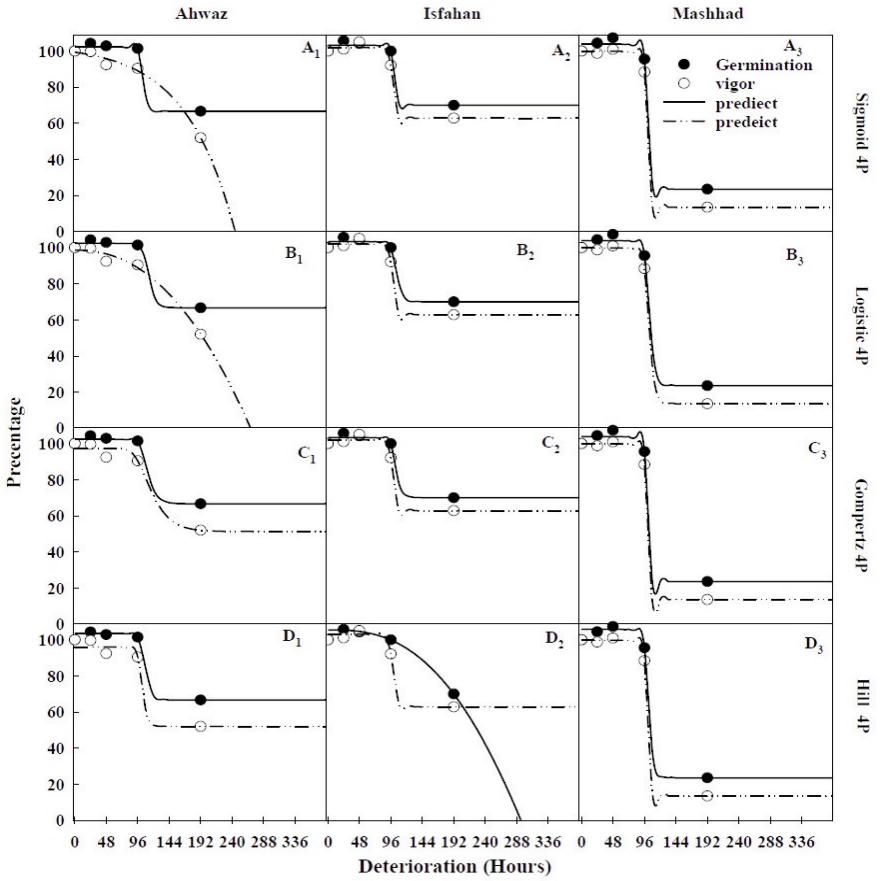

Fig. 5 - Model fit function 4 parameter of process deterioration germination and vigor ecotypes okra (*Abelmoschus esculentus* L.). Points are the percentage of improvements in seed properties compared with control treatment (no aged seeds) (● germination percentage, ○ seedling vigor) and lines are predicted values.

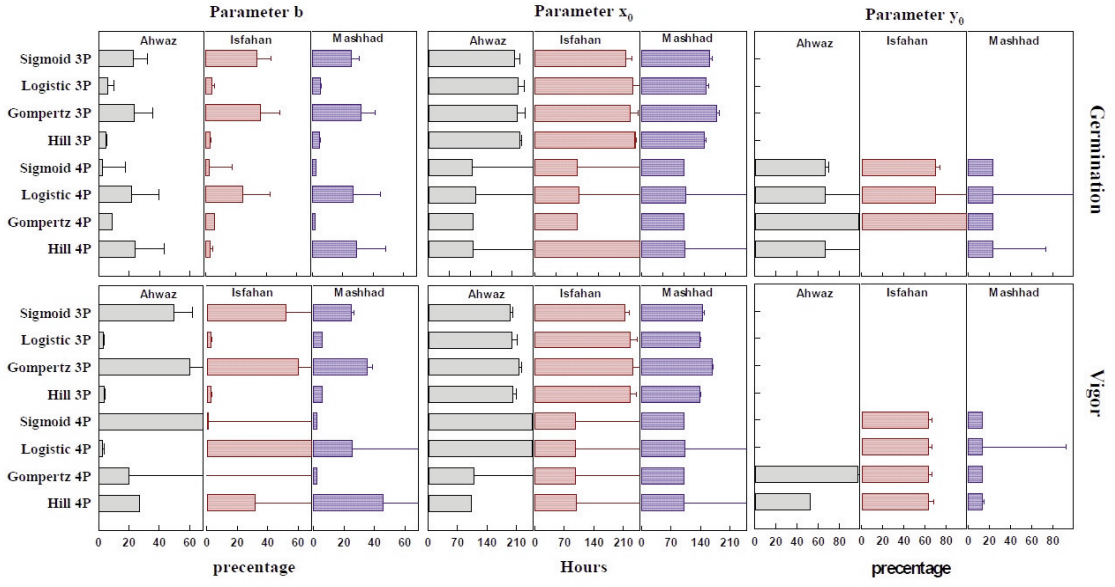

Fig. 6 - Estimate parameter of fit models process deterioration germination and vigor ecotypes okra (*Abelmoschus esculentus* L.). In these models germination and vigor were considered as 100%, and parameter a, for all models was 100%.

models as an unambiguous and strong approach to predict okra seed germination and longevity loss during aging conditions.

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Advances in Horticultural Science

Influence of two training systems on growth, yield and fruit attributes of four apple cultivars grafted onto 'M.9' rootstock

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Abstract: This research was carried out to compare several attributes pertaining to the growth, fruit and yield of four apple cultivars, i.e. 'Golab-kohans', 'Fuji', 'Starking' and 'Delbar estival'. These cultivars were grafted onto M.9 rootstocks trained into 'Guttingen V-slender-spindle (or V-system) and 'Geneva Y-trellis (or Y-system) systems. Compared to the Y-system, it was observed that the V-system caused the trees to yield more fruits, dry matter, ash and total soluble solids (TSS). In contrast, the Y-system caused the trees to have broader trunk cross sectional areas (TCSA), along with higher yield, fruit weight, fruit diameter, fruit length and fruit firmness, compared to trees trained with the V-system. In summary, these results showed that both systems can be employed as promising approaches, but the 'Y-system' appears to be more productive than the 'V-system'. In addition, among the studied cultivars, it seems that the 'Delbar estival' and 'Fuji' were more adaptive to these intensive training systems, especially when considering the fruit traits.

1. Introduction

Intensive training systems are particular layouts that assist orchard managers in improving the productivity of orchards (Ferree and Warrington, 2003). The need to improve training and pruning methods can better fit the natural growing conditions, and this can be associated with higher fruiting performances by the fruit trees (Lauri, 2009). Thus, modern apple orchards are planned on the basis of higher tree density than that of traditional planting systems which use dwarfing apple rootstocks (Ferree and Warrington, 2003). Dwarfing rootstocks are increasingly becoming prevalent among the sectors of the fruit industry. They are an important factor that improve orchard productivity due to their significant effects on agro-morphological characteristics such as the yield

(Barritt *et al*., 1995). The Guttingen-V system, the Y-system (Tatura), the Drilling system, and the Mikado system are the most popular V-shaped canopy systems, and are suggested as promising alternatives to high density orchards (Robinson, 2000). Dwarfing rootstocks, such as M.9 and M.27, are generally employed in V-shaped systems (Ferree and Warrington, 2003). V systems allow better light penetration than other training-shaped trees (Robinson, 2003). The 'Geneva Y-trellis' system is a V-shaped system which uses a Y shaped trellis to support the trees. The 'Guttingen V-slender-spindle' system includes individual conic-shaped trees allowing high tree densities within multiple rows. It has been reported that the Guttingen V causes the production of higher yield per hectare and thinner trunks, compared to the drilling system (Sosna and Czaplicka, 2008). Many investigations have shown that there are significant differences between local and foreign apple cultivars in terms of growth and productivity (Dadashpour *et al*., 2010; Dadashpour *et al*., 2011). Such reports also indicate the same with regard to apricot (Strikic *et al*., 2007) when trained by intensive training systems. Recently, it has been reported that rootstocks and training forms have significant effects on the vegetative growth, yield and fruit traits of apple cultivars (Alizadeh and Pirmoradiyan, 2016). It has been reported that the efficiency of several parameters can be improved by more production or by the reduction in tree size (Fioravanco *et al*., 2016). When apple scions are grafted onto dwarfing and semi-dwarfing rootstocks, they usually produce larger fruits and more yield, compared to when scions are grafted onto non-dwarfing rootstocks (Perry and Byler, 2001; Gjamovski and Kiprijanovski, 2011). Negligible differences have been reported in the cumulative yield among 'slender spindle', 'Hybrid Tree Cone' ('HyTec') and 'vertical axis' (Crassweller and Smith, 2004). Rutkowski *et al.* (2009) studied nine training systems for apple trees, and reported that the growth and yield of trees may be more dependent on genetic traits, while the shapes of trees can modify the skeletal structure of an orchard. To this end, Gonkiewicz (2011) showed that trees having spindle shapes can produce the best yield and fruit weight among the studied pruning systems in sweet cherry. By studying the 'Fuji' apple, grafted onto the M.9 rootstock under five training systems, Ozkan *et al.* (2016) reported that there were significant differences among the studied training systems in relation to canopy volume, trunk-cross sectional area (TCSA), yield, yield efficiency and fruit size.

With 2.8% of the total harvestable area (134,000 ha) and 2.2% of the total production (1.7 million tons) in the world, Iran is among the largest producers of apple after China, USA, Turkey, Poland, India and Italy (Faostat, 2012). The majority of apple orchards in Iran are traditional ones. They are characterized by low tree densities and are commonly grown on seedling rootstocks. However, semi-intensive and intensive apple orchards are recently becoming popular among apple growers. 'Golden Delicious' and 'Red Delicious' are two apple cultivars that are planted in about 90% of cultivated areas. Meanwhile, the early ripening cultivar 'Golab-Kohans' is the most prevalent, native apple cultivar in Iran. It provides the summer demand for fresh apples in the market. Furthermore, 'Granny Smith', 'Fuji', 'Gala', 'Jonagold', and 'Braeburn' are increasingly becoming popular in the country (Gharaghani *et al*., 2015).

As the apple industry in Iran is about to shift dramatically from traditional to modern production systems, e.g. semi-intensive and intensive orchard, it is important and necessary to study the performance of popular apple cultivars on different rootstocks, especially within the context of various training systems. Accordingly, the objective of this study was to evaluate two training systems, i.e. 'Guttingen V-slender-spindle' and 'Geneva Y-trellis', and compare their effects on growth characteristics, yield and fruit quality of four apple cultivars. Their scions were grafted onto M.9 rootstocks in the Alborz Province of Iran.

2. Materials and Methods

Plant materials and experimental design

This research was conducted at an experimental field belonging to a horticultural research station, Karaj, Iran. The duration of the entire experiment took from 2007 to 2010. The average maximum temperature of the region is 13.7°C, with an annual rainfall of 254 mm. The soil in the region is classified as clay-loam. The experiments were arranged as spilt-plot (main plot: training system; split-plot: cultivar) according to a randomized complete block design (RCBD) with four replicates. Four apple cultivars were used, i.e. 'Delbar estival', 'Fuji', 'Golab-kohans' and 'Starking', and their scions were grafted onto dwarfing M.9 rootstocks. All trees were planted in March 2005, and trellis systems were established in June 2006. The trees were trained into two training systems, i.e. 'Guttingen V-slender-spindle' (V-system) (0.9×3.7 m or 3000 trees/ha) and 'Geneva Y-trellis'

(Y-system) (1.6×3.7 m or 1680 trees/ha), based on the relevant protocols described by previous research on apples (Robinson, 2003). Drip-irrigation was scheduled to operate twice a week. The soil was fertilized once in every season and was managed according to the common practice in the region. Trees received their first fertilizers in the second year after planting. They were pruned during the winters, but the amount of wood being removed by pruning was not documented. Fruit thinning was performed if necessary. The fruits were harvested manually. Twenty representative trees within each replicate were selected for sampling and data collection.

Agro-morphological and yield traits

To calculate the Trunk Cross Sectional Area (TCSA), the trunk circumference was measured (20 cm above the graft union) from both sides (north-south) with a hand caliper. This was performed at the end of the growing season in the November of 2007, 2008, 2009 and 2010. The average measurement of the two sides on the trunk were taken to make trunk diameter (R) and "Area= πr^2". A formula assisted in calculating the TCSA in cm^2. In addition, the cumulative yield per tree and per hectare were recorded at harvest time (kg/tree and kg/ha). The yield efficiency was defined as "yield per tree divided by TCSA (kg/cm^2)".

Fruit properties

All attributes pertaining to fruit traits were measured using 5 randomly-sampled fruits from each test tree. Then, their average was recorded. The individual fruit length, the fruit diameter and the ratio of length to diameter (L/D) were calculated by a vernier caliper. The fruits fresh weight was determined using a Mettler PC 8000 scale. In addition, fruit firmness was measured using a penetrometer (Instron Universal Machine, Model 1011) and recorded as $kg.cm^{-2}$. Total soluble solids (TSS) were measured with a Bausch and Lomb Abbe 3L refractometer. Moreover, the dry matter content was determined after the fruits were exposed to a process of drying at 70°C for 48 h. One gram of dry matter was burnt to yield ash in a Gaallankamp furnace at 550°C for 6 h. Titratable acidity (TA) was determined using an Aminex HPX-87H column which operated at 65°C, while 4 mM sulfuric acid was used as an eluent.

Data analysis

The data were obtained by field measurements. Laboratory observations were processed by analysis of variance (ANOVA) using the SAS software and the Duncan mean separation test procedure.

3. Results

Agro-morphological and yield traits

In general, all cultivars had developed a sufficient stem diameter (data not shown). The analysis of variance signified substantial differences among the cultivars and training systems. Tree vigor was affected substantially by training systems. After four years, there were significant differences in TCSA among the four cultivars. 'Golab-kohans' exhibited the highest value of TCSA (17.12 cm^2) (Table 1). The apple trees that were trained by the Y-system showed significantly higher TCSA values (16.41 cm^2) compared to those trained by the V-system which formed thinner trunks (9.80 cm^2) (Table 2). The interaction between

Table 1 - Means comparison of four apple cultivars about studied characteristics in Guttingen V and Geneva-Y trellis systems during 2007-2010

Cultivar	Fruit firmness (kg/cm^2)	Fruit weight (gr)	Fruit diameter (cm)	Fruit length (cm)	L/D	TSS	TA (%)	Ash (%)	Dry matter (%)	Cumulative yield (Kg/tree)	Yield efficiency (Kg/cm^2)	TCSA (cm)
Delbar estival	10.00 b	130.15 b	6.57 b	5.81 a	0.86 a	14.53 a	0.45 bc	0.40 b	20.63 bc	16.4 a	0.41 a	9.58 c
Fuji	14.52 a	148.40 a	6.94 a	5.78 ab	0.83 b	15.33 a	0.68 a	0.35 b	23.89 ab	14.72 ab	0.1 c	14.69 b
Golab-kohans	8.44 c	79.25 c	5.72 c	5.01 c	0.86 a	11.23 b	0.28 c	0.38 b	19.56 c	7.72 c	0.1 c	17.12 a
Starking	14.37 a	143.99 a	6.63 b	5.58 b	0.82 b	14.56 a	0.47 b	0.73 a	24.14 a	10.64 b	0.22 b	10.98 c

Means with same letters are not significantly different. (P>0.05) using Duncan Multiple Range Test.

Table 2 - Properties in Guttingen V and Geneva-Y trellis systems during 2007-2010

System	Fruit firmness (kg/cm^2)	Fruit weight (gr)	Fruit diameter (cm)	Fruit length (cm)	L/D	TSS	TA (%)	Ash (%)	Cumulative yield (Kg/tree)	Cumulative yield (t/ha)	Yield efficiency (Kg/cm^2)	TCSA (cm)
Guttingen V	10.53 b	122.45 b	6.36 b	5.39 b	0.84 a	14.17 a	0.46 a	0.5 a	7.88 b	23.640 b	0.25 a	9.80 b
Geneva-Y trellis	12.90 a	126.69 a	6.54 a	5.69 a	0.84 a	13.55 a	0.47 a	0.43 a	16.72 a	28.089 a	0.22 b	16.41 a

Means with same letters are not significantly different. (P>0.05) using Duncan Multiple Range Test.

training systems and cultivars showed that 'Fuji' had the largest trunk diameter and the largest TCSA (19.98 cm^2) (Fig. 1A). Regardless of the training system, 'Delbar estival' produced the most cumulative yield (16.4 kg/tree) (Table 1). Table 2 shows that the Y-system results in a higher average value of cumulative yield per tree (16.72 kg/tree) and per hectare (28.08 t/ha) than that of the V-system (7.88 kg/tree and 23.64 t/ha, respectively).

The V-system contributed to a higher density of trees (3000 tree/ha), compared to the Y-system (1680 tree/ha). Results show that 'Fuji' and 'Delbar estival' exhibited the most cumulative yield per tree and per hectare, under the Y-system and the V-system, respectively (Figs. 1B and 1C). Concerning the yield efficiency, during the four years, regardless of the training system, the 'Delbar estival' yielded the highest amount of fruit per trunk cross sectional area (Table 1). In addition, the V-system showed a higher yield efficiency (0.25 kg/cm^2), compared to the Y-system (0.22 kg/cm^2). A smaller trunk diameter and a higher tree density per hectare can be reasons for the higher yield efficiency (Table 2). The interaction between training systems and cultivars functioned mostly in determining the yield efficiency (0.57 kg/cm^2) in the 'Delbar estival' through the V-system (Fig. 1D).

Fruit properties

Results showed that the 'Fuji' cultivar yielded the heaviest fruit weight (148.40 gr), whereas 'Golab-kohans' had the lightest fruit (79.25 gr) (Table 1). Trees trained by the V-system (as a denser system in this study) developed fruits with an average lighter weight (122.45 gr), but the apples obtained from the Y-system were slightly heavier (126.69 gr) (Table 2). The 'Starking' cultivar exhibited the heaviest (159.69 gr) and longest fruit (6.1 cm) by the Y-system (Figs. 2A and 2B). In fact, the Y-system caused the 'Starking' to exhibit the maximum fruit length among the four cultivars. The Y-system contributed to the production of fruits that were significantly longer (5.69 cm) than those obtained by the V-system (5.39 cm) (Table 2). In addition, the 'Fuji' yielded the widest fruit (6.94 cm) among the four studied cultivars (Table 1). The Y-system caused a greater fruit diameter (6.54 cm) than the V-system (6.36 cm) (Table 2). Figure 2C shows that the maximum width of fruit (7.1 cm) was recorded in the 'Fuji' by the Y-system. The highest L/D ratio (0.87) belonged to the 'Delbar estival' by the Y-system. In general, the greatest value of fruit firmness was observed in 'Fuji'

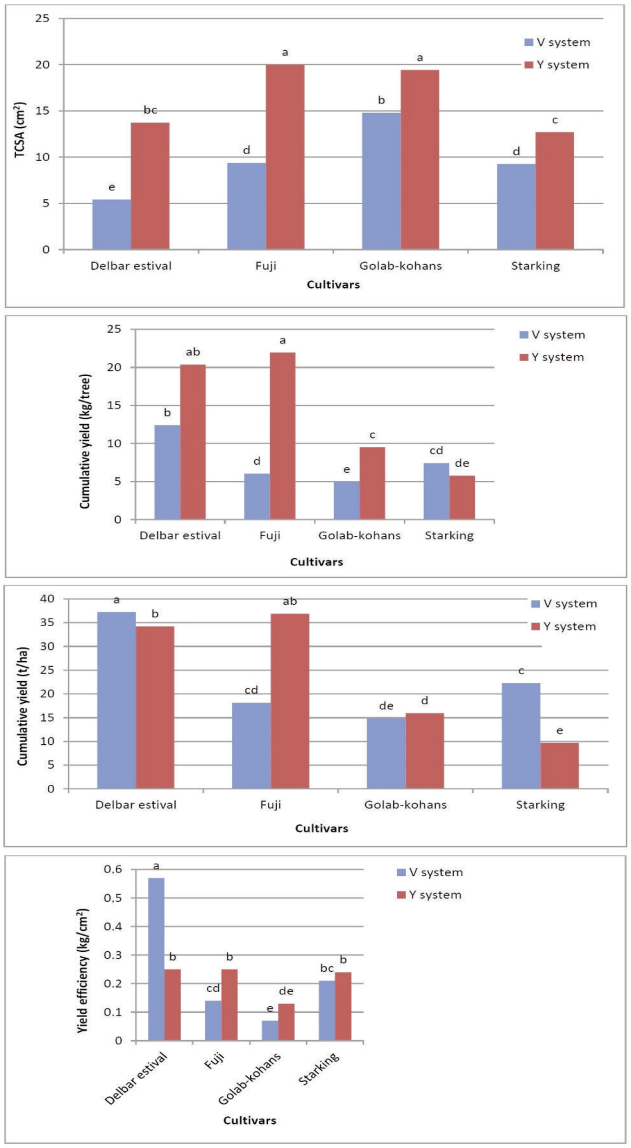

Fig. 1 Interaction of training systems (V-system and Y-system) and four cultivars (Delbar estival, Fuji, Golab-kohans, Starking) on fruit properties.

(14.52 kg.cm^{-2}) and the lowest was observed in 'Golab-kohans' (8.44 kg.cm^{-2}) (Table 1). Also, trees trained by the Y-system yielded fruits with the greatest value of firmness (12.90 kg/cm^2), compared to the function of the V-system (10.53 kg/cm^2) (Table 2). 'Fuji' yielded the firmest fruits (15.96 kg/cm^2) by the Y-system (Fig. 2D). The highest TSS (15.33%) and TA (0.68%) were produced by 'Fuji', whereas the lowest TSS and TA were recorded in the fruits of 'Golab-kohans' (Table 1). The content of TA also differed because of the training systems. The Y-system caused higher TA values in fruits, compared to the V-system, but this difference was insignificant (Table 2) which suggests that the training system had no remarkable influence on the acidity of fruits in this

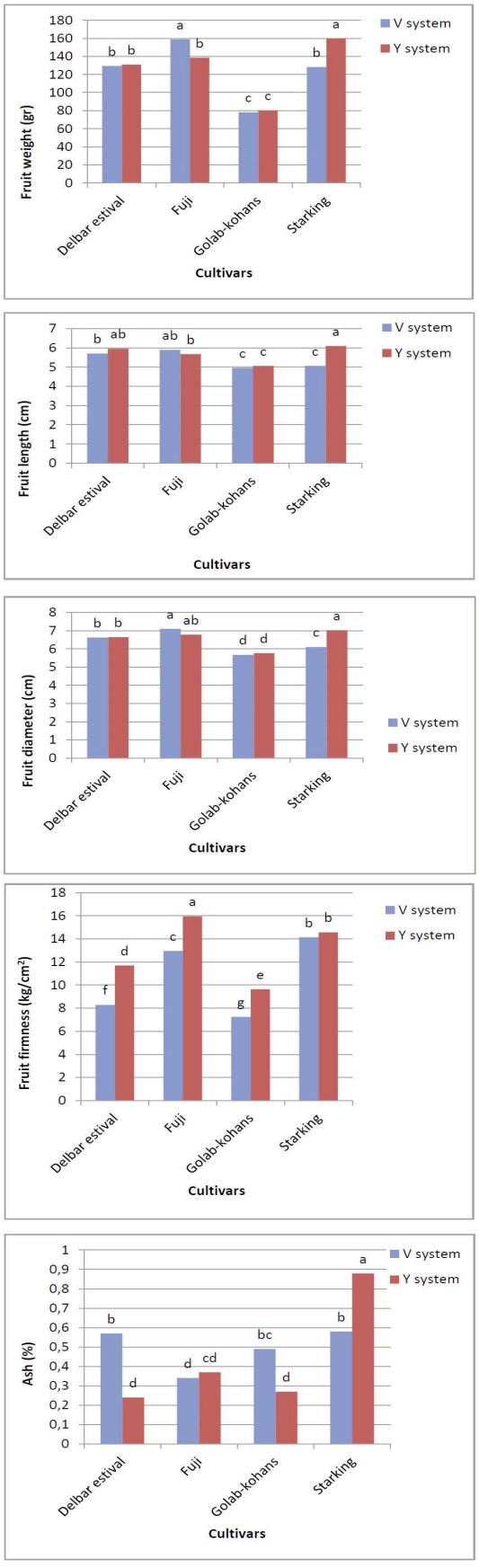

Fig. 2 - Interaction of training systems (V-system and Y-system) and four apple cultivars (Delbar estival, Fuji, Golab-kohans and Starking) on fruit properties.

research. The 'Fuji' yielded fruits with the highest amounts of TSS and TA by the Y-system and V-system, respectively. When comparing the cultivars, 'Starking' had the best results regarding the dry matter of fruits (24.14%) and ash (0.73%) (Table 1). Regardless of the cultivar, the fruits contained more dry matter when the trees were trained by the V-system, compared to training by the Y-system (Table 2). Additionally, 'Starking' yielded the highest amount of ash by interaction with training systems examined in this study (Fig. 2E).

4. Discussion and Conclusions

The results herein suggest that the cultivars and training systems caused differences in the measured characteristics. The occurrence of more tree growth by 'Golab-kohans' may be due to a higher degree of shading in the canopy than in other cultivars (Lo Bianco *et al.*, 2007). In addition to the influence of rootstocks, cultivar vigor can be affected by training systems. A lower TCSA was observed in trees of the V-system. This can be attributed to the competition between adjacent trees which, in turn, was a result of shorter spacing between trees (0.9 m) in comparison with the Y-system (1.6 m). As reported by other researchers (Musacchi *et al.,* 2015; Sosna, 2017), planting the trees closer to each other might have negatively affected the stem diameter in this study. These results are in accordance with the latest findings in the available literature (Robinson, 2007; Ozkan *et al.*, 2016) in which intensive cultivations had remarkable effects on tree growth. The greater yield caused by the Y-system might be due to the larger (wider) tree canopy. This result is in agreement with recent reports which suggest that the number of trees per unit area has a great influence on the yield per tree and per hectare (Robinson, 2007; Ozkan *et al.*, 2016). In general, a more even distribution of fruit-bearing can be observed in apple trees with V-shaped canopies, as trained by the Y- and V-systems, compared to other popular training systems. This has been suggested before by similar research (Sosna, 2017). It is known that the yield efficiency depends on the tree's vegetative vigor and fruit production. When the cultivar has good yield and high TCSA, a lower yield efficiency occurs compared to trees of other cultivars by the same yield and lower TCSA. A lower tree vigor, as caused by the V-system, did not result in a higher yield efficiency. This can be due to a lower yield per tree. In fact, results show that a high-

er yield efficiency can be attained by increasing the number of fruits in each tree or by controlling the tree vigor by dwarf rootstocks. Significant differences in yield efficiency were also reported in a previous study (Fioravanco *et al.*, 2016). It may be assumed that trees on dwarf rootstocks exhibit a weaker vegetative vigor and result in a higher amount of yield (Robinson, 2007). Nonetheless, the differences among cultivars in this study is likely due to the variations in morphological traits, which is in agreement with previous studies (Barritt *et al.,* 1995; Dadashpour *et al.,* 2010). No incremental trend was observed in the fruit weight during the four years, even by the influence of training systems. The contradictory effects of planting density on the fruit weight in this study are consistent with earlier reports (Ozkan *et al.*, 2012; Sosna, 2017). Nonetheless, fruit quality is influenced by many factors such as the specifications of a training system (Robinson *et al*., 1991). Therefore, it is natural to expect variations in the type of influence caused by the two different training systems on the measured traits in fruits. The L/D (≥1) is a criterion used for apple marketing, but all cultivars showed L/D <1 in this study. This observation is probably due to warmer nights in the climatic conditions of the experiment, resulting in insufficient cell elongation. This confirms the results of previous research (Dadashpour *et al.*, 2011). Based on the current discussion, the 'Delbar estival' probably has the highest marketable value in terms of its visual appearance among the cultivars. The denser cultivation of trees in the V-system contributed to the production of fruits with lower amounts of coloration, but this was not substantially different compared to the other training system. The good quality of apples obtained from the V-system was noticed in previous studies (Rutkowski *et al.,* 2009; Dadashpour *et al.,* 2012). It seems that the climatic temperature can affect the fruit firmness. In most of the cultivars, the softest fruits were observed in 2008 (as a cool year in this experiment). However, the relation between temperature and fruit firmness is not fully understood. The Y-system caused firmer fruits, compared to the V-system (Table 2), and this confirms that fruits harvested from the Y-system can be transported with less physical damage. Significant differences in apple firmness support recent findings (Talaie *et al.,* 2011). 'Golab-kohans' was the earliest ripening cultivar and produced the softest fruits (7.25 kg.cm^{-2}) by the V-system (Fig. 2D). The 'Fuji' produced the firmest fruits, probably because of the small fruit size, thereby confirming the findings of previous studies (Drake *et al.,* 1988; Dadashpour *et al*., 2010). In addition, differences in fruit firmness might have been due to genetic variations among cultivars. In addition, it has been reported that fruit firmness is the first edible criterion affecting buyer acceptance (Harker *et al*., 2008).

Considering the fruit sweetness, fruits and leaves that are exposed to higher light intensities may exhibit more TSS (Tustin *et al*., 1988). Also, the different TSS contents among cultivars may result from variations in leaf area, as suggested by previous research (Hudina and Stamper, 2002) or by a presumably higher canopy shading of cultivars which produce fruits of lower TSS (Garriz *et al.,* 1996, 1998). Although the TSS was not significantly affected by the two training systems, the V-system caused slightly higher levels of TSS than the Y-system (Table 2). Among the cultivars, the 'Fuji' produced the sourest fruits. These results show that acidity, in general, varies with cultivar, confirming previous studies (Platon, 2007; Dadashpour *et al*., 2010). The highest amount of TA was observed in fruits of the 'Fuji' cultivar. This may have resulted from less shading in the tree canopy or because of good nutritional conditions. In general, the 'Starking' cultivar produced the highest amount of dry matter, thereby confirming previous claims regarding the differences among cultivars in this regard (Lata, 2007; Palmer *et al*., 2010). In addition, the dry matter content varies among cultivars, and different training systems cause variations in the dry matter. The dry matter can vary from fruit to fruit and from training system to training system, in agreement with a previous study (Palmer *et al*., 2010).

In conclusion, the 'Delbar estival' exhibited better results under intensive training systems, whereas 'Golab-kohans' and 'Fuji' showed the best growth characteristics. In general, the Y-system was better than the V-system when considering the majority of characteristics. The two cultivars 'Fuji' and 'Delbar estival' were more adaptable to intensive training systems in Karaj's climatic conditions.

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Efficiency of AFLP markers to detect genetic variation in *Phthorimaea operculella* (Lepidoptera: Gelechiidae) offspring irradiated males

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Key words: AFLP technique, IST technique, *Phthorimaea operculella*.

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Abstract: AFLP technique was used to evaluate the genetic variation among normal and partially sterilized potato tuber moth males. Mating experiments were carried out to obtain partially sterilized males and their descending offspring. Then, 316 AFLP bands were amplified using eight primer combinations of which 33.8 were polymorphic 85.5%, which varied from 68.57% to 100%. The UPGMA dendrogram generated for the AFLP data revealed that irradiated and unirradiated male samples were clustered into two groups, and the offspring of F_1 and F_2 of unirradiated parents were clustered into one group. Moreover, the progeny of F_1 and F_2 of irradiated parents clustered into three groups. No specific DNA marker could identify the irradiated males; however, there was a clear genetic variability between examined individuals. Thus, the AFLP technique could be utilized to study genetic variations among individuals of the same line. The AFLP markers could enhance the monitoring system of mass-released insects program when inherited sterility technique is applied against potato tuber moth.

1. Introduction

The potato tuber moth *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae) is a cosmopolitan pest on potato crop, causing an annual yield reduction of 50 to 100% in some country around the world (Ahmed *et al.*, 2013). Insecticides are widely used to control this pest, but these methods have many drawbacks like high cost, nonselective and environmentally unfriendly. Moreover, insects could develop resistance to insecticides (Harba and Idris, 2018). Therefore, more environmentally friendly methods are required. The inherited sterility technique (IST) was suggested as an alternative control method to compact *P. operculella* (Makee and Saour, 1997; Larraín *et al.*, 2009). Because of no-practical methods are available to separate the adult moths by gender, the males and females are mass-reared, irradiated with low sterilizing doses of gamma radiation, then released within the targeted area (Eyidozehi *et al*., 2015). Moths irradiated with low doses live longer, stronger fliers and mate more frequently than moths irradiated with higher radiation doses

(Vreysen *et al*., 2016). However, a dose of 400 Gy induced almost 90% sterility in irradiated males while, a complete sterility in *P. operculella* females was achieved by 200 Gy dose (Makee and Saour, 2004). Furthermore, the costs of using IST program are likely to be more acceptable in terms of monetary expenditures and efficacy, as reported by Edgington and Alphey (2017), when they released dominant-lethal strain *Aedes aegypti* (L.) (Diptera: Culicidae) mosquitoes. The cost-effective improvements to the IST programs are required by applying modern genetic methods (Leftwich *et al.*, 2018). RAPD, AFLP, microsatellites and ESTs are popular DNA marker systems used in insect genetic research (Singh *et al.*, 2017). They are used as monitoring systems of insects mass-release programs to improve the application of IST against insects (Oliva *et al.*, 2012; Edgington and Alphey, 2018). In this study, AFLP technique was employed to investigate the genetic variation among the offspring of partially sterilized males of *P. operculella.*

2. Materials and Methods

Inherited sterility experiment

P. operculella insects used in this study were obtained from our laboratory stock cultures. They were reared on wax coated potato slices, maintained at a constant temperature of 25±1˚C, with 70±5% relative humidity, and 12 hour light-darkness cycle as described by Makee and Saour (2004). Fifty couples of females and males were placed in 350 ml transparent plastic boxes with filter papers as an oviposition site. A 10% sucrose solution was provided as food source. Both females and males were kept together until death. The eggs were removed daily, counted, and left until hatching. From the 50 reared couples only two were chosen depending on their fecundity (number of eggs per female), and fertility (percentage egg hatch). All the newly hatched larvae of two couples choosing were reared on small-waxed potato pieces, and the pupae were collected. The couple, with most pupae, was chosen to be the first family for tracking to the F_1 and F_2 progeny. Males were divided into two groups, the first male group was used as a control (♂ N x N ♀), and the second group was irradiated with a 150 Gy in a gamma cell supplied with a Co-60 source rounded the cylindrical (15x25 cm^2) irradiation chamber (Isslcdo-vatel Gamma Irradiator, Techsnabexport Co. Ltd. USR). The average dose rate at the time of irradiation was approximately 40.12 Gy/min with a factor of homogeny (max:min dose ratio) of about 1.05 and the absorbed dose was calibrated with Fricke solution. During this treatment, adult females were kept individually in small plastic tubes inside the irradiation source. The second males group was individually mated with normal virgin females (♂ T x N ♀). All F_1 and F_2 generations were reared on small waxed potato pieces as mentioned above. Fecundity and fertility of the F_1 and F_2 generation were recorded. Adult male parents were kept for DNA extraction and AFLP analysis.

DNA extraction and AFLP analysis

Six DNA isolation protocols of *P. operculella* males from adult stage were used to obtain a good quality and quantity of DNA for AFLP analysis (M1: Beye and Raeder, 1993; M2: Blanchetot, 1991; M3: Favia *et al*., 1994; M4: Harrison *et al*., 1987; M5: Marchant, 1988; M6: Moeller *et al*., 1992) (Reineke *et al*., 1998). The M5-modified protocol was the most appropriate to produce a high quality and quantity of DNA from one adult moth. From each adult moth of 4-5 mg, an 8 to 12 µg pure genomic DNA was obtained.

The AFLP protocol was carried out as reported by Shoaib *et al*. (2008). DNA from all samples was digested with *Eco*R1 and *Mse*I restriction enzymes (0.125 U/µl). Selective amplification reactions were performed using eight primer combinations and the amplified fragments were separated by gel electrophoresis. The sequences of eight primers combinations and adapters used in this study are presented in Table 1. AFLP data analysis for each primer pair, the numbers of polymorphic and monomorphic bands were determined. Each gel from the AFLP experiments was scored as presence (1) or absence (0) of a specific band for every sample. Percentage of polymorphism was calculated as the proportion of polymorphic bands over the total number of bands. Allelic polymorphic information content (PIC) was calculated using the formula of Botstein *et al*. (1980). Data for all the 8 primer combinations were used to estimate the genetic distances among analyzed individuals on the basis of the number of shared amplification products by using the Nei and Li, (1979) method. A dendrogram was generated using the Unweighted pair group of arithmetic means (UPGMA) by Statsoft program (2003).

3. Results

The data revealed that the first and the second

Table 1 - Sequences of oligonucleotide adapters and primers used in the pre amplification step and the selective AFLP primers combinations

Name	Reaction	Code	Sequence
EcoRI adapter	Ligation		5¢-AATTGGTACGCAGTCTAC3¢
			3¢- CCATGCGTCAGATGCTC-5¢
MseI adapter	Ligation		5¢-TACTCAGGACTCAT-3¢
			3¢-GAGTCCTGAGTAGCAG-5¢
EcoRI	Preamplification	E	5¢-GACTGCGTACCAATTC3¢
MseI		M	5¢-GATGAGTCCTGAGTAA3¢
EcoRI +A	Selective amplification	E-A	5¢-GACTGCGTACCAATTCA-3¢
EcoRI +G		E-G	5¢-GACTGCGTACCAATTCG-3¢
EcoRI+ C		E-C	5¢-GACTGCGTACCAATTCC-3¢
EcoRI+ T		E-T	5¢-GACTGCGTACCAATTCT-3¢
MseI + C		M-C	5¢-GATGAGTCCTGAGTAAC-3'
MseI + T		M-T	5¢-GATGAGTCCTGAGTAAT-3'
MseI + A		M-A	5¢-GATGAGTCCTGAGTAAA-3'
MseI + G		M-G	5¢-GATGAGTCCTGAGTAAG-3'

* Families selected for AFLP analysis.

couples were the best. The fecundity and fertility of the two couples were (111/103) and (95/88) (total eggs/ hatched eggs), respectively (Table 2). The first couple (89 pupae, no. of males and females 37 ♂/ 35 ♀) was selected to be the first family. Table 2, 3 show the F_1 and F_2 generations of irradiated and unirradiated males that resulted from seventeen males of this family, which were irradiated with 150 Gy dose and seven males were kept as a control. Table 2, 3 show the families of irradiated (T) and unirradiated (N) males which were selected based on the fecundity and fertility of F_1 and F_2 generations, and presenting in a marker (*). All purified genomic DNA of *P. operculella* samples submitted to AFLP analysis (Table 4). Eight primer pairs successfully amplified DNA fragments from the genomic of 17 samples. However, 316 fragments were scored with an average of 85.5% polymorphic bands per primer combination. The percentage of polymorphism detected by individual primer combination ranged from 68.57% for E-AAG/ M-CTA primer combination to 100% for E-AAC / M-CTG primer combination (Table 5). The ratio of number of fragments produced by primer pairs were 39.5.

The UPGMA dendrogram generated for the AFLP data shows that irradiated and unirradiated males samples were clustered into two groups. Hence, the offspring of F_1, and F_2 of unirradiated parent clustered into one group. While, the progeny of F_1 and F_2 of irradiated parent clustered into three groups. The first group include female parent, the second include the male parent, and the third one include all F_2 progeny that were produced from irradiated male parents (Fig. 1).

Table 2 - Inherited sterility technique experiments and the families of F_1 generations selected for AFLP analysis

Tow couples were chosen from 50			
No. of families	No. of eggs	Eggs hatching	No. of pupea
*1	111	103	89
2	95	88	77
Irradiated F_1 males (♂ N/ ♀ N)			
No. of families	No. of eggs	Eggs hatching	No. of ♂\♀
1	41	25	1\6
2	Death	-	-
3	4	0	0/0
4	29	14	2\1
5	11	2	1\1\
6	48	37	8\1
7	Death	-	-
8	6	3	1\1
9	48	20	5\1
10	38	26	14\1
11	3	3	2\1
12	204	140	45\12
*13	131	70	22\8
14	25	4	2\1
15	5	3	0\1
*16	206	146	61\17
*17	45	14	6\2
Unirradiated F_1 males (♂ N/ ♀ N)			
*18	127	67	8\11
19	Death	-	-
20	34	18	2\5
21	26	19	0\0
22	23	5	0\0
*23	138	92	38\47
*24	153	153	22\18

* Families selected for AFLP analysis.

Table 3 - F_1 progressed studied families and the families of F_2 generations selected for AFLP analysis

The F_1 progressed studied families		
No. of F_1 families	No. of couples studies	No. of couples sustained
12	6	1
13	1	0
*16	8	6
18	6	4
*23	11	8
24	5	2
Irradiated F_2 males		
No. of F_1 families	No. of cross	No. of eggs	Eggs hatching
*16	2	2	0
	3	52	0
	4	7	0
	5	7	0
	7	7	0
	8	3	0
12	6	23	0
Unirradiated F_2 males			
No. of F_1 families	No. of cross	No. of eggs	Eggs hatching
18	1	17	12
	2	11	45
	3	1	0
	6	8	0
*23	1	23	1
	2	35	29
	3	44	35
	4	28	11
	7	153	63
	8	8	7
	9	166	65
	11	58	27
24	3	80	37
	5	121	67

* Families selected for AFLP analysis.

Table 4 - DNA samples for AFLP analysis

Extraction from	No. of Samples
Female	1
Male	2
Irradiated males of F_1	3-apr
Unirradiated males of F_1	5-giu
Mix samples of DNA 8-9-11-12	7
Unirradiated males of F_2	8-9-11-12
Mix samples of DNA 14-15-16-17-18	13
Irradiated males of F2	14-15-16-17-18

Table 5 - Percent polymorphism, band numbers and polymorphic bands produced by eight primer combinations

No.	Primers combination	Total No. of bands	Polymorphic bands	Polymorphism %
1	E-ACT x M-CTG	49	46	93.87
2	E-AAG x M-CTA	35	24	68.57
3	E-ACG x M-CAC	51	42	82.35
4	E-ACG x M-CTA	47	42	89.36
5	E-ACA x M-CAT	45	36	80
6	E-AAC x M-CAC	41	34	82.92
7	E-AGG x M-CTC	24	23	95.83
8	E-AAC x M-CTG	24	24	100
Total		316	271	
Average		39.5	33.8	85.5

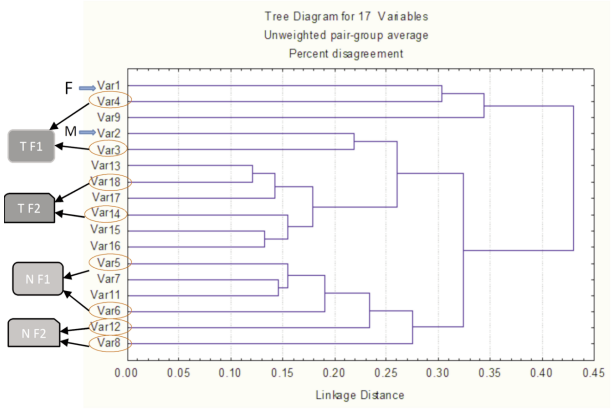

Fig. 1 - UPGMA dendrogram showing genetic relationships among 17 DNA samples of unirradiation and irradiation of P. operculella. Samples are: 1. Female, 2. Male, 3-4. F1 irradiated males, 5-6. F1 unirradiated males, 8-12. F2 unirradiated males, 7. Mix DNA samples of F2 irradiated males 8-12, 18-14, 13. Mix DNA samples of F2 irradiated males 14-18.

4. Discussion and Conclusions

Potato tuber moth, like most of Lepidoptera moths, when exposed to substerilizing doses of gamma rays undergo several physiological, biochemical and genetic changes (Makee and Saour, 2004; Hallman *et al.*, 2013, Sachdev *et al.*, 2017). However, some of the DNA damages due to irradiated male parents are inherited by their progeny (Steinitz *et al.*, 2015). Although, inherited sterility did not occur in *P. operculella* females but infertility of irradiated males and females is irreversible (Makee and Saour, 1997, 1999; Idris *et al.*, 2019). Thus, the sterility in F_1 progeny was more than in its irradiated male parents when IST applied against *P. operculella* (Makee and Saour,

2004). However, the majority of the inherited deleterious effects are expressed in the F_1 generation (Saour, 2014). The potential use of the AFLP technique to discriminate irradiated offspring of partially sterilize males of *P. opercullella* from the unirradiated was the aim of this investigation. Thus, the AFLP-technique using as fingerprinting tools to determine the genetic population structure of potato tuber moth (Medina *et al.*, 2010). Our AFLP data that were obtained from this study shown that no specific DNA marker could distinguish irradiated males from the unirradiated ones, but there was a clear DNA polymorphism between in F_1 and F_2 generations of partially sterilized males of irradiated and unirradiated male parents. Induced DNA damage could have significantly begun at 20 Gy and higher doses as reported by Hambarde *et al*., 2013 on Sf9 Lepidoptera cells. Consequently, it is known, that DNA damages caused by irradiated males at 150 Gy are irreversible and randomly inherited to their offspring (Makee and Saour, 2004; Vreysen *et al.*, 2016). Thus, the DNA damages inherited randomly in F_1 and F_2 generation are not stable when the males exposed to the partially sterility irradiation doses (Sauor, 2014; Kheirallah *et al.*, 2017). Based on these facts, we suggest that DNA changes in F_1 and F_2 generations between irradiated and unirradiated were adequate to be detected by AFLP technique. Additionally, the high percentage of polymorphism between male samples of irradiated and unirradiated reflected the vast diversity genetic level in *P. operculella* males due to a gamma radiation applied doses.

In conclusion, the AFLP-technique revealed to be powerful for studying genetic variation between incest species or between individuals of the same line, which have biological differences induced by several factors such as irradiation. Thus, using AFLP technique in tracking the genetic variation in offspring of partially sterilized males may enhance the effectiveness of the monitoring system in mass-released insects programs when, IST applied against potato tuber moth.

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Long-time storage *Pochota fendleri* seeds with different packaging

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Key words: germination, PET, plastic, seed quality, vigour.

Abstract: The aim of this study was to evaluate the vigour and physiological quality of seeds of *Pochota fendleri* in two weight classes, stored in different types of packaging over a period of 28 months. The experimental design was completely randomised, with treatments arranged in a 2 x 3 x 4 factorial scheme (2 seed sizes x 3 types of packaging x 4 storage periods), with four replications. The stored seeds were evaluated for germination percentage and germination speed index every six months up to 28 months. At 28 months, the percentage incidence of fungi on the seeds was also determined. The seeds of *Pochota fendleri* remain viable and of high quality for a period of 28 months storage. A PET bottle kept in the refrigerator is recommended to store *Pochota fendleri* seeds.

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1. Introduction

A tree species belonging to the family Malvaceae, *Pochota fendleri* (Jacq. WS Alverson) is highly valued in various countries of Central and South America; in Brazil, the State of Roraima is the only area where the species occurs naturally (Smiderle *et al*., 2017). The high rates of deforestation in areas of natural occurrence, caused mainly by the high demand for wood, have classified the species as threatened with extinction (Llamozas *et al.*, 2003; FAO, 2017). In addition to its use as wood in the production of manufactured boards, planks, panels, doors, windows and furniture, the plants of *Pochota fendleri* are also used for the shade and shelter of livestock, for planting living fences, for the manufacture of handicrafts and in the recovery of degraded areas (Briscoe, 1995; Smiderle *et al*., 2017).

Planting Brazilian native species when compared to exotic species, can contribute to the conservation of regional biodiversity, and may also present important technical and economic advantages due to the ease of acclimatising and perpetuating these species (Smiderle and Souza, 2016). However, the reduced number of seeds, the difficulty of their collection in areas of natural occurrence and the lack of basic information on native species make them difficult to cultivate.

One of the most researched and controversial aspects concerns the influence of seed weight on physiological quality; in any one batch, seeds classified as large usually give better performance when compared to small seeds as they have a greater amount of nutrient reserves, which serve as input for the initial development of the plant (Souza *et al*., 2017 a).

Therefore, based on morphological characteristics, germination tests and initial seedling vigour, it is possible to make better decisions about the quality of seed batches, resulting in a reduction in the production time of more-uniform seedlings, and the possibility of successful initial seedling establishment (Pereira *et al*., 2011; Dresch *et al*., 2013; Oliveira *et al*., 2016; Souza *et al*., 2016; Souza *et al.*, 2017 b).

Among the various stages of the seed-production process and seed technology, storage constitutes one of the critical phases and has a great influence on the viability and conservation of the seeds in the batch (Nery *et al*., 2017). Storage is important for maintaining seed quality over time, delaying the process of deterioration, so that the seeds maintain their longevity and vigour until they are sown (Carvalho and Nakawaga, 2000).

Knowing the storage capacity of seeds makes it possible to adopt the proper conditions for each species; however, due to the diversity of forest species, there is still a lack of information on the technology of such seeds in the literature, especially in relation to storage behaviour (Balouchi *et al*., 2017) for the conservation of germinating power (Smiderle *et al*., 2016; Felix *et al*., 2017).

The type of packaging used during storage is also important for maintaining both viability and vigour, since it is directly related to the physiological quality of the stored seeds (Rodrigues *et al*., 2016). The physiological quality of seeds is often evaluated by the standard germination test, which is carried out under optimal environmental conditions to determine the maximum germinating potential and establish a limit for the performance of the batch after sowing (Catão *et al*., 2016).

Conditions able to preserve seed quality for a certain period are essential; however, there is still little information available (Smiderle *et al*., 2018) on seed technology when applied to native species such as *Pochota fendleri*.

The aim of this study was to increase information on the conservation of forest seeds by evaluating the vigour and physiological quality of the seeds of *Pochota fendleri* in two weight classes stored over 28 months in different types of packaging.

2. Materials and Methods

The research was carried out at the Seed Analysis Laboratory and in the forest sector of Embrapa Roraima. The species used in the research was *Pochota fendleri* (Seem.) WS Alverson & MC Duarte, whose seeds were collected for quality analysis from trees at 10 years of age, in the Experimental Area of Embrapa Roraima located in the district of Mucajaí, Roraima (at 2°23'45.31" N and 60°58'44.34" W) during March and April of 2014. The fruit was harvested as soon as it opened. After the seeds were extracted, they were left to dry for 24 hours on a shaded canvas on the ground, and then packed in polyethylene bags and sent to the Seed Analysis Laboratory of Embrapa Roraima located at 02°45'28N and 60°43'54" W, at an altitude of 90 m, in Boa Vista, Roraima, for the experiments to be carried out. The collected seeds were selected and sorted as to weight (small seeds being those that passed through a 4 mm diameter sieve, having a mean individual weight of 0.027 g; and large seeds, those that were retained in a 4.5 mm diameter sieve, with a mean individual weight of 0.048 g). The seeds were then packed in individual paper bags, transparent plastic containers or PET bottles, and stored in a refrigerator at 10°C and a relative humidity of 60%. The temperature and relative humidity were monitored with a thermo hygrometer to obtain a monthly average. The packs of samples were divided into sufficient quantities for later evaluation.

Four samples of 10 previously weighed seeds of *Pochota fendleri* were selected to determine the water content remaining in a drying oven at 105±3°C for 24 h (MAPA, 2009). This determination was repeated at 16 and 28 months. The moisture content was calculated based on the fresh weight of the seeds (MAPA, 2009). The experimental design was completely randomised, with treatments arranged in a 2 x 3 x 4 factorial scheme (2 seed classes x 3 types of packaging x 4 storage periods), with four replications.

Seed characteristics were determined every six months during the 28 months of storage, starting from the fourth month. The tests and methodologies to which the seeds were submitted and evaluated are described below.

The germination test was carried out on four replications of 50 seeds, in plastic boxes (gerbox®) on

germination paper (germitest®) moistened with distilled water at 2.5 times the weight of the paper and kept in a germination chamber at 25±2°C under constant light. The germination test was evaluated by daily counts until the fourteenth day, considering the number of seeds that emitted a root greater than 2 mm (Labouriau, 1983). From the data obtained with the germination test, the germination speed index (GS) was calculated as per the method recommended by Maguire (1962).

The seeds were also characterised for biometry, the small seeds showing mean values of 5.01 mm for length and 3.32 mm for diameter, and the large seeds, 5.49 mm and 4.20 mm. The values were obtained with the aid of a digital calliper.

The means of the variables were submitted to the statistical analysis utilizing the software Sisvar (Ferreira, 2014), with variance analysis and the Tukey test (p≤0.05%). Regression analysis was performed for the time factor (months). The statistical analysis was carried out using the Sisvar software.

3. Results and Discussion

At the time of storage, the seeds of *Pochota fendleri* presented a mean water content of 10.5% for the seeds classified as small, and 8.1% for those classified as large. The results for seed water content after 4, 16 and 28 months storage in the different treatments can be seen in Table 1. The water content of the seeds was determined by the storage conditions, and no statistical analysis was applied to the values. The relative humidity of the three storage containers varied during the 28 months, which contributed to maintaining the seed water content.

The packages prevented or decreased the exchange of water vapour between the seeds and the storage environment. It is worth noting that before storage the seeds presented 98% germination for those classified as large and 85% for those classified as small.

The results obtained in the present study demonstrated a strong relationship R^2= 0.88, for germination percentage in both classes of seed packed in the different containers. For the study under analysis, the seeds classified as small showed inferior performance in relation to germination percentage when compared to the large seeds (Fig. 1A and B), irrespective of container or storage period.

Table 1 - Mean water content (%) in seeds of *Pochota fendleri* for different weight classes, packaging and storage

Packaging	Months			Mean
	4	16	28	
Large seeds				
Paper bag	8.0 a B	7.8 a B	7.7 a A	7.8
Plastic bag	8.1 a B	8.0 a B	7.8 a B	7.9
PET bottle	8.1 a B	8.0 a B	7.8 a B	7.7
Mean	8.05	7.96	7.77	
Small seeds				
Paper bag	10.0 a A	9.2 b A	8.0 b A	9.0
Plastic bag	10.5 a A	10.0 a A	9.0 a A	9.7
PET bottle	10.5 a A	10.0 a A	9.5 a A	10.0
Mean	10.25	9.90	8.79	

In the column, means followed by different letters, lowercase letters between packages and uppercase between sizes, differ by Tukey test at 5%.

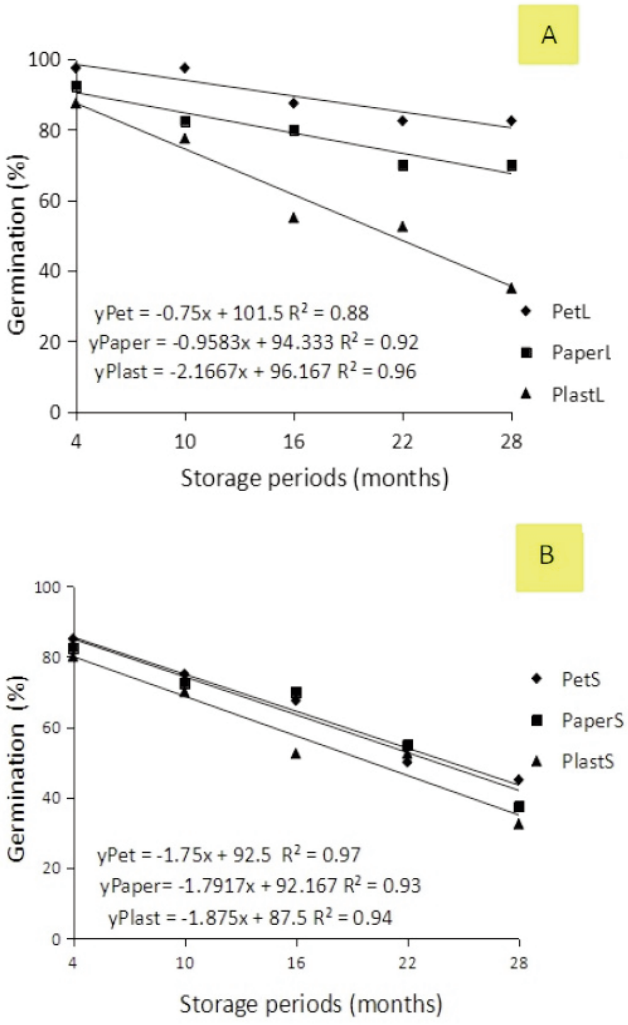

Fig. 1 - Mean values for germination (%) of large (A) and small (B) seeds of *Pochota fendleri* for different packaging and storage periods (months). Pet L= Pet bottles, Large; Paper L= Paper bags, large; Plast L= transparent plastic containers, Large; Pet S= Pet bottles, Small; Paper S= Paper bags, Small; Plast S= transparent plastic containers, Small.

In Table 2, the means of the squares and the levels of significance can be seen by the F test for the characteristics evaluated, at a level of 0.05% probability for all variables studied.

At 28 months storage, the large seeds packed in the PET bottles presented on average a 68% yield in germination percentage when compared to those in the plastic packaging (Fig. 1A).

It was found however, that even after 28 months storage, the large seeds storage Pet bottles (Fig. 1A) maintained a germination percentage 83% below the minimum established for commercialisation, which is 85% (MAPA, 1992).

According to Souza *et al*. (2017 b), for the same species the seeds of greater weight presumably have more reserves, a higher level of hormones and well-formed embryos, and are considered to have greater vigour.

With the small seeds however, there was an expressive linear loss in germination percentage in the three containers during storage. The same results were seen for germination speed index in the small seeds (Fig. 2B), demonstrating that this characteristic can be efficient in detecting differences in vigour among seed size classes in storage.

Evaluating germination capacity in seeds of *Enterolobium schomburgkii*, Horing *et al*. (2012) found that there was a linear decrease in germination percentage after 30 months of storage for seeds packed in waterproof plastic pots but stored under laboratory conditions. Seeds of *Caesalpinia leiostachya* (Benth.) Ducke (pau-ferro) maintained a higher germination percentage when stored for a period of eight months inside the fruit in a natural environment (Biruel *et al*., 2007).

The germination speed index obtained for large seeds stored for up to 10 months in PET bottles was 18.0 (Fig. 2A); however, a greater reduction was found when the seeds were kept in paper or plastic bags (Fig. 2A).

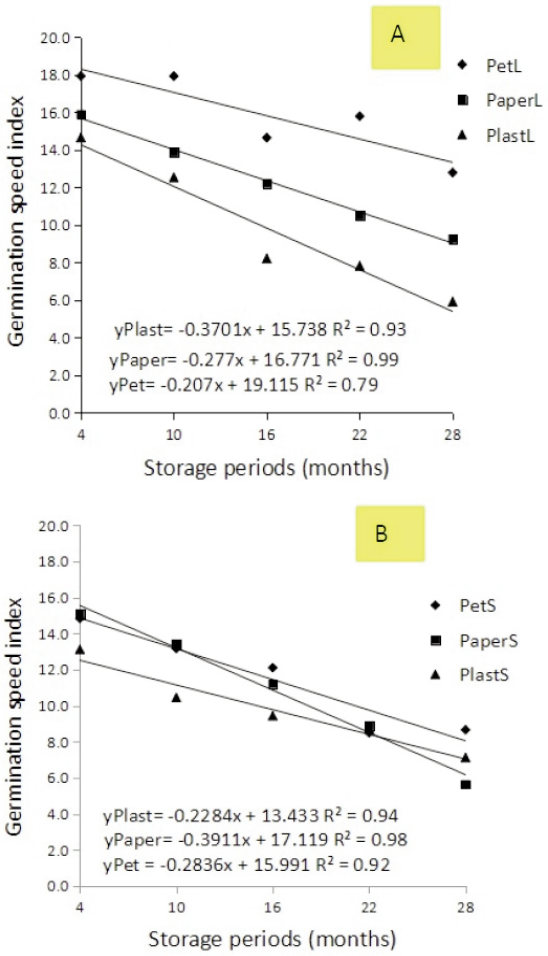

Fig. 2 - Mean values for the germination speed index of large (A) and small (B) seeds of *Pochota fendleri* submitted to different packaging and months of storage. Pet L= Pet bottles, Large; Paper L= Paper bags, large; Plast L= transparent plastic containers, Large; Pet S= Pet bottles, Small; Paper S= Paper bags, Small; Plast S= transparent plastic containers, Small.

Table 2 - Summary of the variance analysis (Mean Squares and significance by the F test), coefficients of variation, and general means obtained for germination (G) germination speed index (GSI) and water content (U%) of *Pochota fendleri* for different packaging and storage periods (months)

Source of variation	DF	Germination (%)	Germination speed index	Source of variation	DF	Water content (%)
Storage periods (SP)	4	6481.7708 **	232.8101 **	Storage periods (SP)	2	4918472 **
Packing (P)	2	3954.1666 **	183.7151 **	Packing (P)	2	2040139 **
Size (S)	1	8066.6666 **	140.9604 **	Size (S)	1	53388889 **
SP x P	8	288.8020 **	3.3093 NS	SP x P	4	0.270347 NS
SP x S	4	272.3958 NS	3.8119 NS	SP x S	2	2287639 **
P x S	2	1379.1666 **	60.6049 **	P x S	1	1075972 **
SP x P x S	8	159.1145 NS	8.0687 NS	SP x P x S	2	0.186597 NS
Error	120	1.237.500	40.788	Error	54	0.183426
Total	149			Total	71	
Coefficient of variation (%)		16.08	17.31	Coefficient of variation (%)		4.87

*significant difference in 0.05 levels; NS= not significant.

It is therefore interesting to classify seeds by size, since the procedure is quick, and can be carried out manually or with the use of specific sieves or even a densimetric table. The use of heavier seeds gives the consumer or producer a greater guarantee of the physiological quality of the material purchased and used in producing seedlings of *Pochota fendleri*, since seed quality assumes a prominent role in the cultivation of forest species, and can be considered one of the principal bottlenecks, especially in the area of seedling production.

In turn, seeds classified as small and packed in paper bags presented an index that was 35% smaller at 28 months than seeds stored in PET bottles. According to Marcos Filho (2005), a reduction in the germination speed index is the first symptom of a fall in seed performance, generally determined by disorganisation of the membrane system. Bello (2005) found an increase in the mean germination time and a decrease in the germination speed index in seeds of *Torresea acreana* (Mirtaceae) over a storage period of 12 months, indicating a reduction in germination speed.

The results obtained for the variables evaluated in the present study indicate that seeds should be stored in PET bottles in a refrigerated environment maintained at 10°C for different periods (months).

There are currently no studies that might support the staggered production of *Pochota fendleri* seedlings in the field or in the nursery. The practice of staggered sowing is not yet carried out by producers of *Pochota fendleri*, but such a method, if based on scientific principles, could make a positive contribution to seedling production, making it possible to produce seedlings at different times throughout the year, as well as providing seedlings of differing quality standards, which would depend on the investment capacity of the producer.

4. Conclusions

The seeds of *Pochota fendleri* remain viable with high physiological quality for up to 28 months storage in PET bottles. Seeds of *Pochota fendleri*, stored in a refrigerator at 8 to 10°C in PET bottles, retain their physiological quality. The vigour of large seeds of *Pochota fendleri* is preserved for a longer time in PET bottles. The use of heavier seeds gives the consumer or producer a greater guarantee of the physiological quality of the material purchased and used in producing seedlings of *Pochota fendleri*, since seed quality assumes a prominent role in the cultivation of forest species.

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Preliminary screening of agricultural feedstocks for anaerobic digestion

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Abstract: The aim of this study is to evaluate the performances in the early stages of biogas production of various unconventional and low inputs crops, such as: kenaf (*Hibiscus cannabinus* L.), amaranthus (*Amarathus cruentus* L.), sorghum (*Sorghum bicolor* L.), and sunflower (*Helianthus annuus* L.). Moreover, according to a circular economy approach, that foreseen the re-use of all the materials, a wide range of agro-industrial residues were tested such as: pomace, olive oil cake, cow milk whey, ewe milk whey, beer residues, jatropha (*Jatropha curcas* L.) oil cake and pelargonium (*Pelargonium graveolens* L.) residues after essential oil extraction. The biogas production was estimated starting from the chemical composition of the substrates as well as through tests in bench's static reactors. The results showed that the use of silage from crops with reduced agronomic requests (kenaf and amaranthus) versus a conventional crop (corn) led to comparable, or even better, biogas production performances during the initial stages. Moreover, the performance of some residues from the milk industry allowed to conclude that the ewe milk whey can be considered a booster feedstock for the first phase of digestion. All the tested substrates produced a digestate suitable, according to the Italian rules, for soil fertilization or amendment.

1. Introduction

Biogas production from anaerobic digestion (AD) for electricity and heat generation represents a significant and well-established opportunity for farmers in the EU countries thanks to several reasons, such as: large technology availability and versatility, very attractive integration of the system in the agronomic practices and rotations, and the availability of incentives provided by the EU-MS to renewable energy generation. For all these reasons the biogas sector in Europe showed a remarkable increase during the last decade, with a global amount of energy produced in 2013 in the EU of 561 x 10^{-9} GJ (EurObserv'ER, 2014).

In this European context, the Italian biogas growth was mostly related

to the agriculture sector and almost 800 power plants based on agricultural biogas were operating at the end of 2012 with a total capacity of 650 MW (Patrizio *et al.,* 2015). The plants are concentrated in the northern part of the country, mainly fed with silage corn and manure, thanks to the livestock sector present there.

However the use of corn as feedstock, although it represents one of the most performing biomass in AD (with an average biogas production of 498 $m^3\ t^{-1}$ and a content of 53% of methane), exposes the biomass chain to the criticism of food-feed conflict. The largest part of the Italian biogas plants are fed with livestock manure and energy crops (62.2%), 17.7% of plants use only livestock manure and 20.1% use only energy crops and cereals (Carrosio, 2013). Nevertheless, the main constituent of the feedstock recipes is still represented by the dedicated crops (Dresseler *et al.*, 2012; Bacenetti *et al.*, 2013).

For all these reasons, the present work aimed at preliminary testing several unconventional biomass for the production of biogas in comparison with a traditional feedstock such as silage corn. These feedstocks belong to two main groups: dedicated crops, such as corn silage (as control), sorghum silage, kenaf silage, sunflower silage and amaranthus silage; and agro-industrial residues such as pelargonium residues, olive oil cake, jatropha oil cake, pomace, beer thrasher, cow milk whey and ewe milk whey.

As regards the agro-industrial residues, some of these have been selected since they are widely available in Europe. In fact, they are originated from processes such as olive oil extraction, wine beer and cheese factory, activities very common in this area.

Concerning the jatropha cake (from *Jatropha curcas* oil extraction process) and the residues of the essential oil extraction from *Pelargonium graveolens*, these two biomasses, requiring tropical climates, well fit to tropic areas, where short energy chain is often necessary due to the lack of grid connection and where biogas production could represent an interesting energetic chance.

The bio-methane potential (BMP) of the selected organic matter can be estimated through chemical analysis followed by the use of algorithms, to approximate the biogas and methane potential yield (Buswell and Symons, 1993), or assessed by a simulating small scale anaerobic digestion in laboratory, either batch wise or continuous (Angelidaki *et al.*, 2009; Kowalczyk *et al.*, 2013; Edward *et al.*, 2015).

The bio-methane potential of a biomass can be estimated from its elemental composition (carbon, oxygen, hydrogen, nitrogen and sulfur content) using the well-known Buswell's formula (1):

$$C_nH_aO_bN_cS_d + \left(n - \frac{a}{4} - \frac{b}{2} + \frac{3c}{4} + \frac{d}{2}\right) \bullet H_2O \rightarrow$$

$$\left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4} + \frac{3c}{8} + \frac{d}{4}\right) \bullet CO_2 + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8} - \frac{d}{4}\right) \bullet CH_4 + c \bullet 0NH_3 + d \bullet H_2S$$

where represent the molar number of each element.

Results are calculated under standard conditions, according to Eq. (2):

$$CH_4 = \frac{\left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8} - \frac{d}{4}\right) \bullet 22.4}{(12n + a + 16b + 14c + 32d)}$$

where represent the molar number of each element and 22.4 is the coefficient that express the volume of 1 mole of perfect gas at standard conditions (0°C and 1 atm).

But several factors influence the estimation of Buswell's formula, such as the particles size, the retention time, the temperature, any unbalance in the biomass recipe, the recalcitrant molecules that might occur in the biomass, etc. and, in order to overcome these difficulties, a wide range of tests has been carried out during the last decades to detect the biomass bio-methane potential production. Bio-Methane potential (BMP) tests are very useful for this scope, as they provide a measure of the maximum amount of biogas or bio-methane produced per gram of volatile solids (VS) contained in the organic feedstock (Esposito *et al.,* 2012; Thomsena *et al.,* 2014).

The BMP methodology requires to control the substrate chemical composition, the operating temperature, the inoculum, the length of the trial and the output characterization (biogas and digestate composition).

Owens and Chynoweth (1993) and Angelidaki and Sanders (2004) proposed a BMP method based on a batch process, with a good stability due to the equilibrium between the symbiotic growth of the principal metabolic groups of bacteria; the methodology chosen for the present study refers to Angelidaki *et al.* (2009).

The use of dedicated crops for the biogas process fuelling is well known and a large amount of data is available in scientific literature since the '50s (Reinhold and Noak, 1956), showing a wide range of methane yield such as: VS 200-450 $m^3\ t^{-1}$ CH_4 corn, VS 384-426 $m^3\ t^{-1}$ CH_4 wheat (Schievano *et al.*, 2009; Wu *et al.*, 2010), VS 236-428 $m^3 t^{-1}$ CH_4 sorghum

(Windpassinger *et al.*, 2015), VS 242-324 m^3 t^{-1} CH_4 straw, VS 298-467 m^3 t^{-1} CH_4 grass (Zauner and Küntzel, 1986; Weiland, 2010; Wu *et al.*, 2010), VS 177-400 m^3 t^{-1} CH_4 sunflower (Schittenhelm, 2010; Weiland, 2010) and VS 355-409 m^3 t^{-1} CH_4 hemp (Heiermann *et al.*, 2009; Nges *et al.*, 2012) however, new crops are emerging in recent years (Molinuevo-Salces *et al.*, 2013; Mast *et al.*, 2014; Corno *et al.*, 2015).

Examples of crops not yet widely exploited for biogas production are kenaf (*Hibiscus cannabinus*) and amaranthus (*Amaranthus cruentus*). The first one represents a major feedstock for cellulose pulp (Nishinoa *et al.*, 2003) or for green building component production (Deka *et al.*, 2013) while amaranthus well fit to temperate environments, where short days varieties of tropical origin can enhance the biomass growth rather than the seeds production.

There is a common pre-treatment step among different biomass crops: the after harvest silage process. This is essential to simulate the usual practice in actual biogas plants because the process allows the biomass storage for several months.

As regards the by-products considered in the present article, no literature is available for geranium (*Pelargonium graveolens*), where leaves remaining after essential oil extraction can be used for AD. Similarly, jatropha (*Jatropha curcas*) cake, after oil extraction, could represent an interesting feedstock in some tropical areas.

No specific EU regulation or rules are actually available for the use of digestate as fertilizer or amendment, although its potential utilization can also reduce dependence on energy intensive mineral fertilizers, to further mitigate GHG emissions (Pöschl *et al.*, 2010). In Italy the only limitation is expressed by the Decreto del Ministero dell'Agricoltura 7.4.2006 (MPAF, 2006) stating that if the feedstock is animal manure also the digestate could be considered appropriate as fertilizer, otherwise its utilization is uncertain. Recently a new Italian rule (Legge, 7.10.2012, n. 134) declared that if the digestate is not originated from waste, it can be classified as a by-product usable in agriculture as soil amendment, but every Region applies this rule differently. The use of digestate as fertilizer or soil amendment is validated from several scientific articles (Haraldsen *et al.*, 2011; Mantovi *et al.*, 2011; Chen *et al.*, 2012; García-Sanchez *et al.*, 2015) but only a few works in literature are available regarding the digestate from these still unexploited (in most of the cases) sources.

2. Materials and Methods

Feedstock description

An experimental trial was conducted using twelve different substrates, some of which were dedicated crop specifically cultivated for this use and silaged after harvest (biomass), while the others residues came from various agricultural activities (agro-industrial by-products). The following Table 1 summarizes all the crop type and the origin of the different feedstock tested in the experiment.

The crops substrates were corn (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), amaranthus (*Amaranthus cruentus* L.), kenaf (*Hibiscus cannabinus* L.) and sunflower (*Heliantus annuus* L.) cropped in experimental fields in the farm owned by the Istituto Tecnico Agrario (Florence, Italy) and transformed with the silage technique. A field experiment was carried out in 2012 during April - September, in the farm of the University of Florence, Italy. The seeds for the experimental field were collected from different sources (Table 2).

The research fields were located at latitude 43°47'07'' N and longitude 11°13'12'' E, with an altitude of about 40 m above mean sea level. Maximum and minimum temperature were respectively 29.8 and 17°C, with a mean of 24.6°C as recorded by Oregon Scientific WMR300. The soil texture was sandy-loam (67.6% and 20.7% respectively) with pH of 6.5. No fertilization was planned because the pre-

Table 1 - Feedstock description

No.	Feedstock name	Type	Origin
1	Corn	Green Silage	Italy
2	Sorghum	Green Silage	Italy
3	Pelargonium	Residues from distillation of essential oil, dry	Madagascar
4	Kenaf	Green Silage	Italy
5	Sunflower	Green Silage	Italy
6	Amaranth	Green Silage	Italy
7	Olive cake	Residues from mechanical oil extraction	Italy
8	Jatropha cake	Residues from mechanical oil extraction	Madagascar
9	Wine residues	Residues after fermentation	Italy
10	Cow milk whey	Residues after cheese production	Italy
11	Ewe milk whey	Residues after cheese production	Italy
12	Beer residues	Residues after fermentation	Italy

vious crop insisting on that area was a legume (*Phaseolus vulgaris* L.) and the nitrogen soil content was 1.4%.

Table 2 - Dedicated crops description

Species	Variety name	Seed provenance
Zea mays L.	Cisko	Syngenta Ltd
Sorghum bicolor L. (Moench)	Bulldozer	KWS Ltd
Hibiscus cannabinus L.	HIB 35	IPK seed bank
Helianthus annuus L.	PR64H41	Pioneer Ltd
Amaranthus cruentus L.	Perucho	DISPAA seed bank

The experimental scheme adopted was a completely randomized block with 3 repetition and each plot measured 4 x 5 m and was irrigated immediately after seeding, then irrigation plan was not necessary and the rainfall recorded during the cultivation months was 125.47 mm. Weed control was done manually in the early stages of plants growing.

Considering the restricted number of plants, harvest and ensilage were realized manually on 6 September 2012, collected biomass of each crop was chopped by Stayer Trito 1800 shredder and vigorously compacted into nylon bags. All the bags, hardly sealed, were left for two months in a Temperature controlled environment (25°C), to allow the fermentation processes and the conversion of chopped and pressed biomass in silage. After that, samples were collected in vacuum envelopes, by Valko Favola 310 vacuum packaging machine, with a total amount of 500 g for each crop.

The agro-industrial by-products substrates consisted in pomace, olive oil cake, cow and ewe milk whey and beer residues that were obtained directly from agro-industrial districts in the Tuscany Region, Italy (farm, winery, oil mill, dairy).

As regards the by products used as feedstock for AD: the ewe whey was collected from "Società Agricola Bacciotti Giovanna" an organic farm located in Mugello valley into the municipality of Scarperia - San Piero (Florence) that produces both the sheep milk and the diary.

The ewe whey is the residue of Ricotta cheese (ricotta) production, a typical Italian unripe cheese variety obtained through heat-induced (85-90°C) coagulation of whey proteins, after addition of acidifying agents. During this phase, coagulated whey proteins are divided by the liquid part that, in this case of study, represents the feedstock used for the biogas production and was collected in 1 dm^3 sterile plastic container.

The experimental samples of cow milk whey were sampled from "TRE P" diary located in Mugello Valley in the municipality of Scarperia - San Piero (Florence) and the milk used was produced by the "Emilio Sereni" farm located in Mugello Valley as well, where Frisian and Pardo-Alpina races are grown by organic livestock.

The cow milk whey is the liquid residue that separates from the solid mass obtained from the coagulation of casein, during the production of Mozzarella cheese. Mozzarella cooking is accomplished by melting the curd in hot water (60-85°C) and then working the molten curd by manually stretching and kneading, until the desired texture is achieved. During this step, 1 dm^3 of the residual liquid part was collected in sterile plastic container.

The olive cake came from the mill "Il Mandorlo" located in Trespiano (Florence) where the farm has olives trees grown by organic agriculture. This feedstock represents the 'cold extraction' process residue and it is obtained during extra-virgin olive oil production at a temperature below 27°C from mechanical pressing of the olive paste, using a traditional extraction system with hydraulic presses. It is constituted from a mixture of olive endocarp, olive pulp and skin, as well as pomace olive oil plus the water added in the olive mills. The olive varieties used for oil production were Frantoio, Moraiolo, and Leccino. During extra-virgin oil production, 1 kg of olive cake was collected and vacuum-sealed.

Wine residues came from the "Ornina" winery located in Castel Focognano, Arezzo. The winery produces red wine starting from the organic grapes grown in his own vineyards and the varieties cultivated are Sangiovese and Malvasia Nera. Winemaking process is carried out in stainless steel tanks during the fermentation step, then, the following maceration of grapes continues for about 10 days. The by-product of red vinification that was used as feedstock for biogas production, is the "fermented grape marc" composed of skins and seeds: in October 2012, 1 kg of this material was collected and vacuum-sealed.

Beer residues came from "Birra dell'Elba" brewery, located in Elba Island in the municipality of Portoferraio. The feedstocks used for the biogas trials is represented by the residues of the mashing step realized to produce unfiltered and not pasteurized beers, with low fermentation temperatures (8-12°C). In October 2012, 1 kg of these residues were collected and vacuum-sealed.

Pelargonium (*Pelargonium graveolens*) residues after the essential oil extraction and jatropha (*Jatropha curcas* L.) cake were also investigated. Both

these crops were produced in the same area of cultivation: South Madagascar, Ihorombé Region, village of Satrokala (22°19'39 S, 45°42'54'' E; altitude 1025 m above sea level), where the research farm is located with a characteristic rainfall of 1200 mm year^{-1} mostly concentrated between October until March.

The samples were collected during the agronomic season of 2012, during that period the mean temperatures followed the rainfall with the higher value during the winter season (max 26.2°C, Min 17.2°C) and the lower value during the summer (Max 19.7°C, Min. 10.1°C).

The soil texture is sandy-clay (50.6% and 38.9% respectively) with an average pH value of 5.5 and a general limited mass fraction of dry matter of Nitrogen (0.12%) and Carbon (1.97%).

Pelargonium (*Pelargonium graveolens*) was cropped during the agronomic season 2012 - 2013, from December to April in an open-field plantation in double line with a planting density 50.000 plants ha^{-1}. The cuttings, coming from the farm nursery, were irrigated immediately after transplanting for the proper establishment of the crop in the field and after that the water supply was guaranteed with a dripping irrigation system that supply 4 liters day^{-1} of water for each plant. Only organic manures were applied before the crop installation using cow manure.

After 4 months of growing, at the so called balsamic time, the crop was harvested and the essential oil extraction from the biomass was carried out by steam distillation in a 2 tons distillatory plant for 6 hour. The residues coming from this process, roughly 99% of the original biomass (the oil content is approximately 2 g kg^{-1}) is represented by moist leaves and stocks that have been collected and sun-dried for 48 hours before shipping to Italy.

Concerning the jatropha cake, it represents the residual part of the oil extraction process from seeds. The seeds were harvested from a 3 years old plantation of *Jatropha curcas* L. located in the same experimental fields previously described for pelargonium in Madagascar. The plantation was established starting from cuttings grown in the farm nursery for 6 months and transplanted in open field during the agronomic season 2009/2010, no irrigation was performed and the amount of 200 g of NPK fertilizer (10:10:10) was applied for each plant. The unique agronomical practice performed during the cultivation is the second year pruning at 1 m high. The seed harvest was performed during the dry season of 2012 and the oil extraction was carried out following the mechanical extraction method using a bench Komet screw press (Model CA59G) with a pressing capacity of 3-5 Kg seeds per hour and an electric Power of Drive Motor of 1.1 kW.

Both jatropha and pelargonium residues were divided in samples of 100 g and the total amount of 500 g for each crop was prepared for the shipping to Italy. Each sample was collected in a vacuum envelope, by Valko Favola 310 vacuum packaging machine, to avoid alterations during the transport to the Florence laboratory; in addition, the pelargonium residues for AD have been rehydrated adding water 3 days before the beginning of the trial.

Physical-chemical and energetic analysis

Different laboratory equipment and methodologies were utilized for the analysis of substrates that were prepared through a cutting mill (mod. SM 300, Retsch). Moisture (UNI 14774-1:2009) and ash content (UNI 14775: 2010) were measured using a LECO 701 TGA. CHNOS content was measured using a Truespec CHN and S (LECO) (UNI CEN/TS 15104; KTBL, 2015). Data from these analyses were utilized to calculate the theoretical production of biogas for each substrate according to Buswell and Symons (1993).

The digested sludge was analyzed using the same technologies described above in order to estimate its potential use in agriculture as a soil improver. Furthermore the calorific value was measured by Isoperibolic calorimeter AC 500, Leco.

The total solid (TS), the volatile solid (VS) and the ash content of substrates were determined through a STF N-80, Falc stove. Each sample was weighted and placed in stove for 105°C until a constant weight was reached, after that, the dried material was burnt in a muffle furnace at 550°C and used for the determination of the raw ash content. The organic dry matter was calculated by subtracting the raw ash content from the dry matter.

Specific biogas and methane yield

Static reactor description. The static reactor was a 100 cm^3 glass vessel hermetically closed and placed on a heating plate connected to a thermocouple to control the constant heating (about 45°C) with a continuous monitoring of the system temperature. 4 repetitions were simultaneously carried out for each substrate. The measurement and temporary storage for the produced biogas consisted in a graduate syringe (30 cm^3) placed above the cap with the needle inserted into the rubber membrane and sealed with several layers of parafilm.

The ratio between volatile solids (VS) in the substrate and VS in the inoculum was 0.5. The produced amount of biogas was monitored on a daily basis for 10 days and then until there was no more biogas production.

Regular weekly analysis of inoculum and pH level in the reactors were carried out to evaluate the activity of inoculum and the progress level.

The qualitative analysis of biogas production was carried out through samples performed with a glass microsyringe and successive injection in GCMS (Shimadsu) for the gas chromatography determination. The biogas composition was measured with mass spectrometer gas chromatography GC-MS (GC - 2010 and QP 2010, Shimadsu).

Statistical analysis

The data generated in the present work belong to three major groups: i) theoretical biogas production with Buswell's formula; ii) biogas production of the different substrates from static digestion; iii) substrate and digestate chemical characterization.

Each different data was submitted to a specific statistical analysis and therefore: as regards type 1, the accuracy of Buswell's formula was evaluated through the calculation of standard deviation of the delta between the produced and measured biogas, for each substrate. The standard deviation comparison between the substrates was then evaluated with the "chi square" method.

ANOVA analysis on type 2 data was performed on the biogas production on three key moments: BioGas t_1 - the biogas production expressed in mL g^{-1} VS measured at the end of first day of anaerobic digestion; BioGas t_f - the cumulate final biogas production expressed in mL g^{-1} VS measured at day number 9; $IBRt_{(2\text{-}8)}$ - Increasing Biogas Rate (IBR) production calculated with the following formula (3):

$$IBR = \sum_{2}^{8}\left[\frac{Biogt_{tn}}{Biogt_{tn-1}}\right]$$

where $Biogt_{tn}$ represents the biogas production per day.

The fixed model of analysis of variance was applied using the statistical software SPSS IBM, and the significance of the variance was tested with the Tukey's test.

Regarding the biogas production trend during the digestion process, data were evaluated through the analysis of the regression within each different substrates. In addition, a regression analysis of the whole data set was carried out. The linear and the polynomial regression approximation were calculated and their significance tested according to ANOVA analysis and Fisher test. With respect to type 3 data, a simple correlation between the substrate and digestate composition was performed.

3. Results and Discussion

Accuracy of Buswell estimation of innovative substrates for AD production

Based on the chemical composition of the different substrates showed in Table 3, the potential biogas yields have been calculated using the Buswell's formula.

The Buswell estimation was applied to all the feedstock under investigation (Table 4), however, it provided acceptable values compared to bibliography (ASTM, 1998) only for some of these, specifically corn (Oslaj *et al.*, 2019), sorghum (Wannasek *et al.*, 2017), pelargonium (Gamal-El-Din *et al.*, 2012, Marsili Libelli

Table 3 - Feedstock elemental composition

No.	Feedstock	Moisture (%)	Ash (db) (%)	C (db) (%)	H (db) (%)	N (db) (%)	S (db) (%)	O (db) (%)	C/N ratio	VS ratio	TS (%)
1	Corn	81.00	4.31	50.67	6.20	0.83	0.20	37.79	61.05	71.42	19.00
2	Sorghum	80.00	7.65	46.46	5.51	0.96	0.17	39.25	48.40	84.61	20.00
3	Pelargonium	19.00	10.67	46.46	5.19	1.04	0.20	36.44	44.67	94.00	81.00
4	Kenaf	76.01	7.93	57.40	4.06	1.10	0.13	29.38	52.18	97.70	23.99
5	Sunflower	78.70	8.50	62.95	3.14	1.10	0.11	24.20	57.23	90.00	21.33
6	Amaranth	74.00	5.45	41.40	4.66	1.70	0.20	46.68	24.35	77.80	26.00
7	Olive cake	64.60	4.40	63.90	6.88	0.80	0.20	23.82	79.88	95.60	35.40
8	Jatropha cake	52.40	1.96	58.55	6.08	3.40	0.20	29.81	17.22	98.00	47.62
9	Wine residues	69.02	1.14	32.05	8.13	2.20	0.20	56.29	14.57	96.50	30.98
10	Cow milk whey	99.10	0.06	1.13	11.00	0.30	0.00	87.51	3.77	100.00	0.91
11	Ewe milk whey	92.50	0.79	3.41	10.70	0.48	0.00	84.62	7.10	93.30	7.56
12	Beer residues	72.30	0.91	44.00	6.43	1.80	0.05	46.82	24.44	95.60	27.70

Table 4 - Buswell's formula estimation, biogas experimental production and comparison with Corn's biogas yield

No.	Feedstock	Biogas production estimation by Buswell's formula ($m^3\ t^{-1}$)	Trial measurement biogas at day 9 ($m^3\ t^{-1}$)	Biogas yield at day 9 in comparison to Corn (%)
1	Corn	990.51	359.50	-
2	Sorghum	940.83	322.75	- 10.22%
3	Pelargonium	973.02	217.25	- 39.57%
4	Kenaf	1165.39	370.50	+ 3.06%
5	Sunflower	1285.77	150.68	- 58.09%
6	Amaranth	818.29	156.14	- 56.57%
7	Olive cake	1250.31	310.67	- 13.58%
8	Jatropha cake	1117.00	222.25	- 38.18%
9	Wine residues	829.3	66.85	- 81.40%
10	Cow milk whey	21.10	245.93	- 31.59%
11	Ewe milk whey	64.16	483.87	+ 34.60%
12	Beer residues	829.3	248.43	- 30.90%

et al., 2014) and beer residues (Maier, 2015; Mugodo *et al.*, 2017), with a biogas estimated productions equal to 990.51 $m^3\ t^{-1}$, 940.83 $m^3\ t^{-1}$, 973.02 $m^3\ t^{-1}$ and 829.30 $m^3\ t^{-1}$ respectively. For the other silage feedstock, kenaf (Saba *et al.*, 2015), sunflower (Dubrovskis *et al.*, 2012; Adamovics and Dubrovski, 2015; Markou *et al.*, 2017) and amaranth (Sitkey *et al.*, 2013; Minzanova *et al.*, 2018), the Buswell's formula application provides unrealistic values of 1165.39 $m^3\ t^{-1}$, 1285.77 $m^3\ t^{-1}$ and 818.29 $m^3\ t^{-1}$ respectively. Similar high values of 1250.31 $m^3\ t^{-1}$ and 1117.00 $m^3\ t^{-1}$ arise also respectively from the Buswell estimation performed among the olive oil cake (Tekin and Dalgic, 2000; Battista *et al.*, 2015; Valenti *et al.*, 2017) and jatropha oil cake (Staubmann *et al.*, 1997; Grimsby *et al.*, 2013; Jingura and Kamusoko, 2018) while the cow milk whey and ewe milk whey (Battista *et al.*, 2015) record limited and unacceptable low value of 21.10 $m^3\ t^{-1}$ and 64.16 $m^3\ t^{-1}$ respectively. An acceptable (Failin and Restuccia, 2014; Mugodo *et al.*, 2017) level of accuracy could lie on the Buswell estimation performed over wine residues with: 829.30 $m^3\ t^{-1}$.

Therefore the reliability of the biogas production methodology among the substrates was evaluated in comparison with the corn silage biogas production observed during the experiment (Table 4).

A general lower production, when compared to corn silage biogas yield, can be observed among the majority of the feedstock and this reduced production ranges from admissible values of 10.22% and 13.58% of sorghum and olive oil cake respectively, until 81.40% of wine residues.

On the contrary, a positive value of 34.60% is observed from the comparison between corn and ewe milk whey, probably due to the booster effect of this special milk whey. This difference is even wider when the ewe milk whey biogas production is compared to the theoretical biogas production of 16.30 $m^3\ t^{-1}$ expressed by Buswell's formula application.

Finally, as regard the kenaf silage, it showed a limited increase of 3.06% in biogas production at day 9, when compared to the corn biogas production.

Biogas production from static digestion of the different substrates

The ANOVA analysis performed on biogas production data showed a significant effect ($P<0.001$) of the substrate.

As regards the $BioGas_{(t1)}$ (Fig. 1), the substrates that show a significantly higher production at the first day of digestion are sorghum silage, pelargonium residues and beer residues, with a production of 36.75; 35.25 and 35.23 mL g^{-1} VS biogas respectively. On the opposite, a very limited performance in terms of biogas production was observed for cow milk whey, wine residues, amaranth silage and jatropha oil cake with a production of: 1.73, 9.37, 10.00 and 13.37 mL g^{-1} VS of biogas respectively.

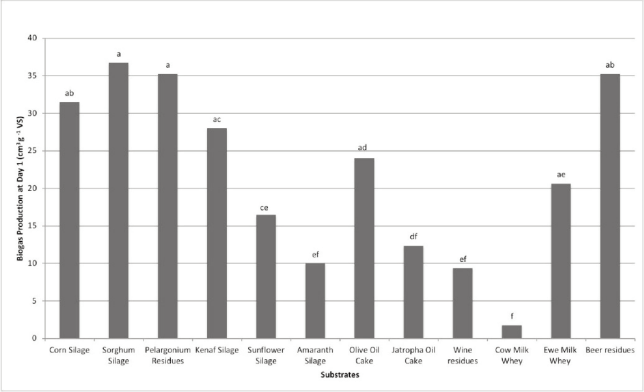

Fig. 1 - $BioGas_{(t1)}$ experimental production ($cm^3\ g^{-1}$ VS) for the different substrates. Different letters means significative differences between substrates according to Bonferroni Multiple Comparisons Test ($P<0.05$).

Among all the remaining substrates, it is possible to identify substrates that produce a large amount of biogas compared to the others such as the corn silage with 31.50 mL g^{-1} VS of biogas. Finally, kenaf silage, olive oil cake, ewe milk whey and sunflower silage generated a reduced quantity of biogas but significantly higher than cow milk whey.

The cumulate final biogas production BioGas $t_{(f)}$ at the end of day 9 (Fig. 2) shows instead a very different behavior. The most productive substrate is the ewe milk whey, with a production of 488.87 mL g^{-1} VS of biogas, while the smaller amounts of biogas were produced from kenaf silage and corn silage, 370.50 and 359.50 mL g^{-1} VS biogas respectively.

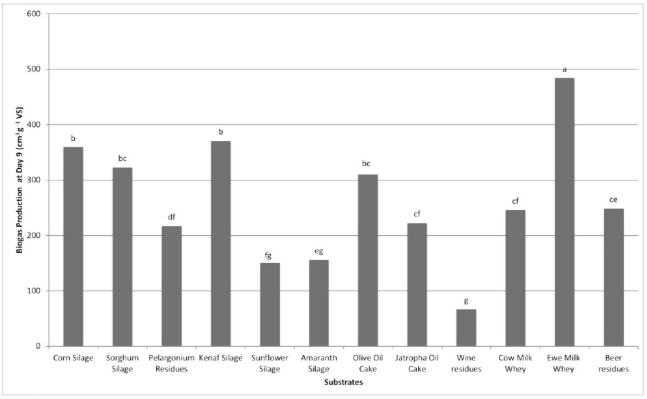

Fig. 2 - BioGas$_{(t9)}$ cumulate production (cm^3 g^{-1} VS) the end of day 9 for different substrates. Different letters means significative differences between substrates according to Bonferroni Multiple Comparisons Test (P<0.05).

At the opposite, the wine residues were the worst performing substrates with only 66.87 mL g^{-1} VS biogas, significantly differing from the majority of the substrates tested during the experimental campaign; only amaranth silage and sunflower silage had a statistically similar behavior, with 156.14 and 150.68 mL g^{-1} VS biogas production respectively. All the other substrates tested show intermediate levels of production: only sorghum silage and olive oil cake were similar to corn silage and kenaf silage.

The IBRt$_{(2-8)}$ ANOVA analysis allowed us to identify the cow milk whey (Fig. 3) as the substrate having the fastest growing rate of biogas production during the intermediate period of anaerobic digestion (IBR=1.59). A good IBR performance was observed also by ewe milk whey (IBR= 1.48) and jatropha oil cake (IBR=1.41) while the pelargonium residues, with an average value of 1.25, achieve lower IBR value similar to beer and wine residues.

Observing the overall tendency of cumulate biogas production (Fig. 4) with the support of the regression analysis that showed significance for the overall

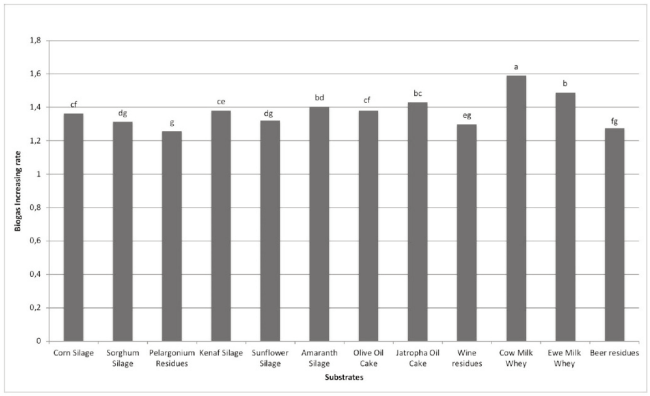

Fig. 3 - IBR$_{t(2-8)}$ values for different substrates. Different letters means significative differences between substrates according to Bonferroni Multiple Comparisons Test (P<0.05).

variance of the regression and, when performed within each substrate, for the linear and polynomial regression as shown in Table 5, it is possible to make some general assumptions.

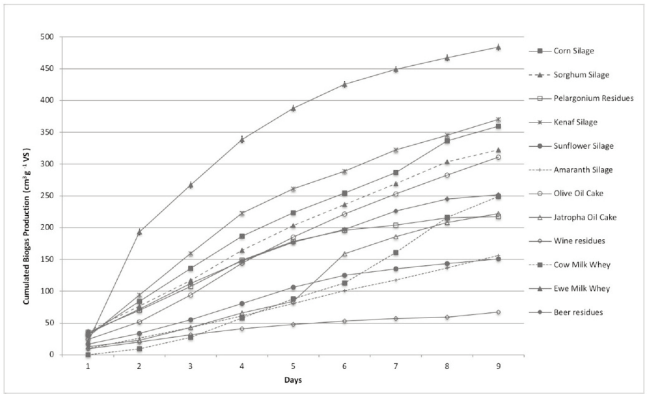

Fig. 4 - Overall tendency of biogas production among the different substrates.

Ewe milk whey substrate showed a cumulate biogas production superior during the entire digestion period, with the exclusion of the first day. The tendency is therefore a fast growth of biogas production during the whole experimental campaign (Table 5, Figs. 4 and 5).

At the opposite, residues showed a limited increase of biogas production during the entire cycle, leading to the lower biogas (Fig. 4).

Kenaf silage, corn silage, sorghum silage and olive oil cake have a similar behavior during the digestion process, with similar final and intermediate biogas yields (Fig. 4).

More complex trends were observed for the remaining substrates; in effect, it is possible to identify three main behaviors:

Beer residues and pelargonium residues showed a

Table 5 - Linear and polynomial regressions equations. R2 values and significance for the different substrate tested

N.	Substrate	Linear regression		Polynomial regression	
		Equation	R^2 (Sign)	Equation	R^2 (Sign)
1	Corn silage	$Y = 40.629\ x + 7.770$	0.976 **	$Y = -1.57\ x^2 + 56.35\ x - 21.03$	0.983 NS
2	Sorghum silage	$Y = 36.671\ x + 8.868$	0.919 **	$Y = -1.33\ x^2 + 50.03\ x - 15.63$	0.925 NS
3	Pelargonium residues	$Y = 23.400\ x + 35.389$	0.864 NS	$Y = -3.07\ x^2 + 54.17\ x - 21.05$	0.940 *
4	Kenaf silage	$Y = 41.904\ x + 22.951$	0.954 NS	$Y = -3.82\ x^2 + 80.18\ x - 47.25$	0.995 **
5	Sunflower silage	$Y = 17.584\ x + 4.861$	0.947 NS	$Y = -1.33\ x^2 + 31.15\ x - 15.52$	0.974 *
6	Amaranth silage	$Y = 18.43\ x + 10.837$	0.702 **	$Y = 0.14\ x^2 + 16.94\ x - 8.11$	0.702 NS
7	Olive oil silage	$Y = 37.249\ x + 12.135$	0.980 **	$Y = -1.05\ x^2 + 47.79\ x - 31.48$	0.984 NS
8	Jatropha oil silage	$Y = 29.567\ x + 36.483$	0.867 **	$Y = 0.91\ x^2 + 20.37\ x - 19.62$	0.871 NS
9	Wine residues	$Y = 6.833\ x + 8.722$	0.317 **	$Y = 0.61\ x^2 + 12.99\ x - 2.56$	0.330 NS
10	Cow milk whey	$Y = 32.300\ x + 59.110$	0.962 NS	$Y = -2.58\ x^2 + 6.49\ x - 11.79$	0.990 **
11	Ewe milk whey	$Y = 52.108\ x + 76.440$	0.843 NS	$Y = -8.36\ x^2 + 135.72\ x - 76.83$	0.954 **
12	Beer residues	$Y = 27.833\ x + 23.194$	0.956 NS	$Y = -1.57\ x^2 + 56.35\ x - 21.03$	0.983 *

** significative for P<0.01; * significative for P<0.05; NS= not significative.

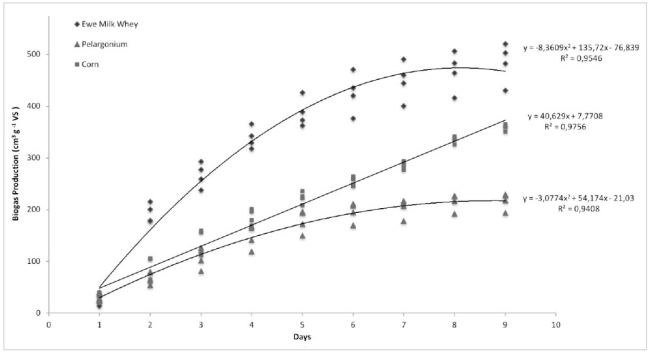

Fig. 5 - Regression (linear and polynomial) of ewe milk whey, corn silage and pelargonium residues.

common trend until day 5, similar to the other groups, and then diverge, with a reduction in the increasing rate of biogas production.

Jatropha oil residues and cow milk whey, that generally showed a low level of biogas production, had an initial poor production until day 5 followed by a certain increase during the second period.

Sunflower silage and amaranth silage showed a limited but continuous increase in biogas production from day 1 to 9.

Digestate chemical characterization

Digestate from the AD of different substrates was chemically characterized (Table 6).

The use of digestate as fertilizer or soil amendment is suggested when an appropriate N content (1-4%) is linked to a C/N ratio between 10 and 20 (Haraldsen *et al.*, 2011; Mantovi *et al.*, 2011; Chen *et al.*, 2012; Garcia-Sanchez *et al.*, 2015): for these reasons, all the different tested feedstocks used were reasonably suitable for this purpose. The high C/N ratio of some of them, such as jatropha cake (19.13) and amaranth (17.56), seems very interesting towards microbial processing soil bacteria.

4. Conclusions

The Buswell's formula effectiveness, estimating the biogas production from various feedstock, was investigated and results of the analysis showed that while it approximates sufficiently well the biogas production rate during the early estimation for conventional feedstock, it does not perform adequately

Table 6 - Digestate elemental composition

No.	Digestate	Moisture (%)	Ash (db) (%)	C (db) (%)	H (db) (%)	N (db) (%)	S (db) (%)	HHV (db) (MJ/Kg)	C/N ratio
1	Corn	95.55	0.89	39.23	5.48	3.11	0.33	16.45	12.61
2	Sorghum	95.40	0.92	39.25	5.54	2.85	0.31	16.57	13.77
3	Pelargonium	95.00	1.00	38.67	5.39	2.73	0.36	16.08	14.16
4	Kenaf	95.26	6.40	40.00	5.13	2.72	0.40	16.65	14.71
5	Sunflower	94.24	5.20	39.00	5.01	2.61	0.32	16.51	14.94
6	Amaranth	94.40	2.00	37.75	5.28	2.15	0.34	15.18	17.56
7	Olive cake	94.50	1.80	39.40	5.34	2.87	0.34	16.45	13.73
8	Jatropha cake	94.60	4.05	39.60	5.36	2.07	0.31	15.99	19.13
9	Wine residues	93.20	4.00	42.97	5.75	2.73	0.32	17.37	15.74
10	Cow milk whey	97.30	3.70	34.35	4.75	2.40	0.38	15.10	14.31
11	Ewe milk whey	96.90	3.75	36.07	5.03	2.73	0.38	14.87	13.21
12	Beer residues	93.50	4.10	41.80	5.55	2.72	0.30	16.87	15.37

when applied to un-conventional substrates, with a general over estimation for the crop silage or crop residue, and with an under estimation for the milk residues. Consequently, the application of the Buswell formula to test new substrates should be limited to a preliminary survey and coupled with a BMP test.

The tests carried out on these new substrates led to several conclusions.

The ewe milk whey represents a good booster product in AD and should thus be coupled in a limited percentage with conventional substrates, such as corn silage or similar.

The cow milk whey did not perform equally well as booster product, despite it shows the best increasing biogas rate (IBR).

Corn and sorghum silage showed a common behavior in AD, as expected.

Kenaf silage gave an interesting and remarkable performance, as this low input crop produces almost the same amount of biogas than corn and sorghum. Therefore, kenaf could represent a promising alternative to conventional corn silage.

The olive oil cake moreover, with its constant and increasing biogas production over the whole period, could be an alternative to kenaf silage but, due to an expected reduction during the following days after day 9, can be use only in limited percentage.

Sunflower and amaranth silage, despite a limited biogas production, might represents the most sustainable crops due to their limited requests of water, fertilizers and labor needs.

Regarding wine and beer residues they both did not perform sufficiently well in anaerobic digestion; its energetic use could be probably better take advantage in direct combustion or in charcoal production.

Specific considerations should be carried out for jatropha oil cake and pelargonium residues, since they both represent a biomass largely available in the tropical areas (Southern Madagascar) and despite the low biogas production, is possible to speculate the creation of a local self-sufficient production of biogas to power the extraction systems in Madagascar.

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Effect of vermicompost on morphological and physiological performances of pot marigold (*Calendula officinalis* L.) under salinity conditions

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Key words: antioxidative enzymes, chlorophyll content, fertilizer, nutrient elements.

Abstract: A greenhouse study was conducted in order to evaluate the interactions of vermicompost and salinity effects on morphology and physiology of pot marigold. The experiment was conducted with vermicompost treatments at five levels (0%, 5%, 10%, 15% and 20%) and salinity treatments at five levels (0, 50, 100, 150 and 200 mM NaCl) in a completely randomized factorial design arrangement with four replications. Results showed that increasing levels of salinity led to decline in leaf area, fresh and dry weights of flower, shoot, and root, N, P, K, Fe, Mg and Zn concentrations, chlorophyll and carotenoid contents, while proline content increased in the plants. APX, SOD, POD and CTA enzyme activities significantly increased with increasing salinity from 0 to 150 mM NaCl, then declined in 200 mM treatment in the plants. Application of vermicompost increased the morpho-physiological indices and mineral nutrient uptake in the plants and could increase the plant yield by alleviating the harmful effects of salinity.

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1. Introduction

Calendula officinalis, known as "pot marigold", is a plant in *Calendula* genus of Asteraceae family. It is perhaps native and widely naturalized further Northern to Southern Europe and elsewhere in warm temperate regions of the world, and it may possibly be planted widely in gardens and landscapes (Gharineh *et al*., 2013). Among ornamental bedding plants, pot marigold is known to grow well under saline conditions. In fact, some pot marigold cultivars that are used as cut flowers or as bedding plants in landscaping can be grown by maintaining the quality of plants under saline conditions with an EC_w of <8 dS m^{-1} (Koksal *et al*., 2016).

Plants are exposed to ever-changing and often unfavorable environmental conditions, which cause both biotic and abiotic stresses such as extreme temperatures, flood, drought, and salinity. Overexploitation of

available water resources as well as environmental factors such as low precipitations, high temperatures and contamination from parental rocks are leading to increases in soil salinization (Aroca *et al*., 2013). Soil salinization is one of the most important agricultural and eco-environmental problems nowadays, which is increasing steadily in many parts of the world. Saline soils have been estimated to occupy more than 7% of the Earth's land surface and it is expected to be increased by up to 50% by the middle of the twenty-first century (Ruiz-Lozano *et al*., 2012). Salinity stress is one of the major abiotic threats to plant life and agriculture worldwide and significantly reduces crop yield in the affected areas. Excessive salt above what plants need limits plant growth and productivity and can lead to plant death. About 20% of all irrigated land is affected by soil salinity, decreasing crop yields. Plants are affected by salinity stress in two main ways: osmotic stress and ionic toxicity. These stresses affect all major plant processes, including photosynthesis, cellular metabolism, and plant nutrition (Aslamsup *et al*., 2011). Amelioration of salt-affected soils can be accomplished through many effective methods, such as water leaching, chemical remediation, and phytoremediation (Qadir *et al*., 2007). The amelioration of salt-affected soils using chemical agents, including gypsum, calcite, calcium chloride and organic matter, is a successful approach that has been implemented worldwide (Sharma and Minhas, 2005; Tejada *et al.*, 2006). According to a study, the application of organic matter conditioners has become a common practice in salt-affected areas in the last several decades and constitutes an important method of soil regeneration and fertility enhancement (Melero *et al.*, 2007). Organic matter is very important for maintaining structural stability in soils as well as improving the physical, chemical and biological properties of soils. Salt-affected soils generally exhibit poor structural stability due to low organic matter. The addition of organic materials (e.g. green, farmyard and poultry manures, compost, food processing wastes, etc.) has been suggested for improving structural stability of soils by many researchers (Tejada *et al.*, 2006). Barzegar *et al.* (1997) found that addition of plant residues improved the water-stable aggregate in soils because of increased organic matter content and decreased soil salinity. The application of organic matter for soil remediation is considered essential for sustainable land use and crop productivity. Given the importance of pot marigold in green space, and their being placed in saline soils in most cultivable regions, so far, enough research has not been conducted to understand morpho-physiological properties of pot marigold under salinity stress conditions and determine the status of vermicompost in reducing the devastating effects of salinity stress in pot marigold. For this purpose, an experiment was conducted to determine the effects of vermicompost on some morphological and physiological characteristics of pot marigold under salinity stress conditions.

2. Materials and Methods

To investigate the effects of vermicompost on some physiological characteristics of pot marigold (*Calendula officinalis* L. cv. Candyman Orange) under salinity stress, a greenhouse experiment was conducted in a completely randomized factorial design including vermicompost at five levels (0%, 5%, 10%, 15% and 20%) and salinity stress at five levels (including 0, 50, 100, 150 and 200 mM NaCl) with four replicates. This study was conducted at the Research Greenhouse of the Department of Horticultural Science, College of Agriculture, Shiraz University, Shiraz, Iran (52°32´E and 29°36´N). After disinfection of pots (20 and 30 cm in diameter and length, respectively), 5%, 10%, 15% and 20% (v/v) vermicompost from Kian Pars Shiraz Company, were dried in the shade and were mixed with four kilograms of a native soil. The main physical and chemical characteristics of the vermicompost and soil mixture are shown in Table 1. Then, four marigold seeds were planted in each pot. The pots containing seeds were kept in a greenhouse with 27/18°C (day/night) temperature, 16 h light conditions, and 35% relative humidity. After germination of seeds one plant was selected in each pot and the three other plants were removed. When the plants reached the four-leaf stage, the plants were treated with four levels of salinity stress.

Table 1 - Some physico-chemical properties of vermicompost and soil

Sample	Vermicompost	Soil
Organic matter %	33	0.41
pH	8.1	7.05
EC (d/Sm)	1.7	0.6
P (%)	1.8	
PWP (%)		5.1
K (%)	1.2	
FC (%)		14
C/N	13	
Total N (%)	1.5	
Soil texture		Sandy-loam

Salinity stress treatment was applied by adding net quantities of sodium chloride (NaCl) to the irrigation water so that pots were irrigated with 50, 100, 150, and 200 mM NaCl containing water based on field capacity of the soil, and the amount of the decreased water obtaining by the salinity treatment resulted in the average electrical conductivity (EC) of 3.62, 6.27, 9.36 and 12.71 dS/m in each level of the treatment, respectively. The control treatment was applied using distilled water. After 35 days of the treatments, the plants were harvested in order to measure morphological and biochemical traits.

Growth parameters

Growth parameters including, flower diameter (mm), leaf area (cm^2) and fresh and dry weights of flower, shoot, and root (g) were measured. For dry weight determination, the shoots, roots, and flowers were dried in an oven at 70°C for 48 h and weighed.

Proline content

The leaf proline content was determined using the method of Bates *et al*. (1973). Proline was extracted from leaf samples of 100 mg weight fresh with 2 ml of 40% methanol. 1 ml of the extract was mixed with 1 ml of a mixture of glacial acetic acid and orthophosphoric acid (6 M) (3:2, v/v) and 25 mg ninhydrin. After 1 h of incubation at 100°C, the tubes were cooled, and 5 ml toluene was added. The absorbance of the upper phase was spectrophotometrically determined at 528 nm. The proline concentration was determined using a standard curve.

Chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents were estimated by the method of Hiscox and Israelstam (1979). Fresh leaf and petal material (1 mg) and 10 ml DMSO were taken in vials and kept in an oven at 65°C for 4 h. Absorbance was read at wavelengths of 665 and 649 for leaves, and 480 nm for petals using a spectrophotometer (Beckman DU 640 B, Fullerton, USA). The following equations were used to calculate each compound.

Chl a= (12.47×A665)-(3.62×A649)

Chl b= (25.06×A649)-(6.5×A665)

Total chlorophyll (mg g^{-1} F.W.) = Chla + Chlb

Carotenoids (mg g^{-1} F.W.) = $\frac{(1000 \times A480) - (1.29Chla - 53.78Chlb)}{220}$

where A stands for the absorbance reading of a sample at 665, 649 and 480 nm of wavelength.

Antioxidant analysis

Fresh leaf samples were homogenized in extraction buffer (0.1 M phosphate buffer pH 6.8) with a mortar and pestle on ice. The homogenate was then centrifuged at 12,000 *g* for 15 min at 4°C and the supernatant was used as the crude extract for the superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase and catalase. The superoxide dismutase (SOD), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT) enzymes were estimated using the methods previously described by Beauchamp and Fridovich (1971), Chance and Maehly (1955), Nakano and Asada (1981), and Dhindsa *et al*. (1981), respectively.

Plant nutrient element analysis

Leaf material was ground to pass 0.5 mm sieve in a cyclone laboratory mill, weighed into ceramic crucibles, ashed overnight at 550°C in a muffle furnace, and the ash was suspended in 2M HCl for determination of mineral nutrients. Then, total nitrogen (N), phosphorus (P) and potassium (K) were determined in the leaf using the Kjeldahal, colorimetrically and ammonium acetate methods respectively., Zinc (Zn), magnesium (Mg) and Iron (Fe) were determined by atomic absorption spectroscopy (Baumard *et al*., 1998).

Statistical analyses

The data were analyzed using one-way analysis of variance at $P < 0.05$ significance with SAS version 9.4 software (SAS Institute Inc., Cary, NC). Fisher's LSD test was conducted to determine the statistical differences among different treatments.

3. Results and Discussion

Leaf area and fresh and dry weight of shoots

The growth (leaf area and fresh and dry weight of shoots) was significantly affected by the treatments (Table 2). The treatment of plants with NaCl significantly reduced the growth parameters and the decrease was proportional to the concentration of NaCl. The highest concentration (200 mM NaCl) was the most deleterious and decreased the leaf area by 53.32%, fresh weight by 72.76%, and dry weight by 48.74% as compared to those of the control plants. However, all the above parameters were significantly enhanced by the vermicompost treatments. The highest and the lowest leaf area and fresh and dry weight of shoots were obtained in the 20% vermicompost and the control treatment, respectively (Table 2). Leaf area and fresh and dry weight of shoots increased (12.03%, 39.52% and 38.96%, respectively) at the 20% vermicompost treatment compared to the control plants. Researches showed

that soil salinity reduces the growth of plant shoots. This water potential reduction in the soil or osmotic effect is due to the presence of salt in the soil which limits the root water absorption (Nguyen *et al*., 2015). In the salt stress, the plant's hormonal system which synthesizes and transmits a number of hormones such as cytokinins is impaired and their transport from roots to the upper parts of the plant is limited. The termination of the hormone transfer from roots to branches and the consequent reduced water absorption capacity can lead to a decline in plant growth. Reduction of leaf area can be explained by decreased cell growth or a reduced cell division rate due to decreased cellular turgor. Decrease in leaf area also reduces the rate of photosynthesis, resulting in decreased fresh and dry weight of plants (Zarei *et al*., 2016). In addition, Alshammary *et al.* (2004) showed that decrease in growth was due to reduction of flexibility, development of cells, and reduction of auxins. Vermicompost, due to its high microbial activity resulting from the presence of fungi, bacteria, yeasts, actinomycetes, and algae, can produce different growth regulators such as auxins, gibberellins, and cytokinins all of which may have positive effects on plant growth and development (Xu *et al.*, 2016; Ullah *et al*., 2018). Thus, the cause of rises in height and leaf area of the plants treated with vermicompost is probably from the stimulation of production of growth regulators including auxins and gibberellins. Furthermore, vermicompost contains humic substances that increase the availability of plant N, P, K, and in particular Zn for the synthesis of tryptophan, a precursor to auxins that are used for rooting and plant growth (Sharifianpour *et al*., 2015; Scaglia *et al*., 2016).

Fresh and dry weight of roots

The fresh and dry weight of roots of the pot marigold plants was lower with the salinity stress treatments as compared to that of the plants under non-saline conditions (Table 3). Fresh and dry weight of roots decreased (51.91% and 77.98%, respectively)

Table 2 - Effect of salinity and vermicompost and their interaction on leaf area and shoot fresh and dry weight

Vermicompost (%)	Salinity (mM)					Mean vermicompost
	0	50	100	150	200	
			Leaf area			
0	36.10±1.33 gh	36.10±0.62 gh	35.12±0.62 i	27.15±0.62 m	15.91±0.62 q	30.05±8.10 E
5	37.05±0.62 f	36.71±0.62 fg	35.01±0.62 gh	27.85±0.62 m	16.61±0.62 q	30.03±8.08 D
10	38.39±0.62 de	38.05±0.62 e	37.16±0.62 f	29.19±0.62 l	17.95±0.62 p	32.14±8.08 C
15	39.28±0.62 bc	38.94±0.62 cd	38.05±0.62 e	30.08±0.62 k	18.84±0.62 o	33.03±8.08 B
20	40.40±1.18 a	40.06±1.18 ab	39.17±1.18 cd	31.20±1.18 j	19.96±1.18 n	34.16±8.08 A
Mean salinity	38.24±1.73 A	37.95±1.65 A	37.06±1.65 B	29.09±1.65 C	17.85±1.65 D	
			Shoot fresh weight (g)			
0	59.92±1.18 h	57.79±1.18 i	54.37±1.18 k	27.19±1.18 n	9.79±1.18 p	44.05±1.92 E
5	60.62±0.62 h	58.49±0.62 i	55.07±0.62 kj	39.11±0.62 m	10.49±0.62 p	44.75±1.92 D
10	70.35±0.82 e	68.22±0.82 f	64.22±0.82 f	48.84±0.82 l	20.22±0.82 o	54.49±1.92 C
15	76.27±0.91 ab	74.14±0.91 c	74.14±0.91 c	54.76±0.91 kj	26.14±0.91 n	60.41±1.92 B
20	77.32±0.75 a	75.19±0.75 bc	75.19±0.75 bc	55.8±0.751 j	16.22±0.75 n	61.46±1.92 A
Mean salinity	68.89±7.67 A	66.76±7.67 B	63.34±7.67 C	47.38±7.67 D	18.76±7.67 E	
			Shoot dry weight (g)			
0	13.18±1.18 gh	12.84±1.18 hi	11.38±1.1 8 jk	8.92±1.18 mn	5.8±1.18 1p	10.42±3.01 E
5	13.88±0.62 fg	13.54±0.62 fgh	12.08±0.62 ij	9.62±0.62 lm	6.5±0.621 p	11.12±2.87 D
10	15.22±0.62 cd	14.88±0.62 de	13.42±0.62 gh	10.96±0.62 k	7.85±0.62 o	12.46±2.87 C
15	16.11±0.62 b	15.77±0.62 bc	14.31±0.62 ef	11.85±0.62 j	8.74±0.62 n	13.35±2.87 B
20	17.23±0.62 a	16.89±0.62 a	15.43±0.62 bcd	12.97±0.62 h	9.62±0.62 lm	14.48±2.87 A
Mean salinity	15.12±1.65 A	14.78±1.65 A	13.32±1.65 B	10.86±1.65 C	7.75±1.6 5 D	

* In each variable, data followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at 5% level. Data represent the mean value ± S.D. the mean of four replicates.

at salinity level of 200 mM NaCl compared to the control treatment. Ionic toxicity, nutritional imbalance, and osmotic osmolality adjustment are the negative effects of salinity stress on plant metabolism. Roots are the organ responsible for absorbing water and minerals, and salinity stress affects plant shoots more than the root system; however, the root system is the first organ that is exposed to salinity stress (Demiral and Türkan, 2005). One of the most effective indices in salinity tolerance is the maintenance of cellular turgor and osmotic regulation due to salt absorption and organic matter production. Plants, for production of organic materials such as glycine betaine, sorbitol, mannitol, and proline, spend a large amount of energy to regulate osmotic resistance in response to salinity stress. As a consequence, plants under salinity stress conditions reduce root efficacy in supplying nutrients and water to other organs. This reduces growth of shoots and dry matter production, and eventually reduces the transfer of nutrients from roots to shoots, and thus leads to a reduction in dry weight of roots and stems of plants (Claussen, 2005; Butt *et al*., 2016; Sattar *et al*., 2016). The vermicompost treatments of the plants grown under stress-free conditions significantly elevated the amount of biomass; the total fresh and dry weightwere 31.37% and 79.04% higher than those of the control plants, respectively. The vermicompost treatments also improved the amount of biomass in the plants that were subjected to the salinity stress treatments (Table 3). Edwards and Burrows (1988) reported that increase in fresh and dry weight of roots depends on increase in the activity of hormonal substances such as auxins, cytokinins, and gibberellins as well as vitamin B12.

Flower diameter and fresh and dry weight of flower

Flower diameter and fresh and dry weight of flower of the pot marigold plants were found to significantly decrease as the salt concentration was raised. These parameters decreased (33.58%, 67.94% and 27.48%, respectively) at the salinity level with 200 mM NaCl compared to the control treatment (Table 3 and 4). Several reports have shown that salinity decreases the flower diameter and fresh and dry weight of flower in (*Zinnia elegans*) (Carter and Grieve, 2010), Madagascar periwinkle (*Cathasanthus roseus*) (Jaleel *et al*., 2008) and garden mum (*Chrysanthemum× morifolium*) (Lee and Van Iersel, 2008). The vermicompost applications significantly

Table 3 - Effect of salinity and vermicompost and their interaction on root fresh and dry weight and flower diameter

Vermicompost (%)	Salinity (mM)					Mean vermicompost
	0	50	100	150	200	
			Root fresh weight (g)			
0	13.33±0.54 bcd	13.25±0.54 bcd	13.18±0.54 cde	9.28±0.54 fg	6.26±0.54 i	11.06±2.96 D
5	13.33±1.32 bcd	13.46±1.32 bcd	12.08±1.32 ij	9.49±1.32 fg	6.30±1.32 i	11.24±3.18 D
10	14.46±0.73 b	14.38±0.73 bc	14.31±0.73 bc	10.41±0.73 f	6.47±0.73 i	12.00±3.34 C
15	16.01±1.24 a	15.93±1.24 a	15.86±1.24 a	11.96±1.24 e	7.85±1.24 h	13.52±3.48 B
20	17.02±0.66 a	16.94±0.66 a	16.87±0.66 a	12.97±0.66 de	8.86±0.66 gh	14.53±3.36 A
Mean salinity	14.87±1.69 A	14.79±1.69 A	14.72±1.69 A	10.82±1.69 B	7.15±1.36 C	
			Root dry weight (g)			
0	6.50±0.54 d-g	6.38±0.54 efg	6.07±0.54 fg	2.17±0.54 jk	0.83±0.54 l	4.39±2.51 D
5	6.71±1.32 e-g	6.59±1.32 e-g	6.28±1.32 fg	2.38±1.32 ijk	0.89±1.32 l	4.57±2.73 D
10	7.63±0.73 cd	7.51±0.73 de	7.20±0.73 def	3.30±0.73 ij	1.30±0.73 ik	5.38±2.76 C
15	9.18±1.24 ab	9.06±1.24 ab	8.75±1.24 bc	4.85±1.24 h	3.43±1.24 i	6.85±3.02 B
20	10.19±0.66 a	10.07±0.66 a	9.76±0.66 ab	5.86±0.66 gh	7.85±0.66 h	7.86±2.87 A
Mean salinity	8.04±1.69 A	7.92±1.69 A	7.61±1.69 A	3.71±1.69 B	1.77±1.28 C	
			Flower diameter (mm)			
0	65.50±1.91 cde	61.50±1.91 def	59.50±1.91 fg	52.50±1.91 i	41.50±1.91 k	55.50±8.20 C
5	62.50±2.08 cde	61.50±2.08 def	59.50±2.08 fg	52.50±2.08 i	41.50±2.08 k	55.50±8.23 C
10	63.25±2.98 bcd	62.25±2.98 c-f	60.25±2.98 ef	53.25±2.98 i	42.25±2.98 k	56.25±8.45 C
15	65.00±3.55 abc	64.00±3.55 bcd	62.00±3.55 def	55.00±3.55 hi	44.00±3.55 kj	58.00±8.62 B
20	67.00±1.41 a	66.00±1.41 ab	64.00±1.41 bcd	57.00±1.41 gh	46.75±1.41 j	60.15±7.85 A
Mean salinity	65.05±2.85 A	63.05±2.85 A	61.05±2.85 B	54.05±2.85 C	43.20±3.03 D	

* In each variable, data followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at 5%level. Data represent the mean value ± S.D. the mean of four replicates.

increased these parameters compared to the control under both absence and presence of the salinity stress treatments. The 20% vermicompost treatment under salinity stress conditions gave the higher values for these parameters than those with the other treatments (Table 3 and 4). The extent of the increase in the values mentioned above was by 8.37%, 56.53% and 6.19 in the 20% vermicompost treatment as compared with those of the control, respectively. Hidalgo *et al*. (2006) stated that using vermicompost fertilizer increased flower diameter and fresh and dry weight of flower in marigold which is consistent with the findings of this study.

Proline content

As shown in Table 4, proline content of the pot marigold plants was markedly increased by the salinity stress treatments. The maximum and the minimum proline content were observed in the 200 mM NaCl and the control treatments, respectively. Proline content increased 447.87% at 200 mM NaCl compared to the control treatment (Table 4). Proline content increase is one of the protective mechanisms of this plant against salinity stress. It has been reported when plants are exposed to salinity stress, the breakdown of proteins and thus the increase of amino acids and amides accelerates, one of which is proline (Iqbal *et al*., 2015). Protein degradation, decrease in proline oxidase enzyme activity, and exacerbation of *P5CS* gene expression are the most important factors affecting proline concentration under stress conditions. Increase in *P5CS* gene expression is one of the most important factors affecting proline concentration under stress conditions (Kubala *et al*., 2015). The proline content was clearly affected by the vermicompost treatments. As compared to the control, the increase was 1.28% at 20% vermicompost. Interaction between levels of salinity and vermicompost resulted in the highest and the lowest proline content in the plants with 200 mM NaCl and 20% vermicompost treatment and with 0 mM NaCl and 0% vermicompost treatment, respectively (Table 4). In this study, the application of vermicompost increased proline content and reduced the damaging effects of salinity stress. Vermicompost increases the amount of N available in plants due to the presence of N in the proline structure, which leads to increased proline synthesis under salinity conditions and increased plant resistance to salinity stress (Rafiee *et al*., 2017).

Table 4 - Effect of salinity and vermicompost and their interaction on flower fresh and dry weight and proline content

Vermicompost (%)	Salinity (mM)					Mean vermicompost
	0	50	100	150	200	
			Flower fresh weight (g)			
0	4.35±0.57 ef	4.28±0.57 ef	3.88±0.57 fg	2.89±0.57 ij	1.04±0.57 m	3.29±1.37 D
5	4.71±0.65 de	4.64±0.65 de	4.24±0.65 ef	3.25±0.65 ih	1.56±0.65 lm	3.68±1.33 C
10	5.07±0.65 d	5.00±0.65 d	4.60±0.65 de	2.37±0.65 jk	1.92±0.65 kl	3.79±1.51 C
15	6.25±0.47 bc	6.18±0.47 bc	5.78±0.47 c	3.55±0.47 gh	2.03±0.47 kl	4.76±1.77 B
20	6.93±0.82 a	6.86±0.82 a	6.46±0.82 ab	3.27±0.82 ih	2.22±0.82 kl	5.15±2.16 A
Mean salinity	5.46±1.14 A	5.39±1.14 A	4.99±1.14 B	3.07±0.74 C	1.75±0.65 D	
			Flower dry weight (g)			
0	1.26±0.07 e-h	1.25±0.07 e-h	1.20±0.07 hi	1.11±0.07 jkl	0.84±0.07 p	1.13±0.07 C
5	1.27±0.06 e-g	1.26±0.06 e-g	1.21±0.06 gh	1.12±0.06 jk	1.05±0.06 lm	1.16±0.07 B
10	1.30±0.05 cde	1. 30±0.05 cde	1.23f±0.05 gh	1.14±0.05 ji	0.88±0.05 op	1.18±0.07 AB
15	1.30±0.01 cde	1.30±0.01 cde	1.28±0.01 e-g	1.07±0.01 klm	1.04±0.01 m	1.20±0.08 A
20	1.40±0.05 a	1.38±0.05 ab	1.36±0.05 bcd	0.96±0.05 n	0.92±0.05 no	1.20±0.09 A
Mean salinity	1.31±0.22 A	1.29±0.13 A	1.25±0.16 B	1.08±0.10 C	0.95±0.16 D	
			Proline content (µmol g-1 F.W.)			
0	4.19±0.12 k	4.31h±0.12 ij	4.76±0.12 f	10.32±0.12 d	23.18±0.12 b	9.35±7.47 D
5	4.20±0.11 k	4.32±0.11 hi	4.77±0.11 f	10.33±0.11 d	23.19±0.11 b	9.36±7.47 D
10	4.22±0.10 k	4.35±0.10 h	4.80±0.10 f	10.35±0.10 d	23.22±0.10 b	9.39±7.47 C
15	4.28±0.08 j	2.40±0.08 g	4.85±0.08 e	10.41±0.08 c	23.27±0.08 a	9.44±7.47 B
20	4.30±0.07 ij	4.42±0.07 g	4.87±0.07 e	10.43±0.07 c	23.30±0.07 a	9.47±7.47 A
Mean salinity	4.24±0.10 E	4.36±0.10 D	4.81±0.10 C	10.37±0.10 B	23.23±0.10 A	

* In each variable, data followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at 5%level. Data represent the mean value ± S.D. the mean of four replicates.

Chlorophyll content

The plants treated with NaCl exhibited a significant decrease in chlorophyll content and the greatest damage was caused by 200 mM NaCl treatment. In comparison with the control, chlorophyll content decreased by 28.12% with 200 mM NaCl treatment (Table 5). Whereas, the vermicompost treatments reversed the adverse effects of NaCl and caused a significant increase in chlorophyll content in the salt-treated plants (Table 5). The highest value of chlorophyll content in leaves (18.71%) was recorded in the vermicompost-treated plants over the control plants. Usually, decreasing chlorophyll when plants face stress conditions may be due to an alternation in N metabolisms in relation to the production of compositional compounds such as proline, which are used in osmosis regulation, because an increase in proline production causes glutamate to less involve in the chlorophyll biosynthesis pathways (Jaleel *et al*., 2008; Håkanson and Eklund, 2010). In addition, increased oxidative stress that is caused by reactive oxygen species damage to the chloroplast structure reduces the concentration of chlorophyll. Reduction of chlorophyll content has been reported in plants under salinity stress conditions due to the activity of chlorophyllase enzyme. Furthermore, some growth regulating agents such as abscisic acids and ethylene stimulate the activity of this enzyme (Ali *et al*., 2004; Zhao *et al*., 2007). The application of vermicompost significantly increased chlorophyll content in the leaves in the present study. Vermicompost increases the synthesis of chlorophyll under salinity stress conditions by providing nutritious elements such as Fe, Zn, Mg, and N directly and indirectly (Nadi *et al*., 2011; Narkhede *et al*., 2011).

Total carotenoid content

The content of carotenoid decreased in the plants that received 50, 100, 150 and 200 mM NaCl concentrations. The content of carotenoid was the lowest (37.16% over control) for the plants that received the highest level of the salinity treatment (200 mM NaCl) (Table 5). The vermicompost treatments not only improved the production of total carotenoid under saline-free conditions, but also successfully ameliorated the adverse effects caused by the salinity stress treatments on the plants (Table 5). The content of carotenoid increased in the vermicompost-treated plants grown with the 20% level by 1.30% compared to that in the control plants. Carotenoids act as

Table 5 - Effect of salinity and vermicompost and their interaction on chlorophyll content, total carotenoid content and superoxide dismutase

Vermicompost (%)	Salinity (mM)					Mean vermicompost
	0	50	100	150	200	
	Chlorophyll content (mg g^{-1} F.W.)					
0	2.09±0.11 k	2.06±0.11 l	1.97±0.11 m	1.77±0.11 r	1.46±0.11 v	1.87±0.26 E
5	2.17±0.11 g	2.14±0.11 i	2.05±0.11 l	1.85±0.11 p	1.54±0.11 u	1.95±0.26 D
10	2.24±0.11 e	2.21±0.11 f	2.12±0.11 j	1.92±0.11 o	1.60±0.11 t	2.02±0.26 C
15	2.27±0.11 d	2.24±0.11 e	2.15±0.11 h	1.95±0.11 n	1.64±0.11 s	2.05±0.26 B
20	2.44±0.11 a	2.41±0.11 b	2.32±0.11 c	2.12±0.11 j	1.81±0.11 q	2.22±0.26 A
Mean salinity	2.24±0.15 A	2.21±0.15 B	2.13±0.15 C	1.92±0.15 D	1.61±0.15 E	
	Total carotenoid content (μg·g^{-1} F.W.)					
0	12.48±0.92 k	12.46±0.92 l	12.43±0.92 o	12.35±0.92 r	7.82±0.92 w	11.51±2.06 E
5	12.50±0.92 i	12.49±0.92 j	12.45±0.92 m	12.37±0.92 q	7.84±0.92 v	11.53±2.06 D
10	12.52±0.92 f	12.51±0.92 h	12.48±0.92 k	12.40±0.9 2p	7.87±0.92 u	11.55±2.06 C
15	12.57±0.92 d	12.55±0.92 e	12.52±0.92 g	12.44±0.92 n	7.91±0.92 t	11.60±2.06 B
20	12.63±0.92 a	12.61±0.92 b	12.58±0.92 c	12.50±0.92 i	7.97±0.92 s	11.66±2.06 A
Mean salinity	12.54±0.82A	12.52±0.82 B	12.49±0.82 C	12.41±0.82 D	7.88±0.82 E	
	Superoxide dismutase (Ug^{-1} F.W.)					
0	146.00±1.92 m	152.00l±1.92 m	156.00±1.92 kl	440.00±1.92 d	104.00±1.92 q	199.60±1.25 C
5	155.50±0.80 kl	161.50±0.80 ijk	165.50±0.80 ghi	449.50±0.80 c	113.50±0.80 p	209.10±1.24 B
10	15.50±0.80 jkl	163.50±0.80 hij	167.50±0.80 f-i	451.50±0.80 bc	115.50±0.80 o	211.10±1.24 B
15	163.50±0.80 hij	169.50±0.80 e-h	173.50±0.80 ef	457.50±0.80 ab	121.50±0.80 n	217.10±1.24 A
20	165.50±0.80 ghi	171.50±0.80 efg	175.50±0.80 e	459.50±0.80 a	123.50±0.80 n	219.10±1.24 A
Mean salinity	157.60±1.22 D	163.60±1.22 C	167.60±1.22 B	451.60±1.22 A	115.60±1.22 E	

* In each variable, data followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at 5%level. Data represent the mean value ± S.D. the mean of four replicates.

helper pigments in chloroplasts, but their most important role is antioxidant properties. Because of the oxidative stress caused by salinity stress in plant tissues, carotenoid activity in both antioxidant enzymatic and non-enzymatic systems decrease (Pant *et al*., 2009). In similar studies in summer savory (*Satureja hortensis* L.) (Najafi and Khavari-Nejad, 2010), wheat (*Triticum vulgare* L.) (Reddy and Vora, 2005), and marjoram (*Origanum majorana*) (Baatour *et al*., 2010), reduction of carotenoids under salinity conditions has been reported. In a study consistent with the findings of the present study, Ayyobi *et al*. (2014) stated that vermicompost increased carotenoid content in peppermint (*Mentha piperita* L.) leaves.

Enzyme activities

The activities of APX, POD, CAT and SOD in pot marigold plants were significantly affected by the salinity stress and vermicompost treatments. With the increasing extent of salinity stress, the activities of APX, POD, CAT and SOD increased markedly and then significantly decreased (Table 5 and 6). APX, POD, CAT and SOD enzyme activities increased (12.08%, 13.67%, 36.08% and 186.54%, respectively) in the plants at salinity level of 150 mM NaCl compared to those in the control plants. Usually one of the biochemical changes that occur in plants under stress conditions is the accumulation of reactive oxygen species such as superoxide, hydrogen peroxide, and radical hydroxyl, all of which are highly toxic and reactive, and disrupt the normal metabolism of the cells. These radicals create secondary oxidative stress, through peroxidation of lipids, resulting in membrane degradation, protein degradation, deactivation of enzymes, elimination of pigments, and disruption of DNA, leading to serious damage to the structure of cells and eventually to the whole plant. One strategy of plants to counteract this stress is the accumulation of antioxidant enzymes (Kang *et al*., 2014). Similar results have been reported in pot marigold (*Calendula officinalis* L.) (Hemmati *et al*., 2018) and chickpea (*Cicer arietinum* L.) (Sadak *et al*., 2017). The vermicompost treatments significantly enhanced the activities of APX, POD, CAT and SOD under salinity stress conditions, and the increases in the activities of APX, POD, CAT and SOD were 2.90%, 21.08%, 2.41% and 9.76% in the plants under the 20% vermicompost treatment compared with those in the control plants (Table 5 and 6). Similar results

Table 6 - Effect of salinity and vermicompost and their interaction on catalase, peroxidase and ascorbate peroxidase

Salinity (mM)	Sanility					Mean vermicompost
	0	50	100	150	200	
			Catalase (Ug^{-1} F.W.)			
0	31.65±1.58 r	31.85±1.58 p	32.16±1.58 m	43.18±1.58 e	20.78±1.58 w	31.92±7.4 E
5	31.80±1.58 q	32.00±1.58 o	32.31±1.58 k	43.33±1.58 d	20.93±1.58 v	32.07±7.4 D
10	31.85±1.58 p	32.05±1.58 n	32.36±1.58 j	43.38±1.5 8 c	20.98±1.58 u	32.13±7.4 C
15	32.05±1.58 n	32.26±1.58 l	32.56±1.58 h	43.58±1.58 b	21.19±1.58 t	32.33±7.4 B
20	32.41±1.58 i	32.61±1.58 g	32.92±1.58 f	43.94±1.58 a	21.54±1.58 s	32.69±7.4 A
Mean salinity	31.95±1.43 D	32.16±1.43 C	32.46±1.43 B	43.48±1.43 A	21.08±1.43 E	
			Peroxidase (Ug^{-1} F.W.)			
0	68.40±0.93 o	69.07±0.93 no	70.80±0.93 mn	78.62±0.93 gh	41.48±0.93 t	65.68±12.99 E
5	70.75±3.98 nm	71.42±3.98 lm	73.15±3.98 kl	80.97±3.98 ef	43.83±3.98 s	68.03±13.43 D
10	75.03±3.98 jk	75.71±3.98 ij	77.44±3.98 hi	85.26±3.98 c	48.12±3.98 r	72.31±13.43 C
15	77.59±3.98 hi	78.27±3.98 gh	80.00±3.98 fg	87.82±3.98 b	50.67±3.98 q	74.87±13.43 B
20	82.25±3.98 ef	82.93±3.98 de	84.66±3.98 cd	92.48±3.98 a	55.33±3.98 p	79.53±13.43 A
Mean salinity	74.80±5.96 C	75.48±5.96 C	77.21±5.96 B	85.03±5.96 A	47.89±5.96 D	
			Ascorbate peroxidase (Ug^{-1} F.W.)			
0	869.60±1.51 s	875.40±1.51 q	880.40±1.51 n	976.10±1.51 e	459.60±1.51 x	812.20±1.85 E
5	873.20±1.51 r	878.90±1.51 p	883.90±1.51 m	979.60±1.51 d	463.20±1.51 w	815.80±1.85 D
10	879.60±1.51 o	885.40±1.51 l	890.40±1.51 k	986.10±1.51 c	469.60±1.51 v	822.20±1.85 C
15	885.40±1.51 l	891.10±1.51 j	896.10±1.51 h	991.80±1.51 b	475.40±1.51 u	827.90±1.85 B
20	893.20±1.51 i	898.90±1.51 g	903.90±1.51 f	999.60±1.51 a	483.20±1.51 t	835.80±1.85 A
Mean salinity	880.20±1.6 D	885.90±1.6 C	890.90±1.6 B	986.60±1.6 A	470.20±1.6 E	

* In each variable, data followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at 5%level. Data represent the mean value ± S.D. the mean of four replicates.

have been reported in cowpea(*Vigna unguiculata*), rice (*Oryza sativa* L.), and tall fescue (*Festuca arundinacea* Schreb.) (Cavalcanti *et al*., 2004; García *et al*., 2014; Adamipour *et al*., 2016).

Nutrients in leaf tissue

N, P, K, Fe, Mg and Zn concentrations significantly declined by the increasing salinity stress level from 0 to 200 mM NaCl (Table 7 and 8). Their concentrations

Table 7 - Effect of salinity and vermicompost and their interaction on nitrogen, phosphorus and potassium

Vermicompost (%)	Salinity					Mean vermicompost
	0	50	100	150	200	
			Nitrogen (%)			
0	2.01±0.04 ghi	1.99±0.01 hi	1.90±0.01 j	1.01±0.01 op	0.97±0.01 q	1.58±0.49 E
5	2.03±0.02 g	2.02±0.009 gh	1.93±0.009 j	1.04±0.009 o	1.00±0.009 pq	1.60±0.49 D
10	2.15±0.04 e	2.10±0.01 f	2.01±0.01 ghi	1.12±0.01 m	1.08±0.01 n	1.69±0.49 C
15	2.40±0.08 c	2.38±0.02 c	2.29±0.02 d	1.40±0.02 k	1.36±0.02 l	1.97±0.49 B
20	3.01±0.02 a	2.99±0.01 a	2.90±0.01 b	2.01±0.01 gh	1.97±0.01 i	2.58±0.49 A
Mean salinity	2.32±0.38 A	2.30±0.38 B	2.21±0.38 C	1.32±0.38 D	1.28±0.38 E	
			Phosphorus (%)			
0	0.15±0.005 ijk	0.14±0.005 lm	0.12±0.005 no	0.11±0.005 p	0.08±0.005 r	0.12±0.02 E
5	0.15±0.006 hi	0.15±0.006 jkl	0.13±0.006 mn	0.11±0.006 op	0.09±0.006 qr	0.13±0.02 D
10	0.16±0.009 gh	0.15±0.009 ij	0.14±0.009 klm	0.12±0.009 no	0.10±0.009 q	0.13±0.02 C
15	0.19±0.006 e	0.18±0.006 f	0.16±0.006 g	0.15±0.006 ij	0.12±0.006 no	0.16±0.02 B
20	0.24±0.005 a	0.23±0.005 b	0.22±0.005 c	0.20±0.005 d	0.18±0.005 f	0.22±0.02 A
Mean salinity	0.18±0.03 A	0.17±0.03 B	0.15±0.03 C	0.14±0.03 D	0.11±0.03 E	
			Potassium (%)			
0	3.93±0.14 fgh	3.89±0.14 ghi	3.84±0.14 hi	2.93±0.14 mn	2.11±0.14 p	3.34±0.74 D
5	4.11±0.18 d-h	4.07±0.18 d-h	4.02±0.18 e-h	3.11±0.18 lm	2.29±0.18 op	3.52±0.75 C
10	4.22±0.12 de	4.18±0.12 def	4.13±0.12 d-g	3.22±0.12 kl	2.40±0.12 o	3.63±0.74 C
15	4.63±0.22 b	4.59±0.22 bc	4.54±0.22 bc	3.63±0.22 ij	2.81±0.22 n	4.04±0.76 B
20	5.32±0.31 a	5.28±0.31 a	5.23±0.31 a	4.32±0.31 cd	3.50±0.31 jk	4.73±0.78 A
Mean salinity	4.44±0.54 A	4.40±0.54 A	4.35±0.54 A	3.44±0.54 B	2.62±0.54 C	

* In each variable, data followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at 5%level. Data represent the mean value ± S.D. the mean of four replicates.

Table 8 - Effect of salinity and vermicompost and their interaction on zinc, magnesium and iron

Vermicompost (%)	Salinity					Mean vermicompost
	0	50	100	150	200	
			Zn (mg/kg)			
0	91.47±0.47 efg	91.42±0.47 fg	91.34±0.47 fg	90.35±0.47 i	88.88±0.47 k	90.69±1. 10E
5	91.78±0.51 def	91.73±0.51 def	91.65±0.51 def	90.66±0.51 hi	89.19±0.51 jk	91.00±1. 10D
10	92.14±0.32 d	92.09±0.32 d	92.01±0.32 de	91.02±0.32 gh	89.55±0.32 j	91.36±1. 06C
15	93.25±0.86 c	93.20±0.86 c	93.12±0.86 c	92.13±0.86 d	90.66±0.86 hi	92.47±1. 28B
20	95.44±0.51 a	95.39±0.51 a	95.31±0.51 a	94.32±0.51 b	92.85±0.51 c	94.66±1. 11A
Mean salinity	92.82±1. 56 A	92.77±1. 56 A	92.69±1. 56 A	91.70±1. 56 B	90.23±1. 56 C	
			Mg (mg/kg)			
0	1.69±0.09 efg	1.64±0.09 e-h	1.60±0.09 e-h	1.46±0.09 ghi	1.23±0.09 i	1.52±0.18 D
5	1.72±0.06 efg	1.69±0.06 efg	1.65±0.06 e-h	1.51±0.06 f-i	1.28±0.06 i	1.57±0.17 CD
10	1.82±0.12 cde	1.79±0.12 c-f	1.75±0.12 d-g	1.61±0.12 e-h	1.38±0.12 i	1.67±0.20 C
15	2.10±0.20 c	2.07±0.20 c	2.03±0.20 cd	1.89±0.20 cde	1.66±0.20 e-h	1.95±0.24 B
20	3.38±0.58 a	3.35±0.58 a	3.31±0.58 a	3.17±0.58 ab	2.94±0.58 b	3.23±0.54 A
Mean salinity	2.14±0.70 A	2.11±0.70 A	2.07±0.70 A	1.93±0.70 B	1.70±0.70 C	
			Fe (mg/kg)			
0	122.05±1.68 ij	121.92±1.68 ij	120.69±1.68 j	115.50±1.68 k	100.27±1.68 m	116.09±8.60 E
5	124.19±1.96 hi	124.06±1.96 i	122.83±1.96 ij	117.64±1.96 k	102.41±1.96 m	118.23±8.65 D
10	128.42±2.33 g	128.29±2.33 g	127.06±2.33 gh	121.87±2.33 ij	106.64±2.33 l	122.45±8.72 C
15	154.19±2.69 d	154.06±2.69 d	152.83±2.69 d	147.64±2.69 e	132.41±2.69 f	148.23±8.80 B
20	196.53±4.15 a	196.40±4.15 a	195.17±4.15 a	189.98±4.15 b	174.75±4.15 c	190.56±9.24 A
Mean salinity	145.07±29.02 A	144.94A±29.02B	143.71±29.02 B	138.52±29.02 C	123.29±29.02 D	

* In each variable, data followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at 5%level. Data represent the mean value ± S.D. the mean of four replicates.

decreased (44.82%, 36.06%, 40.99%, 15.01%, 20.56% and 2.79%, respectively) in the plants at 200 mM NaCl compared to those in the control plants. N, P, K, Fe, Mg and Zn concentrations in the leaf tissues of pot marigold increased significantly with increasing vermicompost composition in the media compared to those in the control plants. The highest and the lowest concentrations of the elements were observed in the 20% and the 0% vermicompost treatments, respectively (Table 7 and 8). Furthermore, N, P, K, Fe, Mg and Zn concentrations in leaves increased 46.20%, 80.32%, 4.61%, 64.14%, 112.50%, and 4.37% in the plants with the 20% vermicompost treatment compared to those in plants with the control treatment, respectively. Interaction between the levels of salinity and vermicompost resulted in the highest and the lowest nutrient concentrations in the plants with the 0 mM NaCl and 20% vermicompost and with the200 mM NaCl and 0% vermicompost treatments, respectively (Table 7 and 8). Plant growth is reduced in salt-affected soil because of the excess uptake of potentially toxic ions. Soil salinity is characterized by high amounts of Na, Mg, Ca, Cl, HCO_3^- and B ions in soil which have negative effects on plant growth. Eventually, high salt concentrations in soil reduce the absorption of nutrients of plants which negatively affects the fertility of the soil (Zhao and Ren, 2007). In this study, the increase of vermicompost levels increased the mineral nutrition. Vermicompost contains humic substances that have multiple effects in the soil. It may improve soil properties such as micronutrient transport and availability (Aşık *et al*., 2009). Chen and Aviad (1990) summarized the effects of humic substances on plant growth and mineral nutrition, pointing out the positive effects on the uptake of macro- (such as N, P, and K) and micro-elements. Vermicompost may enhance the uptake of nutrients and reduce the uptake of some toxic elements of plants under salinity stress conditions. Chamani *et al*. (2008) reported that addition of vermicompost to soils resulted in increased mineral contents in the substrate and higher concentrations P, Ca, Mg, Cu, Mn and Zn in shoot tissues of red clover and cucumber.

Correlation analysis

The results of the Pearson correlation analysis between vermicompost abundance and the measured morphological traits and elements showed a positive and significant correlation. Therefore, a positive relation between the increases of the amount of vermicompost and improvement in the morphological traits and measured elements can be inferred. According to the results, shoot fresh weight compared to shoot dry weight (r=0.89**) and flower diameter (r=0.57**) showed the highest and the lowest correlations among the morphological traits, respectively (Table 9). In the measured elements under the vermicompost treatments, the highest correlation was observed between shoot fresh weight and K content (r=0.81**) while the lowest correlation was seen in Mg content (r=0.67**) (Table 9). This improvement can be attributed to the presence of macro-micro elements, the release of elements in the soil and soil amendment of physical and biological conditions which have been previously reported in other plants

Table 9 - Correlation coefficients for evaluated traits on pot marigold in vermicompost treatment

Traits	Shoot FW	Shoot DW	Leaf area	Root FW	Root DW	Flower diameter	Fruit FW	Fruit DW	Zn	Fe	Mg	Ca	K	P	N
Shoot FW	1														
Shoot DW	0.891**	1													
Leaf area	0.886**	0.992**	1												
Root FW	0.798**	0.799**	0.777**	1											
Root DW	0.798**	0.799**	0.777**	1.00**	1										
Flower diameter	0.574**	0.603**	0.586**	0.519*	0.519*	1									
Fruit FW	0.811**	0.795**	0.801**	0.618**	0.618**	0.334 NS	1								
Fruit DW	0.618**	0.728**	0.695**	0.734**	0.734**	0.536*	0.417 NS	1							
Zn	0.789**	0.883**	0.875**	0.864**	0.864**	0.623**	0.778**	0.722**	1						
Fe	0.809**	0.836**	0.822**	0.825**	0.825**	0.624**	0.818**	0.686**	0.951**	1					
Mg	0.679**	0.797**	0.781**	0.764**	0.764**	0.538*	0.733**	0.749**	0.927**	0.907**	1				
Ca	0.789**	0.883**	0.875**	0.864**	0.864**	0.623**	0.778**	0.722**	1.00**	0.951**	0.927**	1			
K	0.818**	0.851**	0.842**	0.799**	0.799**	0.561**	0.778**	0.765**	0.911**	0.929**	0.884**	0.911**	1		
P	0.815**	0.847**	0.835**	0.815**	0.815**	0.554*	0.831**	0.698**	0.937**	0.981**	0.921**	0.937**	0.938**	1	
N	0.805**	0.834**	0.814**	0.831**	0.831**	0.604**	0.810**	0.704**	0.956**	0.989**	0.933**	0.956**	0.916**	0.981**	1

FW = fresh weight; DW= dry weight.
NS= Not significant, * and **: Significant at 5% and 1% respectively.

(Bachman and Metzger, 2008). In this correlation analysis, peroxidase activity (r=0.79**) and proline content (r=0.44**) had the highest and the lowest correlations with shoot fresh weight. Further, insignificant correlations were observed in carotenoid content and catalase enzyme activity (Table 10). It seemed the positive correlations in activities of antioxidant enzymes, chlorophyll and proline content in the plant was due to increased concentrations of the elements which could be provided by vermicompost. Because in the structure of the mentioned traits, there are a variety of macro- and micro- elements that vermicompost provides directly and indirectly to plants (Hidalgo *et al*., 2006). The results of a simple correlation analysis between the morphological traits and the studied elements under salt stress conditions showed that shoot fresh weight had a positive and significant correlation with all the morphological indices and concentration of elements. In this analysis, the highest and the lowest correlations were seen in leaf area (r=0.99**) and fresh flower weight (r=0.91**), respectively (Table 11). Also, the highest correlation of shoot fresh weight was observed in Fe content (r=0.98**) and the lowest correlation was found in N content (r=0.88**) (Table 11). There are many reports of positive correlations between plant yield and concentration of elements under salinity stress conditions. Increase in soil pH, reduction in the amount of absorbent elements in the soil, increase in toxic elements of soil such as Cl and Na, and osmotic stress in the soil that prevents water absorption are the most critical factors for this phenomenon in plants (Edwards *et al.*, 2010). Analysis of correlations of shoot fresh weight with chlorophyll and proline con-

Table 10 - Correlation coefficients for evaluated traits on pot marigold under salinity stress conditions (above diameter) and vermicompost treatment (below diameter)

Traits	Shoot fresh weight	Chlorophyll	Proline	Carotenoid	Ascorbate perodidase	Peroxidase	Catalase	Superoxide dismutase
Shoot fresh weith	1	0.913 **	-0.904 **	-0.874 **	0.872 **	0.990 **	0.905 **	0.861**
Chlorophyll	0.664 **	1	-0.992 **	0.857 **	-0.856 **	0.888 **	0.888 **	0.626**
Proline	0.444 **	0.176 NS	1	0.854 **	0.680 **	-0.816 **	0.714 **	0.571**
Carotenoid	0.059 NS	0.488 *	0.454 *	1	0.801 **	0.651 **	-0.544 *	-0.346 NS
Ascorbate peroxidase	0.490*	0.064 NS	0.038 NS	-0.435 NS	1	0.760 **	0.407 NS	-0.085 NS
Peroxidase	0.794 **	0.480 *	0.540 *	0.093 NS	0.746 **	1	0.458*	-0.020 NS
Catalase	0.131 NS	0.444 *	-0.341 NS	0.252 NS	0.430 NS	0.317 NS	1	-0.006 NS
Superoxide dismutase	0.514 *	0.793 **	-0.210 NS	0.131 NS	-0.002 NS	0.241 NS	0.355 NS	1

NS= Not significant, * and **: Significant at 5% and 1% respectively.

Table 11 - Correlation coefficients for evaluated traits on pot marigold in salinity stress conditions

Traits	Shoot FW	Shoot DW	Leaf area	Root FW	Root DW	Flower diameter	Fruit FW	Fruit DW	Zn	Fe	Mg	Ca	K	P	N
Shoot FW	1														
Shoot dw	0.939**	1													
Leaf area	0.995**	0,956**	1												
Root FW	0.966**	0.886**	0.961**	1											
Root dw	0.923**	0.868**	0.921**	0.986**	1										
Flower diameter	0.986**	0.977**	0.991*	0.952**	0.919**	1									
Fruit FW	0.918**	0.830**	0.906**	0.844**	0.798**	0.865**	1								
Fruit dw	0.942**	0.947**	0.948**	0.901**	0.855**	0.972**	0.779**	1							
Zn	0.938**	0.947**	0.956**	0.850**	0.802**	0.941**	0.923**	0.892**	1						
Fe	0.985**	0.940**	0.987**	0.914**	0.851**	0.977**	0.909**	0.948**	0.963**	1					
Mg	0.895**	0.910**	0.803**	0.762**	0.897**	0.919**	0.856**	0.969**	0.912**	0.857**	1				
Ca	0.938**	0.947**	0.956**	0.850*	0.802**	0.941**	0.923**	0.892**	1.00**	0.963**	0.969**	1			
K	0.981**	0.961**	0.991*	0.954**	0.928**	0.982**	0.915**	0.927**	0.966**	0.967**	0.932**	0.966**	1		
P	0.936**	0.877**	0.911**	0.933**	0.926**	0.919**	0.843**	0.858**	0.804**	0.879**	0.756**	0.804**	0.894**	1	
N	0.885**	0.870**	0.892**	0.941**	0.976**	0.886**	0.809**	0.796**	0.831**	0.811**	0.781**	0.923**	0.817**	0.891**	1

FW = fresh weight; DW= dry weight.

NS= Not significant, * and **: Significant at 5% and 1% respectively.

tent, and antioxidant enzyme activity showed significant positive and negative correlations. From the viewpoint of biochemistry, most of significant correlations were found in content of chlorophyll (r=0.91**), proline (r= -0.90**), carotenoid (r= -0.87**), in activity of ascorbate peroxidase (r=0.87**), peroxidase (r=0.99**), catalase (r=0.90**), and superoxide dismutase (r=0.86**) compared to the shoot fresh weight (Table 10). There were also few negative correlations in which proline and carotenoid content can be attributed to their function in dealing with osmotic stress in the plants due to increased salinity in the soil. In similar studies, positive correlation of antioxidant enzyme activity under salinity stress conditions in German chamomile (*Matricaria recutita* L.), sunflowers (*Helianthus annuus* L.) and basil (*Ocimuum basilicum* L.) have been reported (Baghalian *et al*., 2008; Heidari *et al.*, 2011; Heidari, 2012).

4. Conclusions

Salinization of soil is a serious land degradation problem and is increasing steadily in many parts of the world, particularly in arid and semiarid areas. Soil salinity affects the establishment, growth, and development of plants leading to huge losses in productivity. Vermicompost is one of the major organic fertilizer which can improve growth and salinity tolerance with containing plenty nutrition elements, hormones, and organic materials. In this study, increase in salinity stress significantly led to decline in morphological and physiological indices of pot marigold. Application of vermicompost under salinity conditions increased morpho-physiological indices in this plant. Vermicompost increased the activity of the antioxidant system, the content of proline and chlorophyll in the plant by increasing the nutrients in the soil environment that could increase the plant yield and alleviating the harmful effects of salinity.

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Biometry and vigor of seeds of *Myrciaria dubia* (Kunth) McVaugh

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Key words: biometric characterization, camu-camu, class size, fruit growing, rate of emergency, water content.

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

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Abstract: Camu-camu has aroused the interest of various industries like natural preservatives, ice creams, juices, jellies, wines, natural dyes, but there is little technical information about the seeds. The objective of this work was to determine the biometric, physical and vigor characteristics of camu-camu seeds. Seeds originating from native populations of Roraima were used. The biometry was determined and the data were analyzed in Excel spreadsheet and calculated the mean, median, variance, standard deviation and seeds classified as small, medium and large, based on mass. The vigor was determined by electrical conductivity, seedling emergence, emergence velocity, plant height, stem diameter, in a completely causal design, with four replicates of 25 seeds. The average results for width, thickness, length, individual mass, volume, weight of one thousand seeds and number of seeds per kilogram showed large variability. The size of the seed has direct correlation with vigor, large seeds have greater vigor.

1. Introduction

The camu-camu, *Myrciaria dubia* (Kunth) McVaugh, is a small fruit tree of the Myrtaceae family, popularly known as araçá-dágua, azedinho, camocamo and caçari (Smiderle and Sousa, 2008). It occurs naturally in the Amazon region, growing naturally along the banks of rivers, streams, channels and lakes (Chagas *et al*., 2012; Souza *et al*., 2017). The fruit of the camu-camu is used in the preparation of juices, jellies, alcoholic drinks and ice creams, among others (Yuyama, 2011; Sousa *et al*., 2013). The main property of the camu-camu is the high vitamin C content, around 6,112 ± 137.5 ascorbic acid 100 g^{-1} of pulp, better than most cultivated plants (Yuyama, 2011). The skin of the camu-camu when fresh contains high levels of anthocyanin and ascorbic acid (Villanueva-Tiburcio *et al*., 2010). The interest aroused by the camu-camu is due to its bioactive compounds and biological activity of antioxidant action, which reduce lipid peroxidation, revert high levels of total cholesterol and triacylglycerols, and increase the levels of HDL-cholesterol (Gonçalves, 2012).

Seed characteristics are important in the study of any species, as they give an understanding of the dispersion and establishment of seedlings. The classification of seeds by size or weight is a strategy to be adopted in the standardisation and emergence of seedlings, and to obtain seedlings of similar size or greater vigour (Carvalho and Nakagawa, 2012). Therefore, the biometry of fruits and seeds have a diagnostic value in differentiating species within a genus, and can contribute to their recognition (Pereira and Ferreira, 2017).

In order to find the ideal size class for the multiplication of different plant species, biometry is used to determine physiological quality, but the results have differed considerably, even when the seeds are from the same species. Biruel *et al*. (2010) found that larger seeds of *Caesalpinia leiostachya* displayed a greater percentage and speed of germination; different results to those reported by Queiroga *et al*. (2011), who studied peanut seeds and found that smaller seeds had a higher germination percentage.

Seeds of different sizes showed the same high germination percentage, with small seeds being the most viable, and medium and large seeds yielding seedlings that were more vigorous (Vendramin and Carvalho, 2013; Smiderle *et al*., 2016).

This ambiguous situation may be one of the reasons for two or more seed sizes being produced by the same species. Variations in size were seen in seeds collected in an area of natural occurrence in the State of Roraima, Brazil. In view of the above, the aim of this work was to determine the biometry, physical characteristics and vigour of seeds of the camu-camu.

2. Materials and Methods

The experiment was carried out at the Seed Analysis Laboratory of Embrapa Roraima. The camu-camu fruit used in the experiment were harvested from native plants located on the banks of the Jatapú River (0° 41.072" N and 59° 18.046" W) at an altitude of 144 m. Here the plants develop in rocky soil in the district of Caroebe in the State of Roraima, and are distributed along the river in various populations and subpopulations.

After harvesting, the fruits were packed in plastic bags to prevent damage from crushing, and carefully transported to the laboratory, where they were selected for lack of damage, and then homogenised and sanitised. The mature selected fruit were then depulped and washed in running water, using a sieve for the complete removal of any pulp adhering to the seeds.

Physical characterisation of the seeds

The seeds were evaluated for their physical characteristics.

Seed biometry. The individual thickness, length and width of 600 seeds were measured using a calliper, with the results expressed in mm. The length was measured along the longitudinal axis of the seed; the width was measured perpendicular to the length, considering the broad face of the seed, which corresponds to the median line; and the thickness, by again measuring perpendicular to the length, but on the smaller seed face, corresponding to the median line including the two cotyledons. The following were then obtained:

Seed volume (mm^3): multiplying the three dimensions (width, length and thickness), without considering the actual shape of the seed.

Individual seed weight: obtained by weighing each seed.

Based on these measurements, the seeds were separated into three size classes (large, medium and small).

1000 seed weight (g). Six samples of 100 seeds were used for each size class. The samples were weighed on a 0.001 g precision balance and the weight determined by multiplying the result by 10.

Number of seeds per kilogram. After determining the thousand-seed weight (TSW), the number of seeds per kilogram was calculated using the "rule of three".

Water content (%). Carried out using the standard oven method at 105±3°C for 24 hours. Four replications of 10 seeds were used, where each sample was wrapped in aluminium and placed in an oven. After 24 hours, the samples were removed from the oven and placed in a desiccator for cooling, and then weighed on a 0.001 g precision balance. The water content was calculated based on the difference between the wet and dry weights, applying the formula proposed in the Rules for Seed Analysis (MAPA, 2009), with the result expressed as a percentage.

The biometric data were analysed in an Excel spreadsheet. The mean, median, variance and standard deviation were all calculated.

Determining the vigour of small, medium and large seeds of the camu-camu

The experiment was carried out in a greenhouse at Embrapa Roraima, in Boa Vista. The experimental design was completely randomised (CRD), with three treatments comprising the size classes (small, medi-

um and large), and four replications of 25 seeds. Seeds with a weight between 0.66 g and 0.84 g were considered small, those with a weight between 0.92 g and 1.20 g were considered medium, and those with a weight between 1.27 g and 1.60 g considered large. After classification, the following analyses were carried out:

Electric conductivity (EC). the seeds were weighed on a 0.0001 g precision balance and then immersed in 75 mL of water for 24 hours at a constant temperature of 25°C. The electrical conductivity was then measured using a digital conductivimeter. The results were expressed in $\mu S\ cm^{-1}\ g^{-1}$ of seed.

Seedling emergence. The test was carried out following procedures described in the Rules for Seed Analysis (MAPA, 2009). The tests were conducted in trays containing 50% sand + 50% sawdust as substrate moistened with water up to 60% of its retention capacity, using four replications of 25 seeds distributed at a depth of 2.0 cm. The test was performed in a nursery of 50% Sombrite shade screen. To evaluate the non-germinated seeds, they were removed from the substrate, cut down the middle with pruning shears and identified for viability and senescence. The formula used for calculation was:

$$E = (N/A) \times 100:$$

Where E = percentage emergence; N = number of emerged seedlings; A = total number of seeds placed for emergence.

Speed of seedling emergence. Established by means of a daily count of emerged seedlings, the index being calculated from the expression:

$$SE = (E1/N1) + (E2/N2) + ... + (En/Nn), \text{ where:}$$

SE = speed of seedling emergence; E1 = number of seedlings emerged at the first count; N1 = number of days elapsed until the first count; E2 = number of seedlings emerged at the second count; N2 = number of days elapsed until the second count; n = last count.

Plant height. Evaluated four months after sowing with the aid of a graduated rule (cm). Measured from the cotyledon node to the end of the first pair of leaves in normal seedlings identified at the end of the test for seedling emergence.

Stem diameter. Evaluated four months after sowing using a digital calliper (mm). Determined at the insertion of the cotyledon.

The data for water content, electrical conductivity, seedling emergence, speed of emergence, plant height and stem diameter were submitted to the Lilliefors test for normality. They were then submitted to analysis of variance (ANOVA), using a completely randomised design with four replications, and the mean values compared by Tukey's test at 5% probability using the SISVAR software (Ferreira, 2014).

3. Results and Discussion

From the results, it was seen that there was a significant difference in the biometric variables of the analysed seeds, and it was possible to separate them into three classes according to size: small, medium and large. The data for variance, standard deviation and coefficient of variation, and the mean results for individual weight, width, thickness, length and volume of the camu-camu seeds are shown in Table 1.

The biometric data shown indicates that the studied population was accurately sampled, since the values for variance were low (<1) for each of the characteristics under study. The values for standard deviation shown in Table 1 indicate a low sample variation for each of the characteristics under evaluation.

The values for the coefficient of variation demonstrate the low variation in the variables, considering the mean value of the characteristics. However, variation can be seen when the seeds are sorted by size.

Table 1 - Biometric characteristics of camu-camu seeds from native populations in the State of Roraima, classified by size class

Class	Mean	Variance	Standard deviation	Coefficient of variation (%)
		Weight (g)		
Small	0.79	0.002	0.04	5.65
Medium	1.11	0.004	0.06	5.90
Large	1.45	0.008	0.08	6.12
		Width (mm)		
Small	12.04	0.541	0.73	6.11
Medium	12.90	0.147	0.38	2.97
Large	14.42	0.233	0.48	3.34
		Thickness (mm)		
Small	5.54	0.065	0.25	4.59
Medium	6.11	0.213	0.46	7.56
Large	6.74	0.329	0.57	8.51
		Length (mm)		
Small	15.54	0.620	0.79	5.07
Medium	17.42	0.206	0.45	2.61
Large	19.14	0.342	0.58	3.05
		Volume (mm^3)		
Small	1,035.77	6,144.378	78.38	7.57
Medium	1,371.98	6,104.830	78.13	5.69
Large	1,859.47	20,759.298	144.08	7.75

Similar results were found in camu-camu seeds from the banks of the Anauá River in Roraima, with a weight which ranged from 0.80 g to 1.46 g, and greater results than the seeds of populations from the Rio Urubu, which ranged from 0.56 to 0.78 g (Souza *et al*., 2017). According to Gonçalves *et al*. (2008), species with wide geographic distribution may present differences in their characteristics due to the effects of adaptation and to origin.

The variation in seed size may interfere with their physiological quality, still poorly researched in forest species (Oliveira *et al*., 2009; Smiderle *et al*., 2016). The three classes of seed size displayed significant differences for thousand-seed weight (TSW) and the number of seeds per kilo (NSK) with a low coefficient of variation, showing little variation within each size (Table 2).

The initial water content of the seeds in the different size classes at the time the experiment was set up was greater than 35%, with significant differences between the classes (Table 3), however it was lower than that reported by Yuyama *et al*. (2011), who obtained a value for moisture between 45 and 56%, with no effect on germination. According to Braga *et al*. (2012), hydration of the seeds can favour test performance, because seeds that are more humid, within certain limits, germinate more quickly.

Table 2 - Summary of the analysis of variance and comparison of the mean values for thousand-seed weight (TSW) and number of seeds per kilo (NSK) in camu-camu seeds from native populations in the State of Roraima, classified by size

Source of variation	DF	Mean square	
		TSW	NSK
Treatment	2	346,622.68*	435,142.53*
Replication	5	633.44	672.57
Error	10	1,610,651.55	1,318.23
CV (%)		4.16	3.35
Overall mean value		963.88	1,082.76
Class		Mean value	
Small		734.27g	1.362
Medium		943.67 g	1.061
Large		1,213.70 g	825

* significant at 5% probability by F-test.

The different classes of seed displayed variations in the variables under analysis. It was found that values for electrical conductivity depend on the size of the seed, where the large, small and medium seeds presented higher, lower and intermediate physiological quality respectively (Table 3). According to Vieira and Krzyzanowski (1999), the lower physiological potential in small seeds is probably due to the lower organisational intensity of the cell-membrane systems.

The data for electrical conductivity in the small seeds showed a negative correlation with the other variables under study, demonstrating that the highest value for electrical conductivity corresponded to reductions in the percentage and speed of seedling emergence. These results confirm those reported by Vinhal-Freitas *et al*. (2011), and demonstrate that tests of vigour differentiate between seed classes, indicating significant differences between the larger and smaller size classes, where larger seeds showed greater vigour with the smallest values for electrical conductivity.

With the large seeds, the lower value for electrical conductivity was due to a greater organisation of the cell components, since, despite field emergence not differing statistically from that of the small seeds, they showed greater speed of emergence in addition to viability, even when not germinated, as the small seeds that did not emerge had all died.

It is important to point out that despite the differences seen in electrical conductivity between the seed sizes, each seed class had low values for EC, and values for emergence that were higher than the mini-

Table 3 - Summary of the analysis of variance and comparison of mean values for water content (WC, %), electrical conductivity (EC, µS cm^{-1} g^{-1}), emergence (EMERG, %), speed of emergence, (SE, index), seedling height (HT S, cm) and stem diameter (DIAM S, mm) in camu-camu seeds classified by size

Source of variation	DF	Mean square					
		WC (%)	EC µS cm^{-1} g^{-1}	EMERG (%)	SE	HT S (cm)	DIAM S (mm)
Treatment	2	14.67*	1.83*	433.33*	0.021*	79.12*	0.599*
Replication	3	0.08	0.002	5.55	0.000008	0.131	0.0012
Error	6	0.29	0.001	5.55	0.000008	0.049	0.0008
Mean		37.17	3.27	88.33	0.056	15.61	2.13
CV (%)		1.46	1.11	2.67	5.17	1.42	1.36
Class				Mean values			
Small		35.35 c	3.95 c	80 b	0.01 c	11.30 c	1.71 c
Medium		36.99 b	3.22 b	100 a	0.03 b	15.34 b	2.20 b
Large		39.17 a	2.61 a	85 b	0.14 a	20.18 a	2.48 a

* significant at 5% probability by F-test. Mean values followed by the same letter do not differ by Tukey's test at 5% probability.

mum established by the Seed Standards (MAPA, 2009).

As for plant height and stem diameter, the larger seeds promoted the best results, followed by the medium and small seeds respectively. There was a positive correlation between seed size and plant height and stem diameter, i.e. larger seeds gave rise to larger, more vigorous plants. These results differ from those obtained by Souza *et al*. (2017), who reported that seeds from a population of the Anauá River considered small, displayed better results for these characteristics.

Wagner Junior *et al*. (2011) demonstrated that seed size has an effect on the emergence and initial development of jabuticaba seedlings (*Plinia cauliflora*), and that large seeds gave seedlings that were more vigorous. In açaí Silva *et al*. (2017) in organic substrate obtained higher values for plant height and stem diameter when large seeds were used, as well as. large seeds produce more vigorous plants independent of the substrate in *Euterpe oleracea.*

Alves *et al*. (2005) pointed out that in general, larger seeds are correlated with higher rates of initial seedling growth, which increases the probability of success during their establishment, since the rapid growth of roots and shoots allows the plant to take advantage of the nutrient and water reserves of the soil and carry out photosynthesis. Wagner Junior *et al*. (2011) stated that the germination process in many species is influenced by seed size. Consequently, within the same batch, small seeds present lower seedling emergence and less vigour than the medium and large seeds.

4. Conclusions

The physical characterisation of camu-camu seeds shows great variability in weight, width, thickness, length and volume.

The thousand-seed weight and the water content are influenced by size, with values increasing in direct proportion to the size of the seed, while the number of seeds per kilo decreases in inverse proportion.

Medium and large seeds give rise to plants that are more vigorous.

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Impact of aerobic rice-leafy vegetables intercropping systems on weed management

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Abstract: Field experiments were carried out in summer 2017 and 2018 at Zonal Agricultural Research Station (ZARS), University of Agricultural Sciences, GKVK, Bengaluru, India on red sandy loam soil. The main objective was to evaluate the impact of rice grown in aerobic conditions intercropped with leafy vegetables on weed management, wherein the sole rice and intercrops of four leafy vegetables: palak (*Spinacia oleracea* L.), coriander (*Coriandrum sativum* L.), amaranth (*Amaranthus spp* L.), methi (*Trigonella foenum-graecum* L.) were designed in randomized complete block design (RCBD) of 9 treatments replicated four times. The results revealed that the greater the crop biomass, the higher the weed suppression achieved. Sole rice was densely populated by weeds and also had higher weed biomass compared to the intercrops. However, intercrops suppressed efficiently the weeds, increased growth and rice equivalent yield over the sole rice crop. The intercropping systems with leafy palak (spinach) were the most suppressive of weeds. The rice + leafy vegetable palak recorded significantly lower weed density (138.4 no. m^{-2}), dry weight at harvest (148.04 g m^{-2}), higher rice grain, rice straw and palak leaf yield (7651; 9687 and 25508 kg ha^{-1}, respectively) and higher net monetary return (₹ 156269 ha^{-1}) over the sole rice.

1. Introduction

The rice is the world most important staple food for more than a half of the earth's population. It contains high amount of carbohydrates, proteins and calories. More than 90 per cent of rice is cultivated and consumed in Asia, where more than 60 per cent of the world's population lives.

According to FAO, the world rice consumption demand increases as the population increases too. By 2025, the amount of 800 M t of rice grain will be needed for consumption, which is a bit higher than the current rice grain production of 718 M t. The rice production is facing several challenges such as population explosion, urbanization, industrialization, shrinkage of cultivable land, water resources, etc., which make difficult to meet the rice food production demand. Increasing the rice productivity

per unit area is the need of the hour to bridge the gap between production and consumption.

The production of rice in aerobic condition by using the same production method used for other rain fed cereals like maize, wheat, etc., can be one of the resorts to mitigate the challenges. The rice grown in aerobic conditions known as aerobic rice provides several benefits of saving the inputs and human labour resources and reduces the greenhouse gases among the others. However, this rice production system faces a lot of problems including the weed infestation since the weed competition for resources starts from day one. Weeds can reduce the production of rice by 10-100% (Rao *et al*., 2014; Yaduraju *et al.,* 2015). Controlling weeds satisfactorily increases the cost of cultivation of the crop as well as deplete resource base (Buriro *et al.,* 2003). Reduced weed biomass in intercropping systems has been reported by several workers in various field crops such as soybean, maize, sorghum, sunflower, green gram, red gram, groundnut, etc., but they did not explore yet the leafy vegetables as intercrops. There must be continuing attention paid to study the weed dynamics and crop-weed interference in intercropping systems with smother leafy vegetables. More information is needed concerning crop diversification on weed dynamics and weed flora and differential resources consumption by crops and associated weeds.

Most of the farmers of the developing world are small, marginal and are unable to bear the high costs associated in carrying out weed management operations. Chemical weed control creates many problems such as development of herbicide resistant weeds, shifting weed flora and environmental pollution. The crop diversity can improve crop growth (Kirkegaard and Hunt, 2010), thus increasing crop competitiveness and tolerance to weeds (Anderson, 2011). Cropping systems composed of a diversity of crops with different life cycles are a great option to manage weeds and critical component of integrated weed management (Colbach *et al.,* 2014). To ensure safeguard against environmental pollution and to reduce chances of shifting of weed flora and the development of herbicide-resistant weeds, an intercropping system which allows minimum weed infestation and yield losses, appear to have great importance. Although intercropping is practiced to maximize land use, it has also a significant effect in suppressing weed growth. But intercropping system alone is not sufficient to ensure adequate weed management practices, because of diverse canopy coverage occurred by intercrop.

Labour is becoming a scarce and costly input in agriculture. This has resulted in increased technical grade herbicide consumption. Hence, the present thrust in weed studies is to minimize the herbicide use and to formulate integrated management practices by combining non-chemical methods, which are efficient, economically viable and eco-friendly sound. Therefore, based on this background, the field experiments were planned.

2. Materials and Methods

Field experiments were carried out in summer seasons of 2017 and 2018 at Zonal Agricultural Research Station (ZARS), University of Agricultural Sciences, GKVK, Bengaluru, India on red sandy loam soil to evaluate the performance of leafy vegetables smothering efficiency in weed management.

Experimental design and treatment details

The experiment had RCBD design which was composed of 9 treatments replicated 4 times. The treatments included the four of rice intercropped with 4 leafy vegetables namely Rice + amaranth, Rice + coriander, Rice + palak (spinach) and Rice + methi (fenugreek) and the 5 treatments of sole crops including sole rice crop and 4 sole vegetable crops. The experimental site had pH (6.93), EC (0.36 dSm^{-1}), medium in organic carbon (0.58 %), available nitrogen (362 kg ha^{-1}), P_2O_5 (43 kg ha^{-1}) and K_2O (289 kg ha^{-1}).

Land preparation and layout

During January and February 2017, December 2017 and January 2018 the land was tilled with tractor-drawn cultivator followed by passing rotovator to bring the soil to the fine tilth. The plots were arranged as per the experimental design with 50 cm width bunds around each plot.

The gross plot size was 5 m x 3.5 m (17.5 m^2) accommodating 20 rows at a spacing of 25 cm, each row consisted 20 hills of rice plants with an intra-row spacing of 25 cm. One side row and one adjacent row on each side were left as border rows and the remaining 4 m x 2.5 m (10.00 m^2) was retained as the net plot. The spacing between plots and replications were 0.5 m and 1 m, respectively.

Fertilizers application

The recommended dose of farmyard manure (FYM) at 10 t ha^{-1} was applied 15 days prior to sowing and fertilizer dose of 100: 50: 50 kg N, P_2O_5, K_2O ha^{-1}

was applied through urea, single superphosphate (SSP) and Muriate of potash (MoP), respectively. The 50 per cent of N was applied as basal dose and remaining 50 per cent was applied in two splits *i.e.,* at tillering and panicle initiation stage of the rice crop. Whereas, SSP and MoP fertilizers were applied as basal dose at the time of sowing. For all intercrops, only recommended dose fertilizer of base crop was used.

Seeds and sowing

Shallow furrows spaced at 25 cm apart were created using marker during summer 2017 and 2018. The aerobic rice variety used was MAS 946-1. Two rice seeds per spot were dibbled by maintaining the inter and intra row spacing of 25 cm with a seed rate of 7 kg ha^{-1} on 8th March and 13th January for 2017 and 2018, respectively. The leafy vegetable seeds were sowed by the broadcasting method. Amaranth variety used for sowing was Arka suguna, seed rate used for broadcasting was 2.5 kg ha^{-1}. Coriander variety used was DWD-3, seed rate 10 kg ha^{-1} for broadcasting and duration of the crop was 30 days for vegetable and 90 days for grain. It provides green leaf productivity of 15 t ha^{-1}. Palak variety used was Pusa Jyoti with the yield over 200 to 400 quintals per hectare, seed rate used for broadcasting was 13 kg ha^{-1}. Methi variety used was Co-1 variety; seed rate used for broadcasting was 12 kg ha^{-1}. It provides green leaf productivity of 20 t ha^{-1}. The seeds were covered with soil and gently compacted. Irrigation was provided immediately after sowing to encourage uniform germination.

Irrigation

Irrigation was scheduled at 3-4 days interval through drip based on the rainfall, soil and crop appearance during the crop periods. Drip irrigation system was set out which included the pump, filter units, mainline and sub-main lines for each replication and laterals for each plot. The water source was a bore well. Water was pumped through 7.5 HP motor it was conveyed to the main field using 90 mm PVC pipes after filtering through sand and screen filter. From the mainline water was taken to the field through sub-main of 63 mm diameter PVC pipes. From the sub-main, 12 mm laterals were fixed at a spacing of 50 cm. The emitters in the inline laterals were fixed at 40 cm. The discharge rate of emitters was 3 lph. Irrigation was withheld 10 days before the crop attained maturity.

Weed management

Weeds were controlled by manual cleaning. Weed density and weed dry weight were recorded category wise with respect to grassy, broad leaved weeds and sedge weeds at 30, 60, 90 DAS and at harvest. Weed density was recorded in 0.5 m × 0.5 m quadrate randomly at one spot in each plot. Weeds were uprooted, washed with tap water, sun-dried, oven-dried at 65°C for 48 h. After attaining the constant weight, the samples were weighed and expressed in grams per m^2. The weed smothering efficiency (WSE) was worked out by following the below formula:

$$\text{WSE (per cent)} = \frac{(W1-W2)}{W1} \times 100$$

Where, W_1: Weed dry weight in sole rice crop stand plots; W_2: Weed dry weight in intercropped leafy vegetable plots

Uptake of nutrients nitrogen, phosphorus and potassium by weeds and different parts of paddy and leafy vegetables were calculated by multiplying the nutrient content and dry matter of weeds or yield of plant part using the following formula and expressed in kg ha^{-1}.

$$\text{Nutrient uptake by weed} = \frac{\text{Nutrient content (per cent)} \times \text{weed DW (kg ha}^{-1})}{100}$$

$$\text{Nut. uptake by rice plant} = \frac{\text{Nutr. cont. (per cent)} \times \text{Biological yield (kg ha}^{-1})}{100}$$

Rice and vegetable intercrops harvest

The rice crop was harvested on 28thJuly, 2017 and on 14th June 2018 as the ear heads changed into brown color coupled with yellow colored straw in more than 90 per cent of plant population of each plot. All borderlines in every treatment plot were harvested as bulk by leaving net plot area. Later, the net plot area was harvested treatment-wise separately by cutting at 2 inches above the ground, sun-dried for 3 days and threshed. The harvested produce was threshed manually. The grains were winnowed; sun-dried to bring the moisture up to 10-12 per cent and recorded the grain weight treatment-wise. The rice grain and straw dry weight from the net plot was recorded and expressed as kg ha^{-1}. The threshed straw was left in the same field and same plot for sun drying for 10 days. The weight of straw was also recorded treatment-wise and computed for hectare basis. Regarding the leafy vegetables, before harvesting between 30-40 days after sowing, 5 plant samples were taken for dry weight; thereafter the whole plot leaves were harvested, weighed and sold for consumption purpose.

Index of biological efficiencies of intercropping systems

Different system productivity parameters of inter-

cropping systems were worked out. The below are the formulas used.

The Land Equivalent Ratio (LER) is used to decide which crop is suitable among the intercropping components. It denotes relative to land area under sole crop required to produce the same yield as obtained under a mixed or an intercropping system at the same level of management. It is the ratio of land required by the pure crop to produce the same yield as intercrop. The LER was worked out by using the following formula given by Willey (1979).

$$LER = La + Lb = (Ya/Sa + Yb/Sb)$$

Where, LER = Yield of intercrop over yield of pure crop; La and Lb = LER's for the crops a and b; Ya, Yb is the yield of a and b crop grown as an intercrop; Sa, Sb is the yield of a and b crop grown as a sole crop.

When LER >1 intercropping is advantageous, the reverse means the 2 crops are mutually antagonistic.

Rice equivalent yield was also worked out. Normally, crop equivalent yield refers to the yields of different intercrops/crops are converted into the equivalent yield of any one crop based on the price of the produce. Efforts have also been made to convert the yields of different crops into an equivalent yield of the main crop such as rice (Verma and Modgal, 1983).

The rice equivalent yield of an intercropping system was calculated by taking into account the grain yield of component crops and the prevailing market price of both rice and intercrops as follows:

$$REY\ (Kg\ ha^{-1}) = \text{Yield of rice in intercropping system } (Kg\ ha^{-1}) + \left[\text{Yield of Intercrop } (Kg\ ha^{-1}) \times \frac{\text{Market price of intercrop } (Rs\ kg^{-1})}{\text{Market price of rice } (Rs\ kg^{-1})}\right]$$

The production efficiency was also calculated based on the rice equivalent yield and the duration of the cropping system and expressed as kg day^{-1}

$$PE\ (kg\ day^{-1}) = \frac{\text{Rice equivalent yield}}{\text{Duration of cropping system}}$$

Data analysis

To compare the performance of sole rice treatments with the rest of intercrops, an RCBD with 5 treatments was used. Weed density was expressed on a square metre basis and was square root transformed before the Analysis of Variance (ANOVA) as described by Cochran and Cox (1957). Weed biomass was expressed in g m^{-2} and weed density was expressed in numbers m^{-2} before ANOVA. Rice grain, straw and leafy vegetable yield were expressed in ha^{-1} before the ANOVA. Means were separated using Least Significant Difference (LSD) at P<0.05. The productivity of intercropping was assessed by calculating the REY, LER, PE and net monetary return from component crop yields

3. Results

Effect of the rice-leafy vegetable intercropping system on weed density

*T*otal weed density in aerobic rice was significantly influenced by different leafy vegetables intercropping systems at 30, 60, 90 days after sowing (DAS) and at harvest (Table 1). Total weed density at 30 DAS was significantly lower in rice intercropped with palak and sole palak (20.00 and 25.33; 25.08 and 30.88 no. m^{-2} during 2017 and 2018, respectively) as

Table 1 - Total weeds density (no./m^2) in rice as influenced by different intercropping systems during 2017 and 2018

Weed management practices	30 DAS		60 DAS		90 DAS		At harvest	
	2017	2018	2017	2018	2017	2018	2017	2018
T_1: Rice+Amaranth	5.34(29.33)	5.89(34.25)	7.42 (54.67)	7.73(59.23)	8.57(73.33)	8.93(79.25)	8.51(72.00)	8.76(76.30)
T_2: Rice+Coriander	6.75(45.33)	7.16(50.70)	8.81(78.67)	9.14(82.98)	9.95(98.67)	10.24(104.28)	9.89(97.33)	10.04(100.28)
T_3: Rice+Palak	4.32(20.00)	5.06(25.08)	5.71 (34.67)	6.52(42.05)	7.33(53.33)	7.73(59.33)	7.24(52.00)	7.56(56.70)
T_4: Rice+Methi	6.23(38.67)	6.67(43.98)	8.19(66.67)	8.51(72.00)	9.11(82.67)	9.32(86.45)	8.97(80.00)	9.28(85.70)
T_5: Sole Rice	7.77(61.33)	8.19(66.55)	9.74(96.00)	10.24(104)	10.35(106.67)	10.59(111.68)	10.28(105.33)	10.55(110.80)
T_6: Sole amaranth	6.04(36.00)	6.55(42.43)	-	-	-	-	-	-
T_7: Sole Coriander	7.05(49.33)	7.38(53.90)	-	-	-	-	-	-
T_8: Sole Palak	5.08(25.33)	5.60(30.88)	-	-	-	-	-	-
T_9: Sole Methi	6.56(42.67)	7.00(48.50)	-	-	-	-	-	-
S.Em.±	0.46	0.027	0.56	0.026	0.19	0.007	0.16	0.014
CD (P=0.05)	1.36	0.08	1.76	0.08	0.61	0.021	0.51	0.043

Values in parentheses are original values; data analysed using transformation -√x + 0.5.
DAS= Days after sowing.

compared to the sole rice crop (61.33 and 66.55 no. m^{-2} during 2017 and 2018, respectively).

Among different intercropping practices, intercropping of rice and leafy vegetable palak recorded significantly lower density of total weeds at 60 DAS (34.67 and 42.05 no. m^{-2} during 2017 and 2018, respectively). It was followed by intercropping of rice and leafy vegetable amaranths: at 60 DAS (54.67 and 59.23 no. m^{-2}, respectively). However, the sole rice registered a bit higher number of total weeds (96.00 and 104 no. m^{-2}, respectively). The similar trend was noticed at the further crop growth stage of aerobic rice at 90 days after sowing and at harvest.

Effect of the rice-leafy vegetable intercropping system on weed dry weight

The weed dry weight is the useful parameter to assess the extent of weed competition with the crop plants. The total weeds were differed significantly at different growth stages of aerobic rice due to different intercropping practices. The 2 years pooled data are here under depicted in figure 1.

Total weed dry weight in aerobic rice was significantly lower in rice-palak leafy vegetables intercropping systems at 30 DAS, 60, 90 and at harvest (0.25, 115.34, 151.20 and 148.04 g m^{-2}, respectively) as compared to the sole rice crop (1.56, 204.07, 302.09 and 279.08 g m^{-2}, respectively). However, the latter

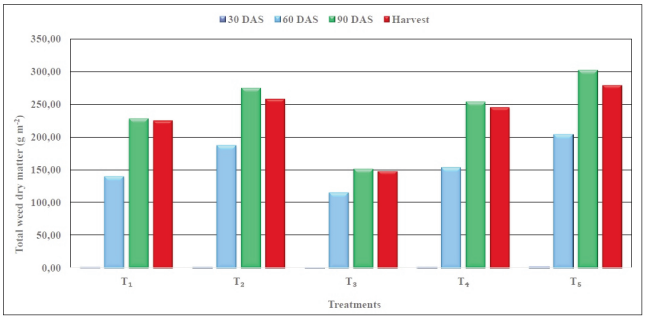

Fig. 1 - Total weed dry weight at different growth stages as influenced by different intercropping systems, pooled data 2017 and 2018. T1: Rice+Amaranth, T2: Rice+Coriander, T3: Rice+Palak, T4: Rice+Methi, T5: Sole Rice.

was followed by intercropping of rice and leafy vegetable amaranths (0.47, 140.15, 228.05 and 225.15 g m^{-2}, respectively).

Effect of the rice-leafy vegetable intercropping system on weed smothering efficiency

Weed smothering efficiency is a measure of the effect of intercropping on the suppression of weeds in comparison to sole crop stand. The data pertaining to the weed smothering efficiency are presented in Table 2.

In the present experiment, the higher weed smothering efficiency was found in intercropping of

Table 2 - Rice grain, straw and leaf vegetable yield, rice equivalent yield (REY), land equivalent ratio (LER), production efficiency (PE), net monetary income and weed smothering efficiency (WSE) in rice as influenced by different intercropping systems, during 2017 and 2018

Practices	Grain yield (kg ha-1)		Straw yield (kg ha-1)		Vegetable yield (kg ha-1)		REY (kg day^{-1})		LER		PE (kg day-1)		Net income (₹ &US$ha-1)		WSE (%)	
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
T_1	6730	5755	8382	8167	14248	13811	11479	10358	1.88	1.82	76.53	69.05	76010₹ 1096 US$	74749₹ 1078 US$	20.07	18.60
T_2	6018	5443	8030	7841	12057	11226	10037	9185	1.82	1.79	66.91	61.24	59357₹ 856 US$	56543₹ 815 US$	8.15	6.88
T_3	7951	7351	9794	9580	25810	25207	16554	15753	2.12	2.15	110.36	105.02	158025₹ 2278 US$	154515₹ 2228 US$	48.09	45.86
T_4	6444	5644	8196	8010	14133	12056	11155	9663	1.87	1.88	74.37	64.42	72095₹ 1039 US$	70098₹ 1011 US$	12.54	11.64
T_5	5978	5403	7382	7174	-	-	-	-	-	-	-	-	-	-	-	-
T_6	-	-	-	-	19010	18407	-	-	-	-	-	-	-	-	-	-
T_7	-	-	-	-	15086	14482	-	-	-	-	-	-	-	-	-	-
T_8	-	-	-	-	32705	32105	-	-	-	-	-	-	-	-	-	-
T_9	-	-	-	-	18743	15893	-	-	-	-	-	-	-	-	-	-
S.Em.±	92.32	183.89	260.38	259.15	1555	1427	684	713	0.09	0.06	4.08	4.19	NA	NA	NA	NA
CD @5%	296.99	572.91	811.20	807.36	4604	4191	2252	2313	0.28	0.21	12.7	13.06	NA	NA	NA	NA

T1: Rice+Amaranth, T2: Rice+Coriander, T3: Rice+Palak, T4: Rice+Methi, T5: Sole Rice, T6: Sole amaranth, T7: Sole Coriander, T8: Sole crop Palak, T9: Sole crop Methi ; NA=Not analysed; (indian rupees: ₹ converted into US$ at the rate of 69.35₹ against 1 US$ on 29th March 2019).

rice and leafy vegetable palak (48.09 and 45.86%, 2017 and 2018, respectively) followed by intercropping of rice and leafy amaranthus (20.07 and 18.60%, 2017 and 2018, respectively).

Effect of the rice-leafy vegetable intercropping system on nutrient uptake by crops and weeds

The weeds withdraw the nutrients that would have normally available to the crop. As the nutrient uptake is increased by weeds on account of higher weed population, the harmful effect could be expected on the crop. When the weed growth is effectively managed through integrated weed management, a decline in nutrient uptake by weeds is a natural consequence. Uptake of major soil nutrients by weeds and crops indicated that the rate of increase in the uptake was proportional to the dry matter production.

The 2 years pooled data on uptake of nitrogen, phosphorus and potassium by rice, leafy vegetables at harvest and weeds at 60 DAS as influenced by different intercropping practices are given in figures 2 and 3. The total nitrogen (153.78 kg ha^{-1}), phosphorous (45.27 kg ha^{-1}) and potassium (152.22 kg ha^{-1}) uptake by rice crop at harvest were significantly higher in intercropping of rice with leafy vegetable palak as compared to sole rice (109.90, 29.49 and 109.25 kg NPK ha^{-1}, respectively). Similar trend was seen with uptake by leafy vegetable crop (Fig. 3) wherein leafy vegetable palak intercropped with rice significantly recorded higher amount of the total nitrogen (101.09 kg ha^{-1}), phosphorous (14.18 kg ha^{-1}) and potassium (38.97 kg ha^{-1}) followed by leafy vegetable amaranthus intercropped with rice (77.08, 8.98 and 28.81 kg NPK ha^{-1}, respectively).

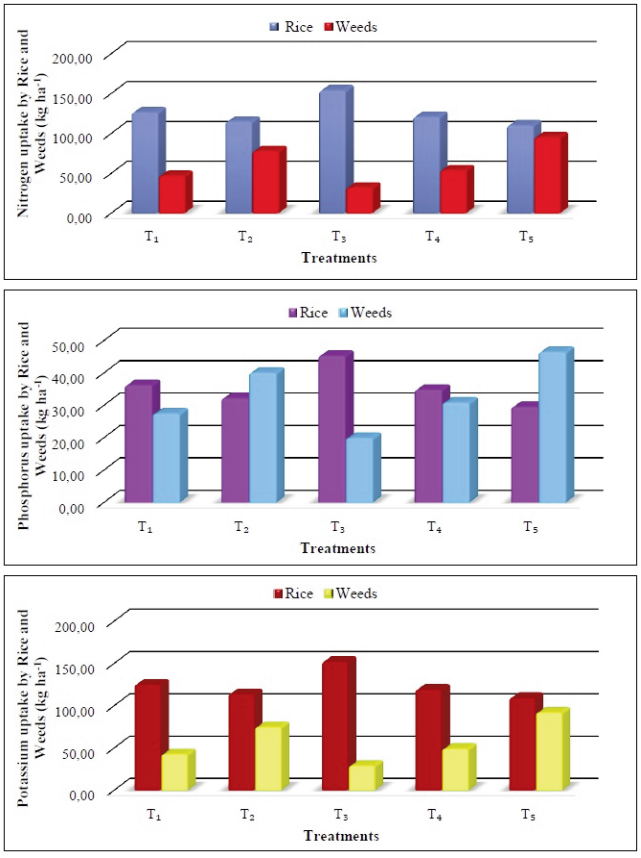

Fig. 2 - Nutrient uptake by rice crop at harvest and weed at 60 DAS as influenced by different intercropping systems. T1: Rice+Amaranth, T2: Rice+Coriander, T3: Rice+Palak, T4: Rice+Methi, T5: Sole Rice.

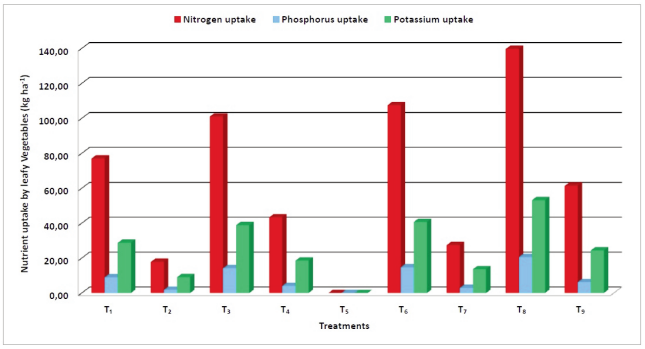

Fig. 3 - Nutrient uptake by leafy vegetable crops at harvest as influenced by different intercropping systems, pooled data 2017 and 2018. T1: Rice+Amaranth, T2: Rice+Coriander, T3: Rice+Palak, T4: Rice+Methi, T5: Sole Rice, T6: Sole amaranth, T7: Sole Coriander, T8: Sole crop Palak, T9: Sole crop Methi.

At 60 days of crop growth (Fig. 2), the nitrogen, phosphorous and potassium uptake by weeds were significantly influenced by different intercropping practices. Significantly lower nitrogen (31.88 kg ha^{-1}), phosphorus (19.90 kg ha^{-1}) and potassium (28.92 kg ha^{-1}) uptake were recorded with the intercropping of rice+leafy vegetable palak. It was followed by intercropping of rice with leafy vegetable amaranth: nitrogen (46.72 kg ha^{-1}), phosphorus (27.39 kg ha^{-1}) and potassium (42.68 kg ha^{-1}) uptake as compared to sole rice (94.93, 46.48 and 91.83 kg NPK ha^{-1}, respectively).

Effect of the rice-leafy vegetable intercropping system on rice and vegetable yield and efficiencies

The rice grain yield, straw yield, leafy vegetable yield, Rice Equivalent Yield (REY), Land Equivalent Ratio (LER), Production Efficiency (PE) and net monetary income differed significantly due to different intercropping practices. The data are accessible in Table 2.

Significantly higher grain yield, straw yield, vegetable leaf yield (7951, 9794, 25810 kg ha^{-1} and 7351, 9580, 25207 kg ha^{-1} for 2017 and 2018, respectively) were recorded in intercropping of rice with leafy veg-

etable palak as compared to grain and straw yield of sole rice (5978, 7382 and 5403, 7174 kg ha^{-1} for 2017 and 2018, respectively). It was followed by intercropping of rice with leafy vegetable amaranth (6730, 8382, 14248 kg ha^{-1} and 5755, 8167, 13811kg ha^{-1} for 2017 and 2018, respectively).

Rice equivalent yield is the best tool to determine the overall productivity potential of an intercropping system. The data presented in Table 2 reflected visible variation in REY among the intercropping systems showing the highest REY (16554 and 15753 kg ha^{-1} in 2017 and 2018, respectively) for intercropping of rice with leafy vegetable palak followed by intercropping of rice with leafy vegetable amaranthus (11479 and 10358 kg ha^{-1}) which was on par with intercropping of rice with leafy vegetable methi (fenugreek) (11155 and 9663 kg ha^{-1}).

The data on Land Equivalent Ratio of different intercropping systems indicated that LER values were greater than one in all the intercropping practices and the range of yield advantage over sole cropping of rice was between 79 and 115 per cent with the highest in case of intercropping of rice with leafy vegetable palak (115 per cent) followed by intercropping of rice with leafy vegetable methi (88 per cent) compared to monocropping of rice.

Significantly higher Production Efficiency was recorded in intercropping of rice with leafy vegetable palak (110.36 and 105.02 kg day^{-1} in 2017 and 2018, respectively) and was closely followed by intercropping of rice with leafy vegetable amaranthus (76.53 and 69.05 kg day^{-1}) in both years.

Net monetary income (indian rupees: ₹ converted into US$ at the rate of 69.35₹ against 1 US$ on 29th March 2019) was higher in intercropping of rice with leafy vegetable palak (**₹** 158025 equivalent to **US$** 2278) ha^{-1}; ₹154515 equivalent to US$ 2228) ha^{-1} for 2017 and 2018, respectively) followed by intercropping of rice with leafy vegetable amaranthus (**₹** 76010 equivalent to US$1096); **₹** 74749 equivalent to US$ 1078) ha^{-1} for 2017 and 2018, respectively).

4. Discussion and Conclusions

The weed population and total dry weight of weeds differed significantly due to different intercropping systems (Table 1, Fig. 1). The decline in weed density and lower weed dry matter accumulation in rice + palak intercropping systems may be attributed to shading effect and competition stress generated by the canopy of leafy vegetable in a unit area having smothering effect on associated weeds, thus preventing the weeds to attain the full growth (Banik and Ravi, 2013). The intercropping system suppressed the weed growth due to their spreading canopy coverage. The increased populations per unit area and crop competition in intercropping were also the possible reason for effective weed control (Jha and Dinesh, 1982; Ibni *et al.* 2005; Abdul *et al.* 2009; Mian *et al.* 2011).

Higher weed smothering efficiency (Table 2) in rice + palak intercropping systems resulted from less space available for the growth of weeds due to quick coverage of ground and more shading effect which led to the lower total weed population and its dry weight. Similar findings were also reported by Musthafa and Potty (2001); Vyas and Kushwah (2008) and Mian *et al.* (2011).

The higher rice grain yield could be attributed to better yield attributing parameters namely higher no. of productive tillers hill^{-1}, higher panicle length, higher panicle weight hill^{-1}, higher total no. grain panicle^{-1}, higher 1000 grain weight and higher harvest index as compared to sole rice. The above increment in yield was attributed to increased growth attributes such as higher total dry matter production and distribution in various parts of the plant and higher leaf area as well. In addition to this, the higher canopy coverage by palak has resulted in a reduction in total weed population which turned the equilibrium in favor of crop for the use of the available resources. Similarly, in the intercropping system, significantly higher fresh leafy vegetable yield (25508 kg ha^{-1}) was recorded in intercropping of rice with leafy vegetable palak followed by intercropping of rice with leafy amaranthus (14029 kg ha^{-1}, respectively). However, the sole leafy vegetable palak registered higher yield (32405 kg ha^{-1}) followed by sole leafy amaranthus (18708 kg ha^{-1}). These findings are in the similar trend with Ibni *et al.* (2005); Ahmed *et al.* (2006); Mian *et al.* (2010).

The higher nutrients uptake by rice crop and leafy vegetables in intercropping of rice with leafy vegetable palak might be attributed to minimum crop-weed competition as a result of higher weed smothering efficiency, gave the better control of weeds from initial stages which led to lower weed population and their dry weight, this helped the crop to grow in weed-free environment and absorb more nutrients from the soil. Hence, resulted in better growth and development of leafy vegetable and rice crops leading to better nutrient uptake. A similar report was also reported by Abdul *et al.* (2009) and

Mian *et al.* (2010).

The lower nutrient uptake by weeds in intercropping of rice with leafy vegetable palak was mainly due to better control of weeds as a result of lower weed competition leading to lower weed dry matter production as also noticed by Ibni *et al.* (2005); Abdul *et al.* (2009) and Mian *et al.* (2010).

The percentage increase over sole cropping of rice as a result of different intercropping systems, however, varied from 46.74 to 76.83 % clearly indicating substantial yield advantage of intercropping. The variation in REY under different cropping systems was ascribed to their variable utilization of soil and agro-resources. Higher yield benefit in terms of REY of intercropping over monocropping of rice has also been revealed by Abdul *et al.* (2009), Mian *et al.* (2011), Nagwa *et al.* (2014), Rayhan *et al*. (2014), and Gurpreet Singh *et al*. (2018).

The data on LER of different intercropping systems indicated that LER values were greater than one in all the intercropping treatments and the range of yield advantage over sole cropping of rice was between 79 and 115 per cent with the highest in case of intercropping of rice with leafy vegetable palak (115 per cent) followed by intercropping of rice with leafy vegetable amaranthus (85 per cent) compared to monocropping of rice was attributed to better utilization of natural resources (land, CO_2 and light). Higher LER in intercropping compared to monocropping of rice was also reported by Abdul *et al.* (2009), Mian *et al.* (2011), Nagwa *et al.* (2014), Udhaya and Kuzhanthaivel (2015) (Table 2).

Significantly higher PE was recorded in intercropping of rice with leafy vegetable palak (107.69 kg day^{-1}) and was closely followed by intercropping of rice with leafy vegetable amaranthus (72.79 kg day^{-1}). The result indicated that the intercrops remained in the field for a shorter time (30 DAS) and yields were also high leading to higher production per day. The similar tendency was noted by Ibni *et al.* (2005); Nazrul and Shaheb (2011) and Rayhan *et al*. (2014) (Table 2).

The higher net income increases in intercropping of rice with leafy vegetable palak was mainly due to the higher rice grain, rice straw yield, rice equivalent and higher leafy vegetable yield which in turn increased gross and net returns. These results are in agreement with the findings of Ibni *et al.* (2005); Abdul *et al.* (2009); Mian *et al.* (2010).

In this experiment, rice crop intercropped with palak as a leafy vegetable was found to be the most efficient practice in smothering the weeds by reducing the weed density and dry weight which significantly increased growth, yield and profitability of aerobic rice compared to the rest of intercrops and sole rice. Since all the leafy vegetable seeds were broadcasted, the use of smother leafy vegetable crops as an intercrop in definite row proportion in aerobic rice is encouraged.

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AHS
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Direct shoot regeneration of three Petunia cultivars

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Abstract: A tissue culture system for acquiring high-efficiency regeneration of Petunia was optimized. Leaf explants of Alvan, Large Flower Alvan (LF Alvan) and Mahalat cultivars of *Petunia hybrida* were cultured separately on MS medium including various concentrations of TDZ and BA without auxin in order to assess direct shoot regeneration. Alvan showed the highest frequency of shoot regeneration (100%) and the highest mean number of shoots per explant (25.33) on MS containing 2 mg/l TDZ. For LF Alvan cultivar the highest percentage of shoot organogenesis (100%) and the highest mean number of shoots per explant (18.20) were observed when MS medium containing 1 mg/l BA was used. With the Mahalat cultivar the maximum rate of direct regeneration was obtained on MS supplemented with 0.5 and 1 mg/l BA (80%). The mean number of shoots per explant (9.63) was obtained when 2 mg/l TDZ was used. Regenerated shoots were successfully elongated (2 to 3 cm in length) and transferred into half-strength MS as the rooting medium supplemented with 0.1 mg/l NAA. The shoots were successfully rooted, acclimatized and transferred to the greenhouse.

1. Introduction

Petunia (*Petunia hybrida)* is well known as an economically important ornamental plant and is grown worldwide for its beautiful and fragrant flowers. Propagation techniques with modern approaches intend to give a hand to scientists to provide demands of ornamental industry (Rout *et al*., 2006). An efficient plant regeneration system is necessary for the successful genetic transformation (Ntui *et al*., 2010). There are several reports for in vitro shoot regeneration of *Petunia hybrida* species from several explants including leaf (Preece, 2000; Ntui *et al*., 2010; Abu-Qaoud *et al*., 2010; Khan *et al*., 2011; Abu-Qaoud, 2012; Burbulis *et al*., 2015), somatic cells (Rao *et al*., 1973) cotyledon (Dulien, 1991), embryo (Dimasi-Theriou *et al*., 1993), protoplast (Auer *et al*., 1992; Auer *et al*., 1999; Abu-Qaoud *et al*., 2010), petal (Razdan, 2003), and microspore (Li *et al*., 2013). Various factors could affect organogenesis in *P. hybrida* such as light (Reuveni and Evenor, 2007), sugar and CO_2 (Qu *et al*., 2007), ethylene

(Dimasi-Theriou *et al*., 1993), nitrogen and calcium (Frett and Dirr, 1996) and also hormonal combinations (Ying *et al*., 2005; Xiao-Feng *et al*., 2009; Xian-Chun, 2010) Petunia regeneration happens directly and indirectly by combinations of auxins and cytokinins in medium culture (Michalczuk and Michalczuk, 2000; Ziv *et al*., 2005).

Adventitious bud formation from somatic cells of *P. hybrida* was induced by exogenous cytokinins such as BA (6-benzyladenine), Zeatin, Kinetin and TDZ (Thidiazuron) (Rao *et al*., 1973; Thirukkumaran *et al*., 2009). It is reported that TDZ acted different from traditional cytokinins and was able to accomplish both the cytokinin and auxin requirements of different plant species for regeneration (Murthy *et al*., 1998; Sanikhani *et al*., 2006). The highest frequency of direct shoot organogenesis of Daady Blue and White Dreams cultivars of *P. hybrida* was obtained on MS medium supplemented with different concentration of TDZ (Abu-Qaoud, 2012). Also, TDZ alone provided the highest percentage of shoot organogenesis and mean number of shoot per explant of *P. hybrida* cv. Mitchell (Thirukkumaran *et al*., 2009). It is also reported that exogenous cytokinin especially BA could control the commitment of Petunia leaf explants to induce shoots in tissue culture (Auer *et al*., 1992; Abu-Qaoud *et al*., 2010). Therefore in this study, we investigated the effect of TDZ and BA as well as genotype on direct shoot regeneration of three Petunia cultivars. This efficient regeneration system is very useful in genetic transformation projects of *P. hybrida*.

2. Materials and Methods

Seed germination

Seeds of three local cultivars of Petunia, Alvan, Large Flower Alvan (LF Alvan) and Mahalat, were sterilized with 70% ethanol for 30s, and sodium hypochlorite solution 1% for 10 minutes. They rinsed 3 times with sterilized water and cultured on MS medium. Seeds were grown under 25 ± 2 °C with 16/8 hour photoperiod, under fluorescent illuminations (40 µmol $m^{-2}s^{-1}$).

Organogenesis

The newly formed leaves were cut 6-8 mm in length, and then cultivated on 5 modified MSmedia: MS medium without hormones (MS_1), MS + 0.5 mg/l BA (MS_2) [Sigma-Aldrich, Steinheim, Germany], MS + 1 mg/l BA (MS_3), MS + 1 mg/l TDZ (MS_4) [Sigma-Aldrich, Steinheim, Germany] and MS + 2 mg/l TDZ (MS_5). Abu-Qaoud *et al*., (2010) got more regeneration when they used 0.8 mg/l BA. Therefore we selected 0, 0.5 and 1 mg/l BA to better estimate BA effect. Also as Thirukkumaran *et al*., (2009) reported more regeneration with 2 mg/l TDZ, we selected 0, 1 and 2 mg/l TDZ to investigate its effect. Moreover, the MS was supplemented with 30 g/l sucrose and solidified with 7 g/l agar [Duchefa, Haarlem and The Netherlands]. The optimum pH of all culture media was considered 5.8 which adjusted with 1N NaOH before sterilization. Then all media were sterilized using autoclave at 121°C for 20 min. Explants were placed on regeneration medium with the adaxial side upward. The cultures were incubated at 25±2°C, with a light to dark period of 16/8 hours under cool-white fluorescent light at 40 µmol m^{-2} s^{-1}. Explants were sub-cultured every two weeks. They were investigated using binocular Stereo Microscope, regarding to the mean number of explants inducing shoots and the mean number of induced shoots and buds per explants after 4-5 weeks on regeneration medium.

Rooting and acclimatizing

Regenerated shoots were transferred into half-strength MS supplemented with 30 g/l sucrose, 0.1 mg/l NAA [Duchefa, Haarlem, and The Netherlands] and solidified with 7 g/l agar. The rooted plantlets rinsed under tap water and planted on the plastic pots with combination of sterile peat moss and perlite mixture (2:1). They kept in greenhouse conditions.

Statistical analysis

The experiment was done based on completely randomized design with three replications and 10 leaf explants in each replication. Data were normalized through arcsin (√x) and (√x+0.5) transformation in SPSS. The normalized data were analyzed using SAS statistical analysis package and were compared via Duncan's multiple range test at $P \leq 0.01$ and $P \leq 0.05$.

3. Results

Effect of BA on organogenesis

Direct shoot formation was obtained in all three cultivars after 4-5 weeks. No regeneration occurred on MS_1 medium which means hormones are necessary to induce shooting (Tables 1, 2). When 0.5 mg/l BA (MS_2) was used no differences in frequency of regeneration was observed among cultivars. The low

Table 1 - Effect of modified MS medium supplemented with different concentration of BA on shoot regeneration from leaf explants of *P. hybrid*

MS Media	Frequency of regeneration			The mean number of shoots per explant		
	Cultivars			Cultivars		
MS_1	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 e	0.00 ± 0.00 e	0.00 ± 0.00 e
MS_2	83.33 ± 2.8 b	83.33 ± 1.8 b	80.00 ± 3.1 b	6.21 ± 0.12 d	13.31 ± 0.85 b	5.05 ± 1.00 d
MS_3	80.00 ± 3.7 b	100.00 ±0.00 a	80.00 ± 1.1 b	10.12 ± 0.41 bc	18.20 ± 0.85 a	7.76 ± 0.56 cd

The values represent the mean ± standard error of three replicates. Different letters are showing considerable differences at P≤0.05.

Table 2 - Effect of modified MS medium supplemented with different concentration of TDZ on shoot regeneration from leaf explants of *P. hybrid*

MS Media	Frequency of regeneration			The mean number of shoots and buds per explant		
	Cultivars			Cultivars		
	Alvan	LF Alvan	Mahalat	Alvan	LF Alvan	Mahalat
MS_1	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 f	0.00 ± 0.00 f	0.00 ± 0.00 f
MS_2	83.33 ± 3.4 b	80.00 ± 1.0 b	66.66 ± 2.1 c	16.25 ± 1.00 b	12.00 ± 1.21 c	6.61 ± 0.08 e
MS_3	100.00 ± 0.00 a	83.33 ± 0.8 b	70.00 ± 1.7 c	25.33 ± 1.02 a	14.31 ± 0.96 bc	9.63.00 ± 0.11 d

The values represent the mean ± standard error of three replicates. Different letters are showing considerable differents at P≤0.05.

mean numbers of shoot per explant (5.05 and 6.21) were observed in MS_2 for Mahalat and Alvan cultivars, respectively. When 1 mg/l BA (MS_3) was used differences were observed in all three cultivars and LF Alvan cultivar showed 100% shoot regeneration (Table 2), with a mean number of 18.20 shoots per explants (Fig. 1 a).

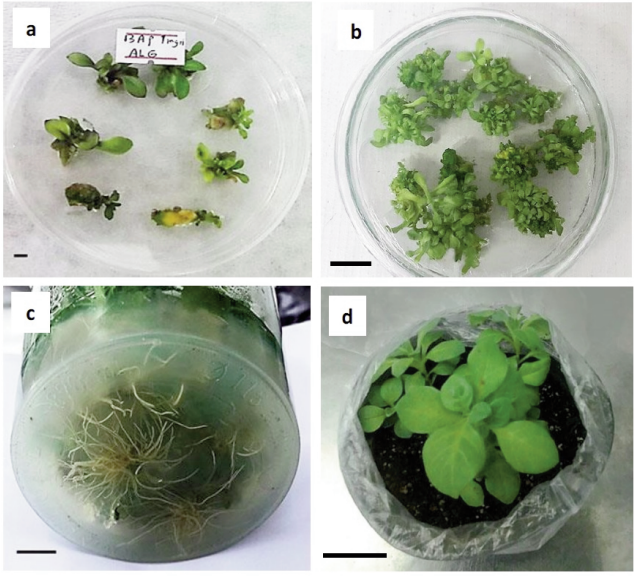

Fig. 1 Plant regeneration from leaf explants of different cultivars of *Petunia hybrida*. (a) Direct shoot regeneration of LF Alvan on MS + 1 mg/l BA (bar: 2 mm); (b) Direct shoot regeneration of Alvan on MS + 2 mg/l TDZ (bar: 4 mm); (c) Root formation after 2 weeks on rooting media (bar: 5 mm); (d) A 4 weeks old plantlet after transfer to the pot (bar: 1 cm).

Effect of TDZ on organogenesis

Significant differences were observed among three cultivars when TDZ concentration was increased (Tables 3, 4). The low shoot regeneration frequency was obtained on MS_4 and MS_5 media for Mahalat cultivar (Table 4). Alvan cultivar showed the highest percentage of shoot regeneration (100%) and mean number of shoots per explant (25.33) on MS with 2 mg/l TDZ (Fig. 1 b) and the lowest one (6.61) was belong to Mahalat cultivar on MS with 1 mg/l TDZ.

4. Discussion and Conclusions

We could show that auxin is not necessary for direct shoot regeneration of three cultivars of *P. hybrida*. It is already reported that the number of shoot per explants dramatically increased when explants exposed to the medium containing BA (Auer *et al.*, 1992). The highest shoot regeneration rate (45%) and the maximum average number of shoots per explant (7.5) from Petunia leaf explants on MS with 2 mg/l BA + 0.5 mg/l NAA has also been reported (Abu-Qaoud *et al.*, 2010). In the current study the highest shoot regeneration frequency in Alvan cultivar and the mean number of shoots per explant in both Alvan and Mahalat cultivars were observed when 2 mg/l TDZ was used which is in conformity with Thirukkumaran *et al.* (2009). The importance of TDZ on regeneration and shoot induction frequency

and the mean number of shoots per explant was also investigated in Daddy blue and Dreams white genotypes (Abu-Qaoud, 2012). This study showed that a cytokinin source of TDZ or BA may be enough for direct shoot regeneration of three mentioned cultivars of *P. hybrida*. Application of TDZ instead of both auxin and cytokinin requirements for organogenesis in the wide range of plant species has been supported (Murthy *et al*., 1998). Probably TDZ tends to make balance among endogenous growth regulators that is essential for inducing specific modes of regeneration. It was found that many factors such as genotype and exogenous growth regulators have the capability to influence on biochemical pathways controlling the endogenous cytokinin content (Krikorian, 1995). In the present study a significant difference in regeneration frequency was observed among studied cultivars probably due to the different level of endogenous hormones. For LF Alvan cultivar, the maximum regeneration frequency (100%) and the highest number of shoots per explants (18.20) were obtained when BA concentration was increased from 0.5 to 1 mg/l while the other two cultivars showed less reaction. These findings confirm the report of Jamshidnia and Sayed Tabatabaei (2013), and Burbulis *et al*., (2015) on differences in shoot regeneration frequency among three different genotypes of Petunia. Here we report an efficient direct shoot regeneration system in *Petunia hybrida* using leaf explants of Alvan cultivar. This cultivar can be considered as a suitable cultivar for transformation experiments.

To conclude, the present study provided an efficient direct shoot regeneration system without auxin in Petunia using leaf explants that could be improve transformation studies.

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Table 3 - Analysis of variance of different concentrations of TDZ on shoot regeneration of *P. Hybrida*

Source of Variation	DF	Mean squares		P-value	
		Frequency of regeneration	Mean number of shoots per explant	Frequency of regeneration	Mean number of shoots per explant
TDZ	2	3.1897 **	11.4806 **	0.000	0.000
Cultivar	2	1.3250**	6.5896 **	0.009	0.004
TDZ × Cultivar	4	0. 8015*	4.2010 **	0.022	0.009
Error	18	0. 215	0.9080		
Total	26				

*, **, significant at 5% and 1% levels, respectively.

Table 4 - Effect of TDZ on shoot regeneration of *P. Hybrida* using Duncan's multiple range test

MS Media	Frequency of regeneration			The mean number of shoots and buds per explant		
	Cultivars			Cultivars		
	Alvan	LF Alvan	Mahalat	Alvan	LF Alvan	Mahalat
MS_1	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 f	0.00 ± 0.00 f	0.00 ± 0.00 f
MS_4	83.33 ± 3.4 b	80.00 ± 1.0 b	66.66 ± 2.1 c	16.25 ± 1.00 b	12.00 ± 1.21 c	6.61 ± 0.08 e
MS_5	100.00 ± 0.00 a	83.33 ± 0.8 b	70.00 ± 1.7 c	25.33 ± 1.02 a	14.31 ± 0.96 bc	9.63 ± 0.11 d

MS_1= MS medium without hormones, MS_4= MS + 1 mg/l TDZ; MS_5= MS + 2 mg/l TDZ.
Means compared using Duncan's multiple range test. The Values represent the mean ± standard error of three replicates. Different letters are showing considerable differents at P≤ 0.05.

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AHS
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The physiological responses of four turfgrass species to drought stress

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Key words: antioxidant, bermudagrass, cool-season turfgrasses, electrolyte leakage.

Abstract: Drought stress is one of the most important factors which reduce turfgrass growth and quality in the area with restricted rainfall or irrigation water supply. Using resistant species and varieties can be a useful management program for reducing irrigation requirement in turfgrass. The present study was carried out to examine the physiological changes of four species turfgrass in response to drought stress conditions. The rhizomes of bermudagrass, and seeds of tall fescue, perennial ryegrass and kentucky bluegrass were cultivated at the greenhouse in PVC pots (20 cm in diam., 20 cm long). After four months, when the seedlings well established, drought stress was applied in 100% field capacity (FC), 75% FC, 50% FC and 25% FC. Proline, electrolyte leakage (EL), malondialdehyde (MDA), relative water content (RWC), chlorophyll, catalase (CAT), superoxide dismutase (SOD) and peroxides (POD) was measured. All species showed an ability to tolerate drought stress, but tall fescue exhibited more tolerance, with a higher RWC and proline content. Tall fescue also revealed higher CAT, SOD, POD activities and lowest MDA, EL. This study found that kentucky bluegrass was more vulnerable to severe water stress, and displayed the highest MDA and EL as compared to the other examined species.

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1. Introduction

Water deficit is the main problem for turf management, especially in arid and semi-arid zones. Turfgrasses play a significant role in the design of urban green spaces, in most cases other plants can not be utilized instead of turfgrasses; therefore it is necessary to find species and cultivars of turfgrass that require little water and are able to maintain their visual quality in drought conditions (Fiorio *et al*., 2012). Increased competition for water has fostered interest in water conservation practices for both warm-season and cool-season turfgrasses. Responses of turfgrass to drought can be viewed in a number of ways. Drought stress will affect visual quality, growth rate and evapotranspiration (ET) (Krishnan *et al*., 2013). Some adaptations and mitigation strategies are necessary to dispose of drought stress. Grass species and cultivars have been found to respond differently to drought stress (Vurukonda *et al*., 2016). Some grass genotype like tall fescue were able to tolerate drought condition in a research it was demonstrated that this kind of grass can be one of the

most suitable plant species to be used for cultivation under arid, semi-arid regions, and areas with limited water supplies or drought conditions (Alam *et al.*, 2018).

Some traits affected by drought stress include relative water contant, electrolyte leakage and some enzyme activities. RWC and EL are indicators for the selection of drought-tolerant plants (Salehi Lisar *et al*., 2012). Reactive oxyen species cause lipid peroxidatin, which leads to damage of cell membrane. Drought stress increased lipid peroxidatin and membrane damage percent in different plants. It is been reported that tall fescue exhibiting a more effective protection mechanism, mitigated oxidative stress and lipid peroxidation by maintaining higher superoxide dismutase (SOD) and catalase (CAT) activities than kentucky bluegrass (Xu *et al*., 2013). There are many reports about increase, decrease, or no change inoxidative enzymes in plants exposed to stress (Fu and Huang, 2001; Ramachandra Reddy *et al*., 2004; Sharma and Dubey, 2005).

Synthesis of compatible solutes such as proline seems to has a central role in osmotic adjustments, preventing or reducing the loss of turgor (Vinocur and Altman, 2005). In fact, studies on several turfgrass species have shown that the free proline concentration increases in leaves with water stress. This phenomenon has been demonstrated in *Festuca arundinacea* Scherb. (Salehi and Salehi, 2012).

Many researches evaluated the physiological adaption or the functional and qualitative response of different cool and warm season grasses to increasing water deficits (Qian and Engelke, 1999; Bastug and Buyuktas, 2003; Fu *et al.*, 2004). Althought all of these studies report that during the drought period, warm-season grasses are more tolerant to drought sress than cool-season grasses, different climatic conditions will influence relative drought tolerance of cool-season and warm-season turfgrass, necessitating regional evaluations. The main goals of this study were to compare the physiological and biochemical responses of cool-season turfgrasses (tall fescue, kentucky bluegrass, perennial ryegrass) and warm-season turfgrasses (bermudagrass) under drought stress condition in Isfahan, iran.

2. Materials and Methods

Plant materials and experimental: This reserch was performed during 2015 to 2016 in Department of Horticultural at Isfahan University of Technology, Isfahan, Iran under greenhouse conditions (32°39' N, 51°40' E). Polyvinyl chloride (PVC) pots (20 cm in diam., 20 cm long) filled with sterilized silt-loam soil, which collected from the Isfahan's landscape. For this experiment seeds of *Festuca arundinacea*. 'Astrix', *Lolium perenne*. 'Numan' and *Poa pratensis*. 'Miracle' were sown and rooted rhizomes of *Cynodon dactylon*. 'Tifway' were planted in 48 PVC pots. Irrigation was applied as needed to prevent any visible drought stress during grass establishment. In general, turfs were watered three times weekly to maintain plants under well-watered condition and soil moisture at field capacity. Plants were maintained at a cutting height of 5 cm and moved once a week using a reel-type mower. A fertilizer (urea) was applied at 5 $g.m^{-2}$ rates once every two weeks to provide nutrients and to facilitate plant establishment before initiation of treatments.

Drought stress treatment and experiment design

This study was carried out as a factorial experiment based on randomized complete block design (RCBD), with two treatments consisted of four levels of drought stress (100%, 75%, 50%, and 25% field capacity (FC)), four turfgrass species (tall fescue, bermudagrass, kentucky bluegrass, and perennial ryegrass), with three replications and 16 pots were used for each replication (numbers of pots= 48). The FC was determined by the gravimetric method, which consists on the difference between the wet soil after saturation and free drainage, and the weight of the dry soil (Cleide de Souza *et al*., 2000). The soil water content was kept at gravimetric water capacity (measured 100%, 75%, 50% and 25% FC were 22.3%, 16.72%, 11.15% and 5.57% respectively) by adding tap water. Drought stress was applied for two months and at the end of the experiment, physiological traits were measured.

Chlorophyll content

Leaf samples were selected randomly from the plants and homogenized in a mortar in 10 ml of 100% acetone. The extract was centrifuged at 2000 rpm for 10 min. Absorbance of the supernatant was recorded at 663, 645 and 450 nm spectrophotometrically. Chlorophyll (Chl) content was determined following the method of (Lichtenthaler, 1987).

Electrolyte leakage: Leaf electrolyte leakage which is used to assess membrane permeability leakage was assayed base on Lu *et al*. (2008) methods. Leaf samples (0.1 g) was placed into a vial with 20 mL of double distilled water. After incubating the samples at room temperature on a shaker (150 g) for 24h, the

electrical conductivity (EC) of the bathing solution (EC_1) was determined. The same samples were then placed in water bath at 100°C for 1h and a second reading (EC_2) was determined after cooling solution to room temperature. The electrolyte leakage was calculated as EC1/EC2 and expressed as percent.

Relative water content

We determined relative water content (RWC) according to the method developed by Ghoulam *et al.* (2002). About 0.2 g of the fresh leaf sample was cut into smaller pieces and weighed (W1). Then the leaf samples were saturated in 100-ml deionized water for 24 h at 4°C and weighed to determine the turgid weight (W2). Finally leaf samples were dried at 70°C for 24 h, and the dry weight was recorded (W3). RWC was determined using the following equation:

$$RWC\ (\%) = (FW\text{-}DW) / (TW\text{-}DW) \times 100.$$

Where FW, DW, and TW are fresh, dry and turgid weights respectively.

Proline content

Proline content measurement was carried out according to a previously described method (Bates *et al*., 1973). Leaves were homogenized in 3% aqueous Sulphosalicylic acid, then centrifuged 5,000 g for 20 min at 4°C. 2 mL of this homogeny solution react acid-ninhdrin and 2 mL of glacial acetic acid in a tube for 1 hour at 100°C and the reaction is torn up in an ice bath and then extracted with 4 mL of toluene. It was kept at room temperature to stabilize. Proline content was measured by spectrophotometer (UV-160A, Shimadzu, Tokyo, Japan) at 520 nm (Bates *et al.*, 1973).

Malondialdehyde

In order to determine the content of malondialdehyde (MDA) in the leaves, 0.1 g of leaf tissues was homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA) for 10 minutes and then was centrifuged at 5,000 g. 1 ml of conventional solution was mixed with 4 ml of thiobarbituric acid (TBA) (0.5% of TBA in 20%). Then the reaction mixture was placed in a hot bath at 100°C for 15 minutes. Finally the mixture centrifuged at 5,000 g for 10 minutes and the amount of MDA was subsequently read by the spectrophotometer at 450, 532 and 600 nm (Wang *et al*., 2008).

Enzyme assay

For enzyme extraction, 0.1 g leaf powder was extracted with 1 ml of sodium phosphates extraction buffer and Triton. The extractions were centrifuged at 12000 × *g* for 30 min at 4°C, and supernatant was collected for enzyme assay. The supernatant was used as a source of SOD enzyme. SOD was measured by a photochemical method (Giannopolitis and Ries, 1977). The reaction mixture (3 ml) contained 0.1 mM EDTA (Ethylenediamine tetra acetate) 0.05 ml HEPES-KOH buffer (pH=7.8), 50 mM Na_2Co_3, 13 mM methionine, 63 µM NBT (Nitro blue tetrazolium) 0.05 ml enzyme extract and 1.3 µM riboflavin. The absorbance was read at 560 nm and one unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT. CAT (EC: 1.11.1.6) activity was assayed in a reaction mixture containing 100 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and enzyme 0.05 ml of aliquot. The decomposition of H_2O_2 was followed at 240 nm (Aebi, 1984). The catalase (CAT) activity is defined in international unit equals (1 unit) as the amount of catalase necessary to decompose 1 µM of H_2O_2 per minute. Activity of peroxides (POD) was determined in a reaction mixture (2.95 ml), which consisted of 75 mM suitable amount of guaiacol, 15 mM H_2O_2, 100 mM phosphate buffer and 0.05 ml of enzyme extract. The absorbance of the supernatant at 470 nm was measured (Maehly, 2006). One unit of POD activity was defined as the amount of enzyme necessary to decompose 1 µM of H_2O_2 per minute.

Statistical analyses

Statistical Analysis System (SAS 9.1) was used for variance analysis and the differences between treatment means were assessed by the least significance difference (LSD) at P= 0.05 probability level.

3. Results

Chlorophyll content

Results from leaf chlorophyll content measurements showed a significant difference between water stress treatments, species and interaction effects ($P \leq 0.01$) (Table 1). Water stress negatively influenced the Chl in all species. Chl content significantly ($P<0.05$) decreased under water stress condition compared to the well-watered treatment (Fig. 1). As shown in figure 1, Lolium perenne in 25% FC showed the lowest chlorophyll content (12.6 mg/g FW) and chlorophyll content was higher at 100% FC in Poa pratensis (44.24 mg/g FW) compare to other species (Fig. 1).

Electrolyte leakage

EL significantly decreased under water stress treatments compared to 100% FC level. The highest EL was manifested under 25% FC level (Table 2). The

Table 1 - Analysis of variance of CHL (chlorophyll content), EL (Electrolyte leakage), RWC (Relative water content), Pr (Proline content), MDA (Malondialdehyde), CAT (catalase), POD (peroxidase), and SOD (superoxide dismutase) activities of turfgrasses species under drought stress

Effect		Mean							
	DF	CHL	EL	RWC	Pr	MDA	CAT	POD	SOD
Block	2	15.00 *	54.31 **	3.93 NS	0.0001 NS	0.01 NS	0.003 NS	0.01 NS	0.34 NS
Species	3	834.1 **	16.08 **	354.15 **	0.003 **	2.09 **	0.07 **	34.89 **	21.18 **
Drought	3	353.86 **	29.72 **	4618.81 **	0.115 **	6.70 **	0.16 **	60.59 **	59.101 **
Drought × species	9	29.38 **	2.61 NS	22.33 **	0.0007 *	0.38 **	0.01 **	4.70 **	8.76 **
Error	30	2.84	1.44	889.86	0.0003	0.02	0.002	0.02	0.86
Coefficient of variation		7.42	1.28	2.33	10.89	9.4	15.45	3.2	9.94

NS= not significant, *P≤0.05, **P≤0.01.

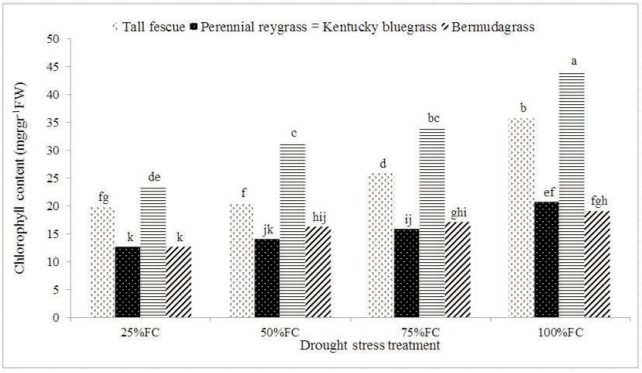

Fig. 1 - Interaction effects of water stress and species on chlorophyll content. Vertical bars (mean ± 1.37) not connected with the same letter represent significant difference between treatments according to LSD test (P<0.05).

EL reached the peak (95.4%) in *P. pratensis*, and the lowest EL (89.3%) was recorded in the *Festuca arundinacea* (Table 2). Interaction of drought stress and species showed no significant effect on electrolyte leakage (Table 1).

Relative water content

The results showed that water stress, species, and their interaction had the significant effect on RWC (P ≤0.01) (Table 1). As shown in figure 2, all species showed high value of RWC under 100% FC. Percentage reduction of RWC under 25% FC, were 43.2, 43.9, 51.1 and 59.6 for tall fescue, bermudagrass, perennial ryegrass and kentucky bluegrass respectively, as compared with well-watered control plants (Fig. 2).

Proline content

Water stress and species both showed significant effect on proline content (P≤0.01) and their interaction effects were significant at 5% level (Table 1). As shown in figure 3, proline concentrations in four species were all increased under drought stress which indicates osmotic adjustment in turfgrasses. *F. arundinacea* at 25% FC level showed the highest proline content (with 0.3 µmol/g FW) whereas lower proline content was shown for well-watered *Lolium perenne*,

Table 2 - Interaction effects of water stress and species and mean comparison on electrolyte leakage

Species	Drought stress treatment	Electrolyte leakage	Mean
Festuca arundinacea	100% FC	89.31 h	93.08 b
	75% FC	92.95 defg	
	50% FC	94.41 bcde	
	25% FC	95.64 ab	
Lolium perenne	100% FC	92.10 fg	93.33 b
	75% FC	92.59 efg	
	50% FC	93.74 bcdef	
	25% FC	94.89 bcd	
Poa pratensis	100% FC	94.66 bcd	95.40 a
	75% FC	94.17 bcde	
	50% FC	95.42 abc	
	25% FC	97.34 a	
Cynodon dactylon	100% FC	91.48 g	92.92 b
	75% FC	92.40 efg	
	50% FC	93.53 cdef	
	25% FC	94.26 bcde	

The values represent the mean ± standard error of three replicates. Different letters are showing considerable differents at P≤0.05.

Poa pratensis and *Cynodon dactylon* (Fig. 3).

Malondialdehyde

According to the results, the MDA content were significantly affected by water stress level, grass species, and their interaction effects (P≤0.01) (Table 1). As the water stress increased, a clear increase in the MDA content has been seen in all species (Fig. 4). As shown in the figure 4, the highest (3.6 µmol g^{-1} FW) and lowest (0.44 µmol g^{-1}FW) amounts of MDA were obtained in *P. pratensis* grown under severe water stress (25% FC) and *F. arundinacea* under control treatment, respectively (Fig. 4).

Enzyme

The results showed that water stress, species, and interaction effects had significant effect on catalase, peroxidase and superoxide dismutase (P≤0.01) (Table 1). As shown in the figure 5, the maximum CAT activity belonged to *F. arundinacea* at 25% FC whereas the minimum CAT activity belonged to *C. dactylon* at

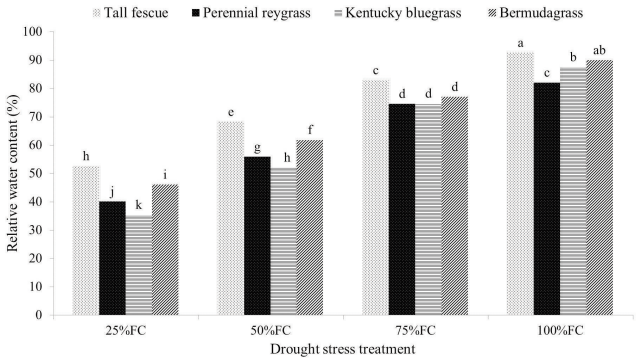

Fig. 2 - Interaction effects of water stress and species on relative water content (RWC). Vertical bars (mean±1.96) not connected with the same letter represent the significant difference between treatments according to LSD test (P<0.05).

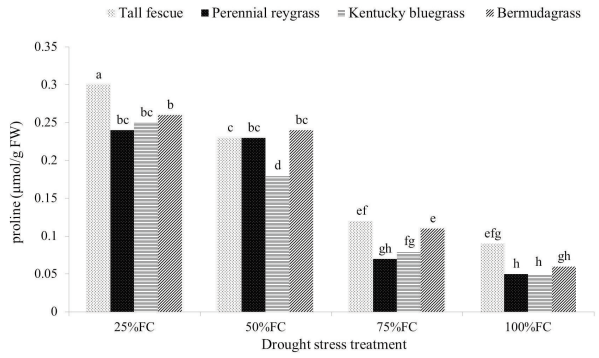

Fig. 3 - Interaction effects of water stress and species on proline. Vertical bars (mean ± 0.01) not connected with the same letter represent significant difference between treatments according to LSD test (P<0.05).

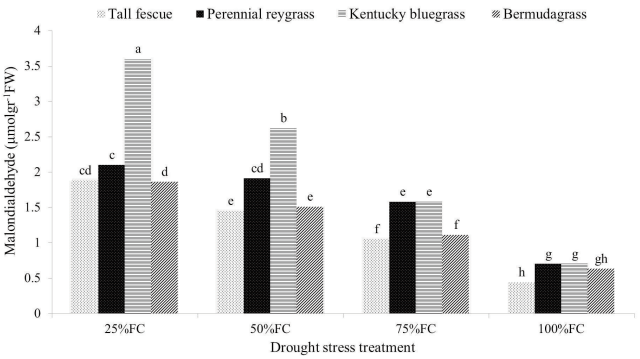

Fig. 4 - Interaction effects of water stress and species on malondialdehyde. Vertical bars (mean ± 0.11) not connected with the same letter represent significant difference between treatments according to LSD test (P<0.05).

100% FC (Fig. 5). Also as shown in figure 6, the highest POD activity was obtained under 50% FC in *F. arundinacea* while the lowest activity was obtained for *Poa pratensis* at 100% FC level., On the whole, POD activities of for species followed a similar pattern under drought stress, which was characterized by a gradual increase until 50% FC level followed by a decline after this treatment (Fig. 6). Moreover, it

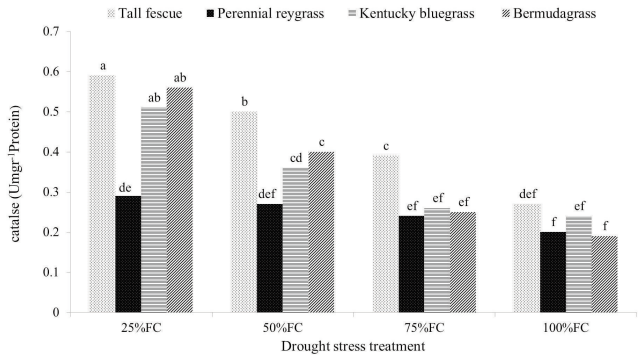

Fig. 5 - Interaction effects of water stress and species on catalase. Vertical bars (mean ± 0.04) not connected with the same letter represent significant difference between treatments according to LSD test (P<0.05).

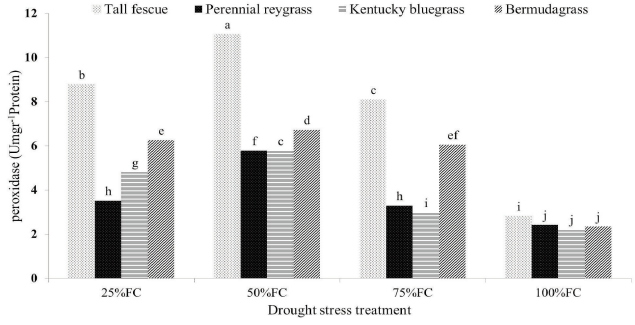

Fig. 6 - Interaction effects of water stress and species on peroxidase. Vertical bars (mean ± 0.12) not connected with the same letter represent significant difference between treatments according to LSD test (P<0.05).

could be obviously seen that SOD activities followed the similar pattern as POD; applying 50% FC in *F. arundinacea* and 100% FC in *L. perenne* scored the highest (15 Umg^{-1}Protein) and the lowest (2.5 Umg^{-1}Protein) respectively (Fig. 7).

Traits correlation

Electrolyte leakage with proline, MDA and CAT showed the significant positive correlation (P≤0.01). Our results showed the significant negative correlation between RWC and EL (P≤0.01). Proline was positively correlated with MDA, CAT, SOD, and POD (P ≤ 0.01). Also, it showed the negative correlation between RWC and chlorophyll at P≤0.01 (Table 3). Our results showed a positive correlation between MDA and CAT (P≤0.01) (Table 3). SOD showed significant positive correlation with CAT and POD (P≤0.01).

4. Discussion and Conclusions

In the present study, increasing in water deficit resulted in an decrease in the plant chl content compared with the plants under well-watered conditions. Chlorophyll content (Chl) in live plants is an important factor in determination of photosynthetic capacity. In the present study, leaf Chl content decreased gradually during the stress periods (Fig. 1). Decreased or unchanged Chl content level during drought stress has been reported in other cultivars, depending on drought duration and severity (Zhang and Kirkham, 1996; Jagtap *et al*., 1998). Under the water deficit stress, chloroplast ultra-structures are the first target to be damaged at the cellular levels since it is the major site of reactive oxygen species (ROS) production (Munné-Bosch and Peñuelas, 2003). An enriched ROS in stressed tissues impairs cellular membrane and organelles which affects the integrity of the cell.

In our study, electrolyte leakage gradually increased with increasing water stress in turfgrass species. Increase of EL with development of drought stress has been reported by many researchers (Guo *et al*., 2006; Liu *et al*., 2008). Electrolyte leakage increase is occurred with the increase of cell permeability (Blum and Ebercon, 1981). Results of a study by Abraham *et al*. (2004) showed at higher electrolyte leakage in *Poa pratensis* and its hybrids under drought stress, while low levels of electrolyte leakage (an indicator of cell membrane stability) were observed in the drought-tolerant plants during drought stress. Fu and Huang (2001) reported that EL and MDA increased simultaneously in five species of kentucky bluegrass which indicated the positive relationship between these traits

In this research, drought stress conditions decresed RWC due to reduced leaf water potential, consistent with results reported by Fu and Huang (2001), Farkhondeh *et al*. (2012). RWC as an indicator of plant water status is one of the most reliable indicators for defining water retention in plants. In this study, RWC was affected significantly by water stress

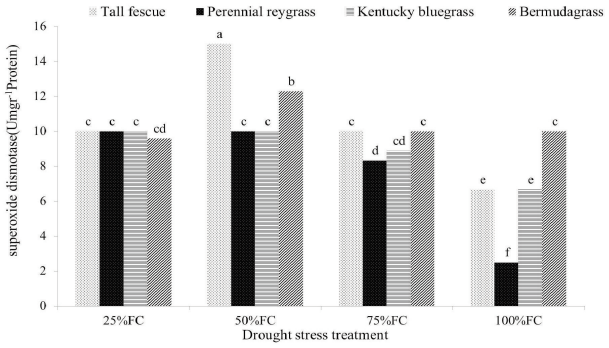

Fig. 7 - Interaction effects of water stress and species on superoxide dismutase. Vertical bars (mean ± 0.75) not connected with the same letter represent significant difference between treatments according to LSD test (P< 0.05).

Table 3 - Pearson correlation coefficients of electrolyte leakage, proline, MDA (Malondialdehyde), SOD (superoxide dismutase), CAT (catalase), chlorophyll, and POD (peroxidase) of turfgrasses species under drought stress

Traits	Electrolyte leakage	Proline	MDA	SOD	RWC	CAT	Chlorophyll	POD
Electrolyte leakage	1							
Proline	0.454 **	1						
MDA	0.59 6**	0.672 **	1					
SOD	0.258 NS	0.575 **	0.354 **	1				
RWC	-0.576**	-0.855 *	-0.890 **	-0.374 **	1			
CAT	0.422 **	0.760 **	0.510 **	0.491 **	-0.587 **	1		
Chlorophyll	-0.029 NS	-0.475 **	-0.242 NS	-0.308 *	0.455 **	-0.205 NS	1	
POD	0.273 NS	0.627 **	0.311 **	0.690 **	0.364 **	0.694 **	-0.274 NS	1

NS= not significant, *P≤0.05, **P≤0.01.

in all species. However, reduction was less pronounced for *F. arundinacea*, which maintained a higher RWC than other species. The amount of RWC in plant with high resistance to drought stress is higher than that of susceptible plants. In other words, plant having higher yields under drought stress should have higher RWC (Liu *et al*., 2002). Under water deficit, the cell membrane is subjected to changes such as penetrability and decrease in sustainability (Blokhina *et al*., 2003). Results of a study by Wang and Huang (2003) showed a decline in the RWC under drought stress, especially in; susceptible cultivars (Wang and Huang, 2003). Bian and Jiang (2009) showed that RWC in *P. pratensis* decreaed during drought stress.

The initial physiological response of plants to drought stress is osmoregulation which decreases water potential and maintains turgor to hold water inside of tissues and absorbing moisture from the environment at the same time, thus finally maintain other physiological activities of the cell (Li *et al*., 2015). The results of the present study clearly showed that proline content increased in all turfgrass species under water deficit compared to the well-watered conditions. Increase of proline under drought stress has been reported by many researchers (Turkan *et al*., 2005; Wang *et al*., 2008). Proline content of *F. arundinacea* under drought stress increased dramatically, which could be a significant factor for maintaining the relative water content. The correlation between proline and antioxidant enzymes had been reported by Morot-Gaudry *et al.* (2001). Since proline can act as a scavenger or reducer of superoxide production, it is normal to find a significant positive correlation seems between proline and antioxidant enzymes. Bian *et al*. (2009) concluded that proline content increased in creeping bentgrass (*Agrostis stolonifera* L.) under drought stress.

Lipid peroxidation has been associated with damages provoked by some environmental stresses (Jaleel *et al*., 2008). The rise in MDA content under different stress conditions showed that drought could induce membrane lipid peroxidation by means of ROS (Moussa and Abdel-Aziz, 2008). In this condition, low concentration of MDA has been associated with drought-tolerant plants (DaCosta and Huang, 2007; Hassan *et al*., 2015). In our experiment, *F. arundinacea*, with low concentration of MDA in different levels of drought stress treatment, showed more tolerance to drought stress. According to other studies (e.g. Sharma and Dubey, 2005; Pan *et al*., 2006; Zlatev *et al*., 2006), drought stress increased MDA concentrations in leaves. Positive correlation between MDA and CAT indicates that the antioxidant enzymes act as a first defensive line to counter oxidative stress in plants. Oxidative stress occurs when the antioxidant defense decreases or the formation of free oxygen radicals increases (Matés *et al*., 1999).

To cope with detrimental effects of oxidative stresses under extremely adverse conditions, plants have developed an antioxidant defense system which includes the antioxidant enzymes SOD, APX, POD, and CAT. The levels of antioxidant enzymes are higher in tolerant cultivars than sensitive ones under various environmental stresses (Wang *et al*., 2009). Accordingly, we observed higher SOD activity in *F. arundinacea* at 50% FC level compared to other species, which suggest that this drought-tolerant grass possess a better reactive oxygen scavenging ability. However, in the 25% FC this trend changed considerably and the amount of SOD decreased. Previous studies have shown that responses of SOD activity to water deficit have varied with drought severity, duration, and species. Zhang and Kirkham (1996) suggested that water stress did not influence SOD activity under moderate stress in sorghum [*Sorghum bicolor* (L.) Moench]. In wheat (*Triticum aestivum* L.), SOD activity increased or remained unchanged in the early phase of drought and then decreased with further water stress (Zhang *et al*., 1995). This reduction in SOD activity could be associated with reduced synthesis or enhanced degradation of the enzyme. SOD converts the toxic O_2^- radicals to H_2O_2 which must be scavenged to O_2 and water by the antioxidant enzyme such as CAT, POD, and APX (Ozkur *et al*., 2009).

Increase in POD activity under various stress conditions has been linked with protection from oxidative damage, lignification, and cross-linking of the cell wall to cope with such adverse conditions (Moussa and Abdel-Aziz, 2008). In our study, drought-induced POD activity in shoot of four species. However, activity of this enzyme in *F. arundinacea* under both control and stress conditions was higher than others species, suggesting a better antioxidant system for removing H_2O_2 by POD. In Kentucky bluegrasss this enzyme activity initially increased and then decreased with development of drought (Fu and Huang, 2001), similar to changes of this enzyme's activity founding *P. pratensis* in the response to drought stress. The activity of POD increased during initial periods of drought stress and decreased as stress intensity increased. The similar trend of POD activity during water stress has been reported in *Poa pratensis*

(Farkhondeh *et al*., 2012).

Catalase is another antioxidant enzyme that scavenges H_2O_2 in cells (Shao *et al*., 2007). High activity of CAT indicated drought tolerance in Chrismas tree (Sharma and Dubey, 2005) and wheat (Simova-Stoilova *et al*., 2010). Fu and Huang (2001) reported that CAT activity was decreased in all studied *Poa pratensis* and *F. arundinacea* cultivars under water stress condition. Accordingly, they concluded that the reduction of CAT activity was supposedly due to inhibition of enzyme synthesis, change in the inhibition of enzyme precursor, or protein degradation under drought stress. In the present research, CAT activity in *F. arundinacea* was higher than other species in different levels of water stress treatment. The high activity of CAT in *F. arundinacea* during drought stresses demonstrated more ability of these species to decomposition of H_2O_2 in stress condition. CAT showed the positive correlation with POD. The correlation between antioxidant enzymes reported in mature leaves of Arabidopsis under drought stress (Jung, 2004). Mercado *et al.* (2004) reported the significant positive correlation between POD activity and RWC content which are in agreement with our results.

According to the results presented here, it can be concluded that among the evaluated species, tall fescue has good tolerance to drought stress. The higher tolerance induced by tall fescue was associated with more efficient osmotic adjustment, which was reflected by the smaller reduction in RWC and cell membrane stability. The identification of these indices is valuable because they can be rapidly assessed and can be used in the early stages of breeding turfgrasses for screening drought tolerant species; however, for efficient selection and better understanding of the mechanisms involved in drought tolerance, biochemical and molecular markers must also be included.

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Effect of salicylic acid on growth, nodulation and N_2-fixation in water stressed chickpeas using ^{15}N and ^{13}C

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Key words: chickpea, N_2-fixation, salicylic acid, water stress, ^{15}N, $\Delta^{13}C$.

Abstract: A pot experiment was conducted to determine the impact of foliar spraying of salicylic acid (SA) on dry matter (DM), carbon isotope discrimination ($\Delta^{13}C$), nitrogen uptake (NY) and N_2-fixation (using ^{15}N) by chickpea plants subjected to three soil moisture regimes (high stress FC1, mild stress FC2 and well-watered FC3). Water stress drastically affected nodulation, DM, NY, N_2-fixation. However, plants responded positively to SA as a means of enhancing growth and overcoming the stress conditions, particularly under FC2 where the measured growth criteria (DM and NY) were relatively similar to those of the FC3. Salicylic acid significantly enhanced amounts of fixed N_2 by 32, 30 and 19% in FC1, FC2 and FC3, respectively. Water stress caused a decrease in $\Delta^{13}C$ values. However, SA increased $\Delta^{13}C$ in water stress treatments, implying that a maximization of DM may occur via an enhancement of CO_2 uptake due to stomatal opening and carboxylation activity. In conclusion, the beneficial effect of SA in enhancing plant performance (growth, N-uptake and N_2-fixation) was affected by soil water content. SA application may be considered an important agricultural practice for the better symbiotic performance in water stressed as well as in well watered chickpeas plants.

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1. Introduction

Chickpea (*Cicer arietinum* L.) is an important pulse legume crop widely grown across the Mediterranean basin where water availability is probably the most limiting factor for crop quality and productivity, comprising economical output and human food supply (Kurdali, 1996; Krishnamurthy *et al*., 2013). Water deficit is a multidimensional stress affecting plants at morpho-physiological, biochemical and molecular levels including inhibition of growth, accumulation of compatible organic solutes, changes in phytohormones endogenous contents, modifications in expression of stress responsive-genes among others (Vasanthaiah and Kambiranda, 2011). In the semi-arid areas of the Mediterranean region, this grain legume is cultivated on a large scale under rain-fed conditions, where water stress occurring during the post-flowering period is considered the major limiting abiotic stress, reducing growth and N_2-fixation (Kurdali, 1996; Kurdali *et al.,* 2002). Thus, increasing N_2-fixation is considered of a

great importance to improve yield and performance of chikpea in the the agricultural systems. Increasing the efficiency of legumes to fix N_2 may be addressed by several approaches including selection of the best plant-microbial combinations and appropriate agricultural practices and managements (Hardarson, 1993; Kurdali *et al.*, 2005). A better plant nutrition (e.g., K, P, Si, etc.) can effectively alleviate the adverse effects of drought (Waraich *et al*., 2011) and hence enhance N_2-fixation (Kurdali and Al-Shammaa, 2010; Kurdali *et al.,* 2013).

Salicylic acid (SA) is one of the endogenous growth regulators that are involved in a range of physiological and metabolic responses in plants (Hayat *et al*., 2010). It coordinates growth and development with plant responses to the environment in a complex signal-transduction network (Aimar *et al.,* 2011). In recent years, SA has been the focus of intensive research due to its function as an endogenous signal mediating local and systemic plant defense responses against pathogens (Rivas-San Vicente and Plasencia, 2011). Moreover, it has, also, been reported that SA is a potential tool in reducing or alleviating the adverse effects of abiotic stress in plants (Khan *et al*., 2015; Amirinejad *et al.,* 2017). Exogenous application of SA has been shown to be beneficial for plants either in optimal or stress environments. Salicylic acid is involved in regulatating various plant metabolic processes and modulating the production of varied osmolytes and secondary metabolites, as well as maintaining plant-nutrient status. Hence, it protects plants under abiotic stress conditions (Khan *et al*., 2015). The effectiveness of SA in inducing stress tolerance depends upon plant species, method of addition, time of application and the concentration (Hayat *et al*., 2010; Gharbi *et al*., 2018). Low concentrations of exogenous SA provides tolerance against damaging effects of stresses on plants, whereas, higher concentrations of SA did not show the same effects (Senaratna *et al*., 2000). Recent evidence highlighted the importance of SA as a regulator of photosynthesis due to its effect on stomatal conductance and the activity of enzymes such as RuBisCO and carbonic anhydrase (Rivas-San Vicente and Plasencia, 2011). Extensive studies have been conducted on the effect of SA application on physiological and biochemical parameters (e.g., gas exchange, stomatal conductance, chlorophyll, photosynthetic rate measurements and enzyme activity estimations) in relation to abiotic stresses (Khan *et al.,* 2003; Khodary, 2004; Hayat *et al.,* 2010; Ghasemzadeh and Jaafar, 2013; Lee *et al*., 2014; Miura and Tada, 2014). During photosynthesis, C3 plants discriminate against the heavy isotope of carbon (^{13}C) leading to a depletion of the plant dry matter in ^{13}C. Carbon isotope discrimination ($\Delta^{13}C$) positively correlates with C_i/C_a (i.e., the ratio of internal leaf CO_2 concentration to ambient CO_2 concentration) and thus provides an integrated measurement of the photosynthesis efficiency in response to environmental conditions prevailing during the plant growth cycle (Farquhar *et al*., 1989). $\Delta^{13}C$ has been intensively studied as a selection criterion for drought tolerance in several C3 species (Farquhar *et al*., 1989). Water stress can alter $\Delta^{13}C$ as a result of its effects on the balance between stomatal conductance and carboxilation (i.e. RuBisCO), (Farquhar *et al.,* 1989). Because of the correlation between $\Delta^{13}C$ and gas exchange values (i.e., C_i/C_a), the isotopic methods represent an alternative to gas exchange measurements. (Farquhar *et al.,* 1989). Carbon isotope discrimination was also used for studying the impact of agricultural practices including fertilizer applications such as nitrogen (Iqbal *et al.,* 2005), potassium (Kurdali and Al-Shammaa, 2010) and silicon (Kurdali *et al*., 2013) on crop performance enhancements under water stress conditions. Accordingly, we hypothesize that $\Delta^{13}C$ can be affected by SA application. Available literature on the relationships between SA and carbon isotope discrimination is very scarce and, to our knowledge, only in one case, the effect of SA on plant water relationships was studied (Barkosky and Einhellig, 1993). On the other hand, the ^{15}N isotope dilution is one amongst several available methods to quantify plant-associated N_2-fixation and provides a valuable mean for evaluating factors affecting N_2-fixation such as drought (Kurdali *et al*., 2002) and salinity (Kurdali and Al-Ain, 2002). Therefore, the objective of this study was to determine the effect of SA on the performance of chickpea plants (growth, nitrogen uptake and N_2-fixation) grown under various soil moisture levels using stable isotopes (i.e., ^{15}N isotopic dilution and ^{13}C isotope discrimination).

2. Materials and Methods

Soil properties and plant materials

The experiment was conducted in pots, each one containing 5 Kg of thoroughly mixed soil collected from Deir AL-Hajar agricultural experiment station, located south east of Damascus, Syria (36° 28’E, 33° 21’ N; altitude 617 m). Some climatic data of the

experimental site during the growing period is shown in Table 1. The main physical and chemical soil properties were: pH 7.80, EC_e 0.31 dSm^{-1}, Soil bulk density was 1.20 g cm^{-3}, organic matter 1.25 per cent, cations (Ca^{++} 2.25, Mg^{++} 0.97, K^+ 0.14 and Na^+ 1.27 mmol L^{-1}), anions (SO_4^{--} 1.27, HCO_3^-1.07 and Cl^- 0.55 mmol L^{-1}), available P (Olsen) 13.40 mg g^{-1}; total N 0.12 per cent, NO_3^- 33.6 mg g^{-1}, NH_4^+28.1 mg g^{-1}. The soil is classified as a clay loam, with an average 57.89% clay, 39.47% silt, and 2.63% sand.

Seeds of chickpea (*Cicer arietinum* L.), and barley as a non-fixing plant were sown. After germination, plants were thinned to two plants per pot. The pots were set outdoors under natural climatic conditions. All pots were protected from rainfall by manually operated shelter equipped with movable sheet of transparent flexible plastic. Since abundant nodules had already been observed on the roots of chickpea plants grown in the area, from which the soil was collected and used for this experiment, the seeds were not inoculated.

Table 1 - Some climatic data during the growing season of the experimental site

Variable	February	March	April	May
Minimum temperature (°C)	4.9	7.7	11.5	14.8
Maximum temperature (°C)	19.5	21.2	29.2	30.5
Relative air humidity (%)	74	67	59	58
ET0 (mm day^{-1})	2.7	3.2	6.3	8.4

Experiment design and treatments

The pots were arranged in a split plot design, with salicylic acid treatments (SA) being the main plots and the irrigation regimes are the sub-main. Salicylic acid C_6H_4(OH).COOH had the following specification: assay min. 99%, melting point 157-162°C, maximum limits of Impurities: chloride 0.01%, sulphate 0.03%, iron 0.002%, heavy metals 0.001%.

Two SA treatments were used: (SA^-, control without SA and SA^+,10^{-5}mol L^{-1}). Within each of the SA treatments, three irrigation regimes, expressed as percent of field capacity(FC), were applied (FC1, high stress 45-50%; FC2, mild stress 55-60% and FC3, well-watering 75-80%). All treatments were replicated four times.

Soil water content in all pots was maintained at around 75% of field capacity from planting up to bud flower initiation (5 weeks after planting). Thereafter, plants were subjected to the above-mentioned soil moisture regimes. Foliar spraying of the plants with salicylic acid (SA) was initiated at the same time of applying water regimes and performed 6 times at 10 days intervals. For non-SA treated chickpeas, plants were sprayed with distilled water set as control (SA^-). Pots were weighed every three days, and water was added to maintain the soil moisture regimes as previously described. The pots were kept weed-free and any drainage was prevented.

^{15}N-Application

An equivalent rate of 25 kg N ha^{-1} of ^{15}N labeled urea (5% ^{15}N atom excess) was applied to chickpea and barley plants to estimate the fractional contribution of nitrogen derived from air (Ndfa, i.e N_2-fixation), soil (Ndfs) and from fertilizer (Ndff), using the isotopic dilution method (Fried and Middelboe 1977). Two equally split applications of N fertilizer (12.5 kg N ha^{-1} for each application) were applied at 2-week intervals starting from complete seedling emergence. This procedure was followed to stabilize the ^{15}N enrichment of the N pool and to minimize N immobilization. Barley was used as a non-fixing reference crop for estimating the N fraction derived from the atmosphere (%Ndfa) in chikpeas, and was similarly treated with the above mentioned treatments (i.e., both of watering regimes and SA application).

Plant sampling and isotopic composition analysis

Plants were harvested twelve weeks after planting. Shoots and nodules were dried at 70°C for 72 h, weighed for dry matter determinations. Shoots were then ground to a fine powder. Total nitrogen was determined by Kjeldahl procedure, and $^{15}N/^{14}N$ isotope ratio was measured using an emission spectrometry (Jasco-150, Japan). The nitrogen fraction derived from the atmosphere (%Ndfa) was calculated using the equation of Fried and Middelboe (1977):

$$\%Ndfa = \left(1 - \frac{atom\ \%\ ^{15}N\ excess_{\ Chikpea}}{atom\ \%\ ^{15}N\ excess_{\ Barley}}\right) \times 100$$

The percent N derived from fertilizer (%Ndff) was calculated using the following equations:

$$\%Ndff = \frac{atom\ \%\ ^{15}N\ excess_{\ plant}}{atom\ \%\ ^{15}N\ excess_{\ fertilizer}} \times 100$$

The percent N derived from soil (%Ndfs) was calculated as follows:

$$\%Ndfs = 100 - (\%Ndfa + \%Ndff)$$

Amounts of nitrogen (mg N $plant^{-1}$) derived from N_2-fixation (Ndfa), soil (Ndfs) and from fertilizer (Ndff) were calculate by multiplying the fractional contribution of each source (%) by nitrogen yield.

The $^{13}C/^{12}C$ ratio ($\delta^{13}C$‰) was determined on subsample of shoots using the continuous-flow isotope

ratio mass spectrometry (Integra-CN, PDZ Europea Scientific Instrument, UK). Carbon isotope discrimination ($\Delta^{13}C‰$) values were estimated using the equation of Farquhar *et al.* (1982):

$$\Delta^{13}C = (\delta\ ^{13}C_{air} - \delta^{13}C_{sample})/(1 - \delta^{13}C_{sample}/1000)$$

where $\delta^{13}C_{air}$ is the $\delta^{13}C$ value in air (-8‰) and $\delta^{13}C_{sample}$ is the measured value in the plant.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) test, and means were compared using the Least Significant Difference (Fisher's PLSD) test at the 0.05 level of probability ($P<0.05$). Moreover, correlation coefficients (r) between $\Delta^{13}C$ and the studied parameters (e.g., DM, NY, per cents & amounts of Ndfa, Ndff and Ndfs) were estimated.

3. Results

Effect of salicylic acid on dry matter and nitrogen yield

Dry matter yield (DM) of chikpea plants was significantly affected by water stress (Table 2). The lowest dry matter value (5.57 g pot^{-1}) was observed in non-SA treated plants grown under the highest water stress level (FC1). Increasing soil moisture from FC1 to FC2, from FC1 to FC3, and from FC2 toFC3 resulted in significant increases in DM by 40%, 64%, and 17%, respectively. The foliar spray of SA had produced appreciable results by increasing dry matter yield under stress conditions and the effect was more pronounced in plants subjected to a mild water stress. At each irrigation regime, the SA increased DM by 13%, 17%, and 6% in FC1, FC2, and FC3, respectively. It is worth mentioning, also, that DM in SA-treated plants grown under moderate water stress (FC2, 9.06 g pot^{-1}) was relatively similar to that of well-watered plants without SA treatment (FC3, 9.12 g pot^{-1}). However, DM of well-plants (FC3) was not significantly enhanced by SA as compared to non-treated SA plants.

The pattern of total nitrogen yield (NY) was relatively similar to that of dry matter yield (Table 2). Water stress significantly reduced the NY and the highest reduction was recorded under the highest stress level (FC1). In non-SA treated plants, the lowest value of NY (129 mg N pot^{-1}) was in FC1. Increasing soil moisture from FC1to FC2, from FC 1to FC3 and from FC2 to FC3 resulted in significant increases in NY by 38%, 58%, and 15%, respectively. The exogenous supply of SA significantly enhanced nitrogen accumulation by 14%, 20%, and 10% in FC1, FC2, and FC3, respectively. The highest amount of TN was observed in well-watered plants (FC3) treated with SA application (223.4 mg N pot^{-1}) representing a 73% increment over the control (i.e., non-treated SA plants in FC1). It is evident from DM and NY data that water stress adversely affected the production of dry matter and nitrogen yield of chikpeas, and exogenous application of SA at 5 mM L^{-1} was successful in alleviating the adverse effect of water stress.

Effect of salicylic acid on nodule dry matter

Effects of SA on nodule dry weight of chickpea plants grown under different water stress conditions are given in figure 1. The lowest nodule dry weight was noted in the highest water stressed plants (FC1). For the non-SA treated plants, increasing soil moisture from FC1 to FC2, from FC1 to FC3, and from FC2 to FC3, resulted in increases in nodule dry weight by 31%, 62%, and 24%, respectively. The exogenous supply of SA significantly enhanced nitrogen accumu-

Table 2 - Total dry matter yield (g pot^{-1}) and nitrogen yield (mg N pot^{-1}) of chickpea plants grown under different water regimes as affected by salicylic acid (SA)

Salicylic acid	Irrigation treatments			
	FC1	FC2	FC3	$LSD_{0.05}$
DM (g pot^{-1})				
SA^-	5.57±0.12 B c	7.77±0.41 B b	9.12±0.31 A a	0.48
SA^+	6.29±0.41 A b	9.06±0.47 A a	9.68±0.40 A a	0.68
$LSD_{0.05}$	0.52	0.76	0.62	
N-uptake (mg N pot^{-1})				
SA^-	129.06±4.68 B c	177.45±2.95 B b	203.33±10.53 B a	10.98
SA^+	146.78±5.11 A c	213.09±4.51 A b	223.41±4.17 A a	7.37
$LSD_{0.05}$	8.47	6.59	13.85	

Means ±SD within a column (capital letter) and within a row (small letter) followed by the same letter are not significantly different ($P<0.05$). FC: water regimes, expressed as % of field capacity (FC1, high stress 45-50%; FC2, mild stress 55–60% and FC3, well-watering 75–80%). SA-: control without SA, SA+ 10-5 Mol l^{-1}.

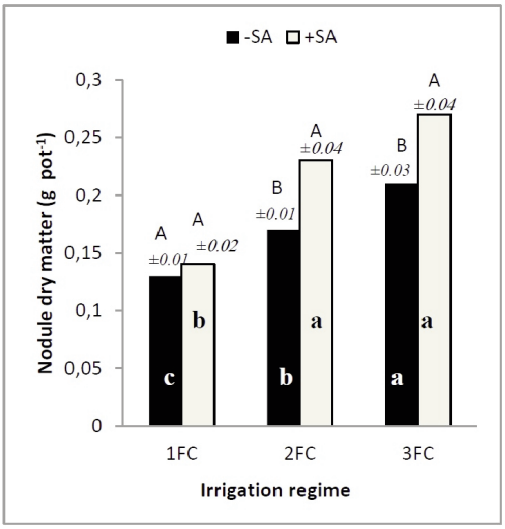

Fig. 1 - Nodule dry matter (g pot^{-1}) of chickpea plants grown under different water regimes as affected by salicylic acid (SA). Columns followed by the same letter are not significantly different (P<0.05); Capital letters (effect of SA in each irrigation regime); Small letters within a row (comparison among irrigation regimes either for SA+ or SA-).

lation by 41% in FC2 and 29% in FC3 watering treatments. However, the enhancement of nodule dry weight in FC1 by SA was not significant (8%). For the SA treated plants, increasing soil moisture from FC1 to FC2, from FC1 to FC3, and from FC2 to FC3, resulted in increases in nodules dry weight by 71%, 93%, and 13%, respectively. These results illustrate the importance of foliar spray of SA in enhancing nodules dry weight of chickpea grown under the different water regimes.

Effect of salicylic acid on nitrogen uptake from the available sources

Nitrogen derived from fertilizer (Ndff), soil (Ndfs) and atmosphere (Ndfa, i.e., N_2-fixation) in chickpeas grown under various watering regimes as affected by SA application are given in Table 3. Regardless of SA application, the proportions of Ndff and Ndfs (%) in chickpea plants significantly decreased as soil field capacity increased. However, the opposite was true regarding the %Ndfa which showed a higher value under optimal irrigation conditions (FC3) compared to water stressed treatments (FC1 and FC2). For the non-SA treated plants, the observed values of %Ndfa were 33, 40, and 43.8% in FC1, FC2, and FC3, respectively. Chickpea plants significantly enhanced their nitrogen fixation (i.e., 39, 43.3 and 47.3% in FC1, FC2, and FC3, respectively) in response to SA application

Table 3 - Proportions (%) and amounts (mg N pot^{-1}) of nitrogen derived from fertilizer (Ndff), soil (Ndfs) and atmosphere, i.e. N_2-fixation (Ndfa) in chickpea plants grown under different water regimes as affected by salicylic acid (SA)

Salicylic acid	Irrigation treatments			$LSD_{0.05}$
	FC1	FC2	FC3	
%Ndff				
SA-	12.62±0.22 A a	11.40±0.18 A b	10.69±0.27 A c	*0.36*
SA+	11.59±0.08 B a	10.77±0.21 B b	10.02±0.19 B c	*0.27*
LSD $_{0.05}$	*0.29*	*0.34*	*0.40*	
Ndff (mg N pot^{-1})				
SA-	16.30±0.74 A b	20.22±0.24 B a	21.73±1.45 A a	*1.52*
SA+	17.01±0.67 A b	22.96±0.84 A a	22.38±0.82 A a	*1.25*
LSD $_{0.05}$	*1.22*	*1.07*	*2.04*	
%Ndfs				
SA-	53.83±0.94 A a	48.60±0.77 A b	45.55±1.14 A c	*1.54*
SA+	49.42±0.36 B a	45.94±0.90 B b	42.71±0.82 B c	*1.17*
LSD 0.05	*1.24*	*1.45*	*1.72*	
Ndfs (mg N pot^{-1})				
SA-	69.48±3.1 A b	86.22±1.0 B a	92.66±6.2 A a	*6.49*
SA+	72.54±2.9 A b	97.91±3.6 A a	95.44±3.5 A a	*5.31*
LSD 0.05	*5.18*	*4.56*	*8.71*	
%Ndfa				
SA-	33.55±1.2 B c	40.01±0.8 B b	43.76±1.4 B a	*1.90*
SA+	39.00±0.5 A c	43.29±1.1 A b	47.28±1.0 A a	*1.45*
LSD $_{0.05}$	*1.53*	*1.79*	*2.13*	
Ndfa (mg N pot^{-1})				
SA-	43.29±1.8 B c	71.01±2.6 B b	88.94±4.2 B a	*4.92*
SA+	57.22±1.7 A c	92.23±1.9 A b	105.6±1.2 A a	*2.58*
LSD $_{0.05}$	*3.06*	*3.97*	*5.38*	

Means ±SD within a column (capital letter) and within a row (small letter) followed by the same letter are not significantly different (P<0.05). FC: water regimes, expressed as % of field capacity (FC1, high stress 45-50%; FC2, mild stress 55-60% and FC3, well-watering 75–80%). SA-: control without SA, SA+ 10-5 Mol l^{-1}.

in all watering regimes. Inversely, both %Ndff and %Ndfs values were decreased by SA treatments. However, amounts of Ndff and Ndfs (mg) increased due to the foliar application of SA in all watering regimes. Likewise, the exogenous supply of SA significantly enhanced the amounts of fixed N_2 by 32%, 30%, and 19% in FC1, FC2, and FC3, respectively. Regardless of SA application, it can be noticed that the amount of fixed N_2 in the well-watered plants (FC3) was almost doubled as compared to those subjected to high stress (FC1). It is, also, worth mentioning that amount of Ndfa in SA-treated plants grown under moderate water stress (FC2) was close or even higher than that of well-watered (FC3) chickpea without SA. These results may illustrate the importance of SA in saving irrigation water and alleviating water stress influences to ensure appropriate yield and N_2-fixation.

Effect of salicylic acid on carbon isotope discrimination

Effect of SA on carbon isotope discrimination (‰$\Delta^{13}C$) in shoots of chickpea plants subjected to different water stress conditions is given in figure 2. Water stress caused a considerable decrease in $\Delta^{13}C$ compared to well irrigated conditions (Fig. 2). For the non-SA treated plants, the mean $\Delta^{13}C$ value in well-watered chickpea (FC3) was 21.29‰, while it significantly decreased to 20.41‰ and 19.88‰ in mild (FC2) and high water stressed (FC1) plants, respectively. The exogenous application of SA increased $\Delta^{13}C$ particularly in high and mild water stressed plants. The lowest $\Delta^{13}C$ value was obtained in FC1 (20.43‰) and increased to 21.34‰ in FC2 and 21.50‰ in FC3, with no significant difference being obtained between the latter two values. On the other hand, correlations between $\Delta^{13}C$ and the studied parameters showed positive relationships with DM (0.953**), NY (0.949**), nodule dry weight (0.92*), %Ndfa (0.948**), and amounts of Ndfa, Ndfs and Ndff (0.957**, 0.91* and 0.91*, respectively). However, negative relationships between $\Delta^{13}C$ and %Ndff and %Ndfs (-0.948**) were observed.

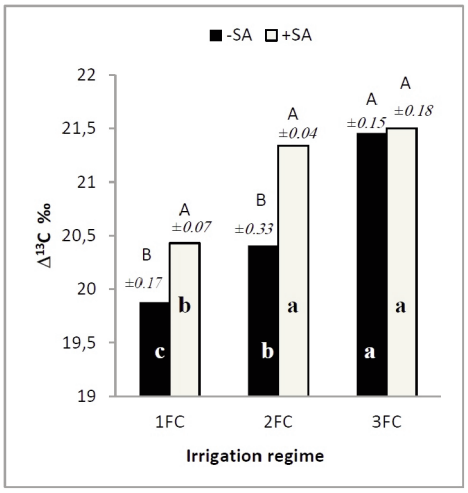

Fig. 2 - Carbon isotope discrimination (Δ13C) in chickpea plants grown under different water regimes as affected by salicylic acid (SA). Columns followed by the same letter are not significantly different (P<0.05); Capital letters (effect of SA in each irrigation regime); Small letters within a row (comparison among irrigation regimes either for SA+ or SA-).

4. Discussion and Conclusions

The results of this study showed that water stress occurring during the flowering period of chickpea plants considerably affected their growth and N_2-fixation. Total dry weight and nitrogen yield decreased significantly in plants as field capacity decreased. Salicylic acid-treated plants exhibited an increase in tolerance to water stress, particularly under mild-stress conditions where the measured growth criteria (i.e., dry matter and nitrogen yield) were relatively similar to those of the non-stressed plants regardless of whether the plants treated with or without SA applications. Gutierrez-Coronado *et al.* (1998) reported a similar increase in the growth of soybean plants in response to salicylic acid treatment. Afshari *et al*. (2013), also, indicated that SA increased the growth and physiological attributes of cowpea under water stress. Moreover, foliar application of SA in drought stressed chickpeas significantly enhanced plant biomass through increasing proline content of leaves (Farjam *et al.,* 2014). Correspondingly, the beneficial role of SA to adverse effects of other abiotic stresses (e.g., heavy metals, salinity and cold..) has been examined in various legumes such as chickpea (Hayat *et al.,* 2014), medic (Palma *et al.,* 2013), lentil (Misra and Saxena, 2009), and common bean (Torquato de Agostini *et al*., 2013).

Water stress is known to decrease nodulation by affecting the establishment of the symbiosis between the host and rhizobia, thereby decreasing the number and the mass of nodules and reducing N_2-fixation (Kurdali *et al.,* 2002). The undertaken study showed that nodulation and N_2-fixation were reduced as soil water content decreased. Such reductions could be attributed to the limitation of photosynthesis and the decrease of photosynthates supply to nodules (Kurdali, 1996). Other physiological factors such as O_2 availability in nodules could be involved with the decline of N_2- fixation. Guérin *et al*. (1991) suggested

that O_2 supply to bacteroids, in water stressed nodules, is restricted either by a limitation of diffusion or by a degradation of leghaemoglobin, and therefore hinders respiration and ATP production, with the final result of reduction of N_2-fixation (Patterson and Hudak, 1996). However, application of SA is found to be beneficial for nodulation and N_2-fixation in plants either grown under optimal or water stress conditions. Several factors are well known to be involved in nodulation and N_2-fixation of grain legumes including the occurrence of effective rhizobium strains, the availability of photosynthesis, nitrogenas activity and its protection against O_2 via increasing of leghemoglobin contents, and the effectiveness of enzymes mediating the export of fixed nitrogen from nodules to plant (e,g., GS, GOGAT, and GDH).

The occurrence of N_2-fixation under various soil water regimes could be explained by the presence of effective indigenous rhizobia strains. On the other hand, it is well known that the high dry matter yield of a given plant species may imply a higher photosynthetic rate (Kurdali and Al-Shammaa, 2010). In a study carried out on soybean, foliar application of salicylic acid enhanced the water use efficiency (WUE) and photosynthetic rate (Kumar *et al*., 2000). Also, Hayat *et al.* (2010, 2012) reported that the exogenous application of SA to chickpea increased the net photosynthetic rate, favoring the production of photosynthates and the nodules obtained a significant proportion of the carbohydrates. The development of healthy nodules in SA-treated plants, as expressed in terms of an increase in nodule dry matter (Fig. 1), is expected to be accompanied by leghemoglobin content increment and consequently increased nitrogen fixation via stimulation of nitrogenase activity (Hayat *et al*., 2012). Moreover, higher leghemoglobin content in SA-treated plants (Balestrasse *et al*., 2004; Hayat *et al*., 2014) might be a consequence of the decrease generation of ROS. Thereby preventing oxidative damage to plants in general and nodules in particular, for cadmium (Cd) stressed chickpea (Hayat *et al.*, 2014) and soybean plants (Balestrasse *et al*., 2004). In this context, Palma *et al.* (2013) reported that SA alleviated the negative effect of salt stress in the *Medicago sativa* - *Sinorhizobium meliloti* symbiosis through the increased level of nodule biomass and the induction of the nodular antioxidant metabolism under salt stress. Similarly, Misra and Saxena (2009) showed that SA could be used as a potential growth regulator to improve salinity tolerance of lentil plants by enhancing proline metabolizing system.

Hayat *et al*. (2012, 2014) reported that, the exogenous application of SA to chickpea plants enhanced the activities of the enzymes involved in nitrogen fixation and assimilation (e,g., NR, GS, GOGAT, GDH and nitrogenase) regardless of whether the plants are grown in the presence or absence of cadmium. On the light of the aforementioned studies, it can be suggested that the beneficial effect of SA on N_2-fixation in chickpea plants might be resulted from enhancing nodulation, leghemoglobin contents and the activity of enzymes involved in nitrogen fixation and assimilation.

In a review paper, Hayat *et al.* (2010) reported that, during the early stages of nodulation, exogenous SA inhibited the growth of Rhizobia and the production of Nod factors by them and also delayed the nodule formation, particularly in plants producing indeterminate nodules, thereby decreasing the number of nodules per plant (Van-Spronsen *et al*., 2003; Mabood and Smith, 2007). In this study, however, since SA was supplied prior to flowering stage of chickpeas (i.e. indeterminate nodule type), it is most likely that nodule formation was established. Therefore, the further benefits of SA were most probably resulted from nodule growth development (DM) and functioning (N_2-fixation).

Carbon isotope discrimination values ($\Delta^{13}C$) in chickpea's shoots were affected by soil water content and SA applications. Water stress significantly decreased $\Delta^{13}C$ values as field capacity decreased. However, the exogenous application of SA increased $\Delta^{13}C$ particularly in high and mild water stressed plants. It has been reported that $\Delta^{13}C$ can reflect the integrated response of physiological processes to environment. Water stress can alter $\Delta^{13}C$ as a result of effects on the balance between stomatal conductance and carboxilation (Farquhar *et al.*, 1989). The lower $\Delta^{13}C$ value in the stressed plants compared to the non-stressed plants implies that C_i/C_a ratios were lower under stress. A lower C_i/C_a ratio could result either from stomatal closure induced by stress or from higher rates of photosynthetic capacity or a combination of both (Condon *et al.*, 2002; Kurdali *et al.*, 2013). Because of the lower dry matter yield in the water stressed plants (FC1-SA^-) compared with FC2-SA^- or with FC3-SA^-, it was unlikely that a higher photosynthetic capacity occurred in FC1. Therefore, the lower $\Delta^{13}C$ value in the high and mild water stressed chickpeas compared to well-watered plants (i.e. SA^-)resulted mainly from stomatal closure induced by stress. The principal components of photosynthesis that influence discrimination are diffu-

sion of CO_2 through stomata and the carboxylation process mediated by Rubisco (O'Leary, 1988). In SA treated plants, the higher ‰$\Delta^{13}C$ value in high and mild water stressed plants comparing with non-SA treated plants were associated with higher dry matter yield (i.e. higher photosynthetic activity). Therefore, the higher $\Delta^{13}C$ following SA applications imply that a maximization of yield may occur via a maximization of CO_2 uptake activity due to stomatal opening (Condon *et al.*, 1987). Janda *et al.* (2014) concluded that the effect of SA on the photosynthetic machinery is indirect, originating from its influence on stomatal conductivity. Moreover, it has been reported that the increases in photosynthetic rates of soybean (Khan *et al.*, 2003) and ginger (Ghasemzadeh and Jaafar, 2013) plants following SA applications were the result of increased CO_2 uptake activity at the chloroplast level (i.e. Rubisco activity), rather than simple increase in stomatal opening, i.e., reduced the resistance to entry CO_2 in the leaves. In stressed plants, Lee *et al.* (2014) reported that the content of rubisco was increased by SA in tobacco plants treated with NaCl. Likewise, Khodary (2004) concluded that SA treatment of salt stressed maize could stimulate their salt tolerance via accelerating their photosynthesis performance (i.e. Rubisco activity) and carbohydrate metabolism. Recently, Gharbi *et al*. (2018) reported that combination of applied SA as a priming agent or concomitantly with NaCl were required to maintain a good water use efficiency in salt-treated tomato plants using carbon isotope discrimination. According to Farquhar *et al.* (1982), a higher $\Delta^{13}C$ values is caused by a higher C_i/C_a ratio due to higher stomatal conductance which leads to higher photosynthetic rate and hence higher biomass, (i.e, positive correlation between $\Delta^{13}C$ and DM, r=0.95**). Accordingly, it can be suggested that the beneficial effect of SA in enhancing dry matter yield of high and mild water stressed chickpeas could be resulted from higher CO_2 uptake activity by Rubisco (i.e., photosynthetic activity) in addition to higher CO_2 uptake at stomatal level (i.e., higher C_i). On the other hand, in FC3, no significant effects of SA were obtained neither on $\Delta^{13}C$ nor on dry matter yield, indicating that the field soil capacity (75-80%) is an optimal watering regime to ensure a good biomass production. Consequently, it can be concluded that SA application can alter $\Delta^{13}C$in water stressed plants (e.g., FC1 and FC2) as a result of effects on the balance between stomatal conductance and carboxylation.

In addition to the positive relationship between $\Delta^{13}C$ and dry matter yield of chickpeas, higher $\Delta^{13}C$ values were also associated with enhancements of %Ndfa (0.948**), amount of N_2-fixed (0.957**) and nodule dry matter yield (0.92*). Hence, it can be suggested that carbon isotope discrimination could be used as an indicator for nodulation and N_2-fixation efficiency. This observation is in harmony with that of Knight *et al.* (1993) who reported a positive correlation between $\Delta^{13}C$ and the amount of N_2-fixed in lentil inoculated with different strains of rhizobia, and with results of Kurdali and Al-Shammaa (2010) in potassium fed lentil grown under water stress conditions.

Salicylic acid can be involved in the regulation of uptake of several plant-beneficial elements (Khan *et al.*, 2015). Exogenously supplied SA can improve plant growth under stresses by stimulating the accumulation of mineral elements including nitrogen (Gunes *et al.*, 2005; Yildirim *et al.*, 2008). In this study, exogenously applied SA to mild water-stressed chickpea resulted in increments of soil nitrogen uptake (Ndfs) as well as amount of N derived from fertilizer (Ndff) and its use efficiency (%NUE). Such increments may support the idea that low SA concentrations (10^{-5} M) can induce nitrite reductase synthesis, which plays a key role in nitrogen metabolism, by mobilizing intracellular NO^{3-} and can provide protection to nitrite reductase degradation (Ghasemzadeh and Jaafar, 2013). Consequently, our finding indicates that the application of SA is beneficial to get improvement in nitrogen uptake and metabolism in plants grown under stressed conditions. Such improvements enhance the protein/enzyme/growth hormone synthesis, developing the metabolic activity and resulting in increasingthe growth and plant productivity (Akhtar *et al.,* 2013). On the other hand, the positive correlations between $\Delta^{13}C$ and total N uptake, amounts of Ndfs or Ndff may illustrate a relationship between photosynthesis and nitrogen metabolism. In addition, the negative relationship between $\Delta^{13}C$ with %Ndfs or %Ndff (0.91*) is also reported by Iqbal *et al.* (2005) in wheat plants grown under rain-fed conditions implying that $\Delta^{13}C$ could be used to predict these parameters. Consequently, it can be suggested that carbon isotope discrimination could be used as an indicator for nitrogen uptake from the available sources. A priority for future research should be elucidation of relationships between carbon isotope discrimination and nutrient use efficiency in various plant species and genotypes, and how these vary in response to environmental conditions and agricultural practices.

The ability of SA to increase chickpea growth, nitrogen uptake and N_2- fixation, ameliorating the adverse effect of water stress, may have significant implications in improving the plant performance and overcoming the growth barrier arising from conditions of limited water availability. A simplified scheme representing the beneficial effects of exogenous application of salicylic acid on growth and N_2-fixation as reported in this study and in the literatures (i.e. Hayat *et al.,* 2010, 2012 and 2014) is shown in figure 3. Further field investigations are required to illustrate the role of salicylic acid in terms of growth, yield, and the time course of N_2 fixation of legumes grown under rain-fed conditions in the semi-arid areas.

To the best of our knowledge, the present study is the first report on the relationship between SA application and carbon isotope discrimination ($\Delta^{13}C$) in N_2 fixing plants (e.g., chickpea). Salicylic acid increased $\Delta^{13}C$ values in water stressed plants, implying that a maximization of dry matter yield may occur via an enhancement of CO_2 uptake due to stomatal opening and carboxylation activity. Moreover, $\Delta^{13}C$ could be used as an indicator for nitrogen uptake, nodulation and N_2 fixation.

Although application of SA is found to be beneficial for plants either in optimal or water stress conditions, its main effect in enhancing plant performance (growth and N-uptake from the available sources) was affected by soil water content as follows:

Under high water stress conditions (FC1), the main effect of SA was on plant growth, nodulation

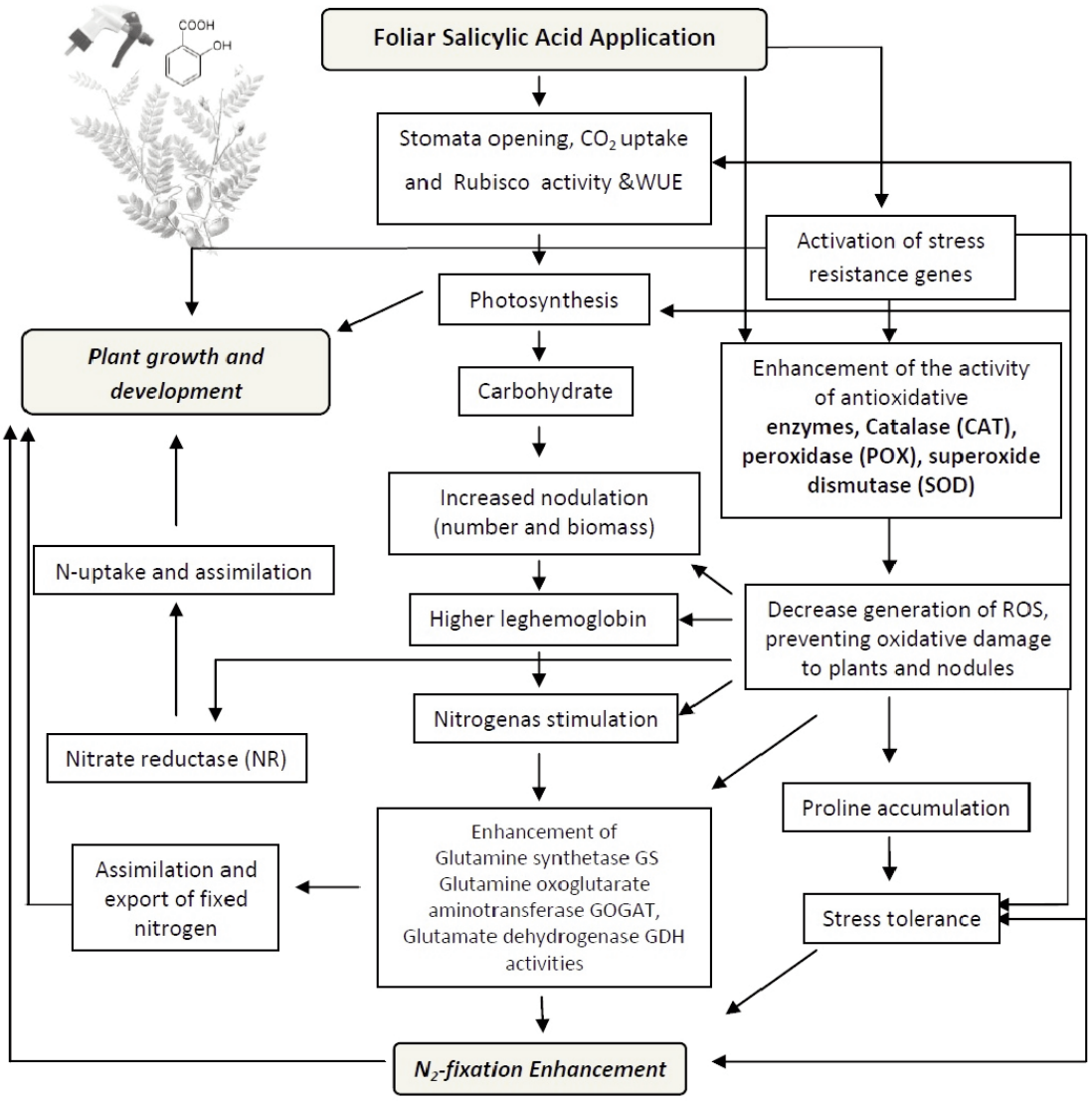

Fig. 3 - Simplified schemes representing the beneficial effects of exogenous application of salicylic acid on growth and N_2-fixation as presented in this study and in the literatures (i.e. Hayat *et al.*, 2010, 2012 and 2014).

and N_2 fixation.

Under mild water-stressed conditions (FC2), SA ameliorated plant growth, nodulation, soil and fertilizer N uptake in addition to fixed N_2.

Under optimal watering conditions (FC3), the beneficial effect of SA was on nodulation and N_2 fixation.

Overall, SA application may be considered as an important agricultural practice for the symbiotic performance (i.e., nodulation and N_2 fixation) in water stressed as well as in well-watered chickpeas plants.

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Barcoding assessment of the *Citrus* species cultivated in eastern Afghanistan

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Keywords: Afghanistan, citrus, *psb*A-*trn*H spacer.

Abstract: The establishment of a modern fruit culture in developing countries requests an accurate evaluation of the preexisting germplasm and its health status. This to prevent the possibility to introduce new germplasm which can be easily prey of the endemic diseases carried by asymptomatic host plants. Therefore, after the identification of cases of citrus plants affected by Tristeza virus, a survey of the germplasm cultivated in the Nangarhar valley and some nearby regions was run. The survey was focused on the identification of the main *Citrus* species widely cultivated using barcoding analysis of conserved sequences located in the plastid DNA. The sequence of *matK* and *rbcl* genes did not show any discriminatory ability while the analysis of the the non-coding *psb*A-*trn*H intergenic spacer (*psbA-trnH)* showed a robust single nucleotide polymorphism (sNP) discriminating *C. aurantium* from *C. sinensis* in all analysed samples. These non-coding regions have no known function; thus, much of the variation may result from the spread of mutations unconstrained by selection. Because nucleotide variation in the psbA-trnH spacer region is high, mutational hot spots maybe useful to detect species-level variations. According to our study, the afghan citrus germplasm belong to the *C. aurantium* species which is commonly used in citrus culture as a rootstock. In Afghanistan it is widely cultivated for fresh consumption, without topworking with selected varieties and this may be the reason why symptoms are often mild and cultivation can be anyhow carried on.

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1. Introduction

In developing countries, farmers are growing landraces or older improved varieties that are not optimized for today's climate or production systems. Therefore, the replacement of the local germplasm traditionally cultivated for long should be carried on by introducing elite varieties in order to complying with the rules of a modern cropping system. However, this change rises concerns about the adaptation of the elite

germplasm to the new conditions which may poses the farmers revenue at risk. The choices to be undertaken are very delicate and consequences may be devastating if not based on preliminary scientific investigations.

Nangarhār (Pashto: رامرګنن ; Persian: رامرګـنن), Laghman and Kunar are three of the 34 provinces of Afghanistan, located in the eastern part of the country. The lowlands in those regions benefit from a semi-tropical climate and have the highest proportion of high cropping intensity irrigated land in the country. The riverine farms, situated along valley bottoms of varying widths, produce a range of crops throughout the year. Semitropical crops such as citrus, sugar canes and henna are produced around Jalalabad. High potential for fruits production occurs in those regions despite the still ongoing conflict. Promoting commercial orchards, establishment through professional nurseries, farmers investment, extension service with technical input from neighboring countries and research are therefore welcome to develop the potential for fruit production and marketing in the Afghan eastern region (Giordani *et al*., 2014). A screening of the health status of the Afghan germplasm of Citrus was undertaken to ensure multiplication of not only the best-selected varieties or ecotypes but also to avoid reproduction and distribution of virus-infected fruit tree. In 2012 a first report showed the occurrence of Citrus Tristeza Virus (CTV) in plants sampled in the National Collection Experimental Farm in Jalalabad (Nangarhar Province) (Rehman *et al*., 2012). A successive investigation showed that CTV was detected in several samples collected in some farms located in the Nangarhar valley, a warning that CTV could spread rapidly if sensitive Citrus cultivars would be introduced in the area. However, Citrus cultivation is still thriving in Nangarhar valley despite the widespread presence of CTV. Different possibilities are therefore to be considered: 1) the local germplasm is made up of low reactive host species which do not heavily show the symptoms of the disease; 2) mild CTV isolates established in the area; 3) an unknown source of resistance to CTV is occurring in the area. Before introducing new rootstocks resistant to such disease, another survey was conducted in order to identify the main species of Citrus. This is to address the choices to be undertaken for the establishment of a modern cropping system according to the market demand and to prevent the diffusion of quarantine diseases. Usually, identification of the cultivated species relies on the use of phenotypic descriptors (UPOV). Phenotypic descriptors are often subjected to the observer's opinion and the environment can deeply affect the behavior of the plant determining errors in the species attribution.

A more reliable system to identify species is based on the genetic analysis and particularly on the DNA barcoding procedure which consists in the comparison of highly conserved sequences located in the ribosomal nuclear (Sun *et al*., 2015) or plastid DNA (Taberlet *et al*., 1991; Penjor *et al*., 2010, Penjor *et al*., 2013). Being highly conserved, such fragments accumulate mutations slowly and it is possible to design universal primers which can be used to distinguish plenty of species. Furthermore, many databases (PubMed, GenBank, OMIM) collect and store millions of sequences, which can be compared by means of specific software (BLAST) with unknown sequences (Zhang *et al*., 2000). When properly queried a DNA database provides, along with the alignment, a similarity index which can be useful for identifying species or even sub-species, depending on the proximity of the taxonomic entities. Therefore, due to the restrictions to accessibility to a conflicting area, a barcoding analysis has been carried out in order to identify the species of Citrus which are commonly grown in the eastern area of Afghanistan in order to better address the choices for modern fruit culture.

2. Materials and Methods

Plant material

Afghan citrus seeds have been collected in three areas where citruses are intensively cultivated (Laghman, Kunar and Nangarhar) in Eastern Afghanistan (Fig. 1). We used seeds because they can

Fig. 1 - Map of Afghanistan showing the provinces where citrus production is located and where the survey was carried on.

be easily transported. Furthermore, most Citrus species are characterized by adventitious embryony and seeds originate individuals which are true clones of the mother plant because rarely zygotic embryo survives. Most sequences for DNA barcoding, being located in the plastid or mitochondrial DNA, are inherited only from the maternal line, therefore germinating seeds produce plants whose organelle genomes are not affected by the pollen donor plant, but are expected to be exactly the same as the seed donor plant. In each area, some plants were selected and batch of seeds have been collected from single fruit. In Table 1 the origin of the seeds is reported. Each batch was labeled and delivered to the Plant Pathology Department of the University of Bologna.

Table 1 - List of the samples coming from Afghanistan

Sample	Province	District	Village	Variety
Af-1 a	Laghman	Mehtarlam	Chardehi	Local
AF-2 a	Laghman	Mehtarlam	Chardehi	Local
AF-5 a	Laghman	Mehtarlam	Chardehi	Local
AF-7 a	Kunar	Asadabad	Landi Tesha	Local
AF-8 a	Kunar	Asadabad	Landi Tesha	Local
AF-9 a	Kunar	Asadabad	Landi Tesha	Local
AF-11 a	Kunar	Asadabad	Landi Tesha	Local
AF-18 a	Nangarhar	Surkhroad	Naghrak	Local
AF-19 a	Nangarhar	Surkhroad	Naghrak	Local
AF-21 a	Nangarhar	Surkhroad	Sabzabad	Local
AF-22 a	Nangarhar	Surkhroad	Sabzabad	Local
AF-limon	Nangarhar		PHDC-Center	Citrus volkaar

The seeds were germinated in pots and leaf samples were collected. For each seed batch, a single seedling has been selected for DNA barcoding analysis. Before starting the present work, Citrus barcoding sequences (ITS 1 and 2,matK, rbcl, psbA-trnH intergenic spacer) were retrieved from GenBank. The dataset was used to compare with the results of sequencing and to assign the analysed samples to a Citrus species. Since the available sequences are few and showed heavy discrepancies, casting doubts about the reliability of the data retrieved online (Bengtsson-Palme *et al*., 2016), we decided to provide robust references by analyzing accessions whose origin was absolutely certain. Therefore we have sampled sour orange along with other *Citrus* species from private and public collections (Vivai Oscar Tintori, Orto Botanico "Giardino dei Semplici" of the University of Florence, Istituto Agronomico per l'Oltremare) in order to be compared with the samples coming from Afghanistan (Table 2).

PCR amplification and primers

Total DNA was extracted from the selected plants following the guidelines of the DNA Invisorb DNA extraction kit producer (Stratec Italy). DNA has been electrophoresed on an agarose gel to validate quality and quantity. Universal primers for PCR amplification, used in the present study have been retrieved in literature (Chen *et al.,* 2010; Luo *et al* 2010; Penjor *et al*., 2013; Mahadani and Ghosh, 2014; Uchoi *et al*., 2016; Wattoo *et al*., 2016; Bailey *et al*., 2018; Zhao *et*

Table 2 - *Citrus* species used as control, coming from public and private collections in Tuscany

Sample no.	Species or cultivar	Province	Origin of the accession
1	*Citrus sinensis* (Washington Navel)	Pescia (Italy)	Oscar Tintori
2	*Citrus sinensis* (ovale calabrese)	Pescia (Italy)	Oscar Tintori
3	*Citrus sinensis* (Tarocco)	Pescia (Italy)	Oscar Tintori
4	*Citrus aurantium*	Lucca (Italy)	Botanical garden
5	*Citrus aurantium*	Firenze (Italy)	IAO
6	*Citrus aurantium* (Foetifera)	Pescia (Italy)	Oscar Tintori
7	*Citrus aurantium* (tipo)	Pescia (Italy)	Oscar Tintori
8	*Citrus aurantium* (dolce del Gargano)	Pescia (Italy)	Oscar Tintori
9	*Citrus aurantiifolia* (Philippines Red lime)	Pescia (Italy)	Oscar Tintori
10	*Citrus aurantiifolia* (Mexico)	Pescia (Italy)	Oscar Tintori
11	*Citrus aurantium*	Firenze (Italy)	Botanical garden
12	*Citrus limon*	Firenze (Italy)	Botanical garden
13	*Citrus mitis*	Firenze (Italy)	Botanical garden
14	*Citrus histrix*	Firenze (Italy)	Botanical garden
15	*Citrus decumana*	Firenze (Italy)	Botanical garden
16	*Citrus grandis*	Firenze (Italy)	Botanical garden
17	*Citrus reticulata*	Firenze (Italy)	Botanical garden
18	*Citrus lumia*	Firenze (Italy)	Botanical garden
19	*Citrus medica*	Firenze (Italy)	Botanical garden

al., 2018) and are designed on the sequence of the internal transcribed sequence 1 (ITS1) and 2 (ITS2) of the nuclear ribosomal DNA, of the maturase K (*matK*), the RuBisCo large chain unit (*rbcl*) and of the predominantly non-coding *psb*A-*trn*H intergenic spacer (Table 3). PCR analyses were performed with a thermal cycler Primus 96 (PeqLab) in a 25 µl volume containing 25 ng total DNA, 1x Taq buffer, 1,5 mM $MgCl_2$, 1 unit GoTaq (Promega) and 200 nM of each primer. PCR conditions were 95°C for 5 min, then 35 cycles at 95°C for 30 seconds followed by 30 seconds at a temperature ranging from 50 to 60°C depending on the chosen primer pair and an extension cycle at 72°C for 50 sec. A final extension at 72°C for 5 minutes was carried on. The amplification products were purified with the Qiaquick PCR purification tubes (Qiagen) and then sequenced. Even though the primers for barcoding are called "universal", the presence of conserved flanking sites complementary to the primers sequence near the variable part and the locus copy number have been shown to account for much of the variability of amplification success. Therefore, the all primers have been tested by PCR, amplifying and sequencing the amplicons using a set of the samples as templates.

To perform a phylogenetic analysis we have used the Mega7 software package (Tamura *et al*., 2013) which compares the sequence calculating a similarity matrix, transforms similarity coefficients into distances and makes a clustering using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. The final output is represented by a dendrogram which clusters the samples.

3. Results

The primers constructed on the sequence of the internal transcribed sequence 1 (ITS1) and 2 (ITS2) resulted in a faint amplification and were therefore discarded, while primers designed on the sequence of the maturase K (*matK*) and RuBisCo large chain unit (*rbcl*) genes were correctly amplified and sequenced. Unfortunately, such sequences did not show any discriminatory ability being completely overlapping for most analysed samples (Mahadani and Ghosh, 2014). On the contrary, the primers of the non-coding *psb*A-*trn*H intergenic spacer showed either good amplification (a fragment of about 525 bp) and bidirectional sequencing output either a good discriminatory capacity. We found a robust single nucleotide polymorphism (sNP) in the *psbA-trnH* spacer (Fig. 2) which is capable to discriminating *C. aurantium* (sour orange) from *C. sinensis* showing this mutation in all analysed samples independently from the origin area.

Despite the limited region of the genome analysed containing, as expected, a low number of mutations, all the samples of *C. aurantium*, independently from their origin, clustered together with a reasonable certainty (Fig. 3) The same occurs for the three *C. sinensis* samples which resulted in a well-

Table 3 - Universal primers sequence for barcoding used in this study

Name	5' → 3' primer sequence	References
matK f	CGTACAGTACTTTTGTGTTTACGAG	Jeanson *et al*., 2011
matK r	ACCCAGTCCATCTGGAAATCTTGGTTC	Jeanson *et al.*, 2011
Rbcl_F1	ATGTCACCACAAACAGAGACTAAAGC	Uchoi *et al.*, 2016
Rbcl_R634	GAAACGGTCCCTCCAACGCAT	Jeanson *et al*., 2011
Rbcl_R724	TCGCATGTCCCTGCAGTAGC	Kress *et al*., 2005
ITS1_5F_746	GGAAGTAAAAGTCGTAACAAGG	Cheng *et al*., 2016
ITS1_4R_746	TCCTCCGCTTATTGATATGC	Cheng *et al.*, 2016
ITS2_S2_F497	ATGCGATACTTGGTGTGAAT	Chen *et al*., 2010
ITS2_S3R_497	GACGCTTCTCCAGACTACAAT	Chen *et al.*, 2010
psbA-trnH_Fw	CGCGCATGGTGGATTCACAATCC	Zhao *et al*., 2018
psbA-trnH_Rev	GTTATGCATGAACGTAATGCTC	Zhao *et al*., 2018
matK1F	ACCGTATCGCACTATGTATC	Penjor *et al*., 2013
matK1R	GAACTAGTCGGATGGAGTAG	Penjor *et al*., 2013
matK2F	ACGGTTCTTTCTCCACGAGT	Penjor *et al*., 2013
matK3F	GGTCCGATTTCTCTGATTCT	Penjor *et al*., 2013
matK2R	AGAATCAGAGAAATCGGACC	Penjor *et al*., 2013
matK3R	ACTCGTGGAGAAAGAACCGT	Penjor *et al*., 2013

Species/Abbrv	Group Name	********** **********
1. Citrus sinensis (Washington Navel)	sinensis	GCGCTAATACTACTAATAAATTACTAAATTTCTAATTTTATTATTAGATTATTAA
2. Citrus sinensis (Ovale Calabrese)	sinensis	GCGCTAATACTACTAATAAATTACTAAATTTCTAATTTTATTATTAGATTATTAA
3. Citrus sinensis (Tarocco)	sinensis	GCGCTAATACTACTAATAAATTACTAAATTTCTAATTTTATTATTAGATTATTAA
4. Citrus limon	limon	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
5. Citrus aurantium (Lucca botanical garden)	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
6. Citrus aurantium (Foetifera)	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
7. Citrus aurantium (dolce del Gargano)	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
8. Citrus aurantium (Tipo)	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
9. Citrus aurantium (Firenze botanical garden	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
10. 9a afghan sample	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
11. 5a afghan sample	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
12. 2a afghan sample	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
13. 21a afghan sample	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
14. 1a afghan sample	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA
15. 11a afghan sample	aurantium	GCGCTAATACTACTAATAAATTACTAAATTTATAATTTTATTATTAGATTATTAA

Fig. 2 - Two haplotypes of *C. aurantium* and *C. sinensis* originated by a single nucleotide polymorphism getting from the sequencing of psbA-trnH intergenic spacer (psbA-trnH).

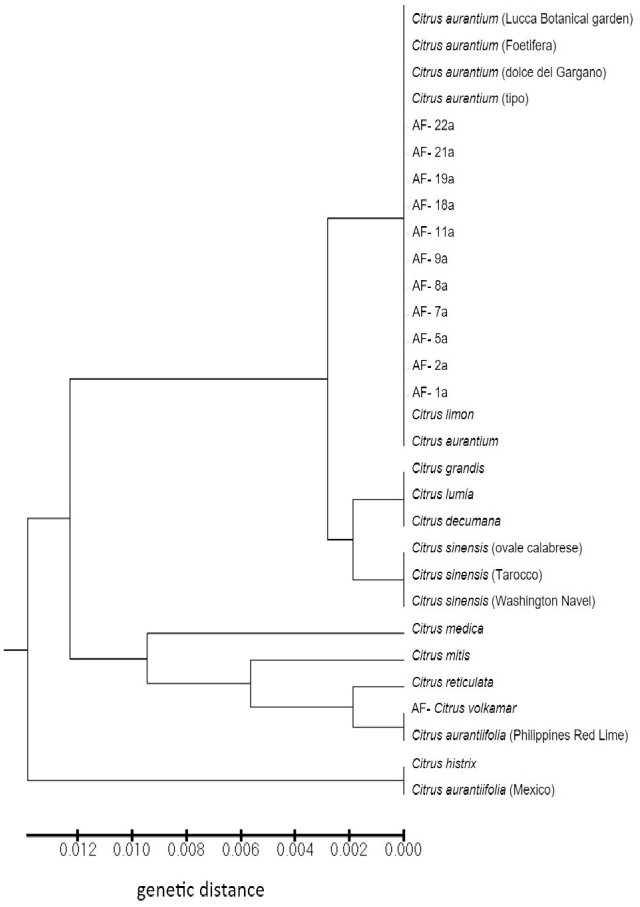

Fig. 3 - UPGMA dendrogram representing the genetic distances among the analysed samples.

separated group while the sample *C. limon* need to be better characterized with additional sequence analysis (Penjor *et al*., 2010, 2013).

4. Discussion and Conclusions

The barcoding analysis of the afghan germplasm and *Citrus* species has been carried out with a set of primers targeting nuclear ribosomal DNA or chloroplast genes (ITS1 and 2, *mat*K, *rbcl* and *psb*A-*trn*H). Apart from ITS 1 and 2, all the primers enabled amplification and sequencing of all the samples. Most of the *Citrus* species were correctly identified even if the comparison of the analysed DNA fragments with the data available in the databases showed several discrepancies. This observation suggested to introduce samples of certain origin in barcoding analysis in order to avoid biases due to errors occurring in the sequences uploaded on the database. According to the results of our analysis, we can state with reasonable certainty that all the afghan *Citrus* samples are sour orange, which is commonly used as a rootstock.

The wide diffusion of sour orange in those regions of Afghanistan is due to the fact that its fruits are consumed fresh (http://anhdo.org.af/wp-content/uploads/2017/06/Citrus-Market-Trend.pdf). The plants are not topworked, as usual, with selected *C. sinensis* varieties. Grafting would have shown heavily the symptoms of Tristeza disease, while this does not occur in ungrafted *C. aurantium* (Gómez-Muñoz *et al*. 2017). In conclusion, stepwise replacement of the orange germplasm in Afghanistan with CTV resistant rootstocks is advisable before grafting with selected orange varieties to prevent CTV spreading. However, the replacement of sour orange, a rootstock characterized by highly desirable agronomic features, with CTV resistant rootstocks should be carried out after devising the whole production chain, starting from the adoption of proper cropping

system (irrigation, fertilization, soil management, etc.) in order to prevent that other endemic diseases can affect the newly introduced germplasm.

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Alleviation of salinity stress by hydrogen peroxide and nitric oxide in tomato plants

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Key words: membrane stability, photosynthetic attributes, relative water content, salt tolerance.

Abstract: Salinity is one of the major abiotic stress factors limiting plant growth and productivity, particularly in arid and semi-arid climates. Hydrogen Peroxide (H_2O_2) and Nitric Oxide (NO) are important signaling molecules in plant response to abiotic stress. In this research the effects of foliar sprays with H_2O_2 (10 mM) and NO (0.1 mM sodium nitroprusside, as a NO donor) on alleviation of Salinity stress (0, 25, 50 and 100 mM NaCl) were investigated in Tomato (*Solanum lycopersicum* L. cv. Falat). Photosynthetic attributes, plant-water relations, membrane stability index and growth parameters were decreased by NaCl treatments. Exogenous H_2O_2 and NO application enhanced salt stress tolerance in tomato plants by improving the photosynthetic efficiency and plant water status as measured by relative water content and membrane stability index. These results were positively reflected by the increase in plant growth under salinity stress conditions. The results of this study described that under the adverse conditions of salinity stress, H_2O_2 and NO could activate the photosynthetic system and improve the physiological attributes in plant growth.

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1. Introduction

Salinity in soil or water is a major problem affecting growth and productivity of many crops, especially under arid and semi-arid conditions. It was estimated that about 20% of the world's cultivated land area and 50% of all irrigated land are salt-affected (Hayat *et al.,* 2013). But, the area of soils with restrictions for vegetable crop production is certainly greater than the area of salinized soils, since a saline soil is generally defined as showing an electrical conductivity (EC) value of the saturation extract (ECe) in the root zone that exceeding 4 dS m^{-1}, while the majority of vegetable crops have a salinity threshold that is 2.5 dS m^{-1} (Machado and Serralheiro, 2017).

Salinity negatively affects plants growth and development through: low osmotic potential of soil solution (water stress), nutritional imbalance, specific ion effect (salt stress) or a combination of these factors (Ashraf, 2004). All of these factors cause morphological, physiological and metabolic modifications in plants, such as a decrease in seed germination, shoot and root length, leaf area, cell membranes stability, inhibition of

different enzymatic activities and photosynthesis attributes (Sairam and Tyagi, 2004; Parida and Das, 2005).

Photosynthesis is one of the physiological processes that is affected by salinity stress (Munns *et al.,* 2006; Chaves *et al.*, 2009). Salinity stress may reduce the photosynthesis rate by decreasing in stomatal factors such as stomatal conductance (Bethke and Drew, 1992; Parida *et al.,* 2004), internal CO_2 partial pressure (Bethke and Drew, 1992; Iyenger and Reddy, 1996) and non-stomatal factors such as inhibition and degradation of photosynthetic pigments (Lee *et al.,* 2004; Chaves *et al.,* 2009), photosynthetic electron transport reactions, quenching ability of excessive energy through chlorophyll fluorescence (Lee *et al.,* 2004), efficiency of Rubisco for carbon fixation (Liu *et al.,* 2011), and photophosphorylation (Stoeva and Kaymakanova, 2008). Adverse effects of salinity on plant growth may also result from impairment of photosynthetic apparatus (Ashraf, 2004).

Hydrogen peroxide and Nitric oxide are bioactive molecule involved in the signaling process within plants (Leshem, 2000; Uchida *et al.,* 2002; Azevedo-Neto *et al.,* 2005; Hung *et al.,* 2005; Li *et al.,* 2011). Researches have shown that hydrogen peroxide and nitroxide at low concentrations, play an important role as signaling molecules (Gechev and Hille, 2005; Quan *et al.,* 2008). Studies have shown that hydrogen peroxide and Nitric oxide are involved in acclamatory signaling triggering tolerance against salt stress (Hayat *et al.*, 2013; Semida, 2016). Azevedo-Neto *et al.* (2005) reported that the pretreatment with H_2O_2 in nutrient solution induces acclimation to salinity stress in maize. Semida (2016) observed that exogenous H_2O_2 application enhanced salt stress tolerance in onion plants by improving the photosynthetic efficiency and plant water status as evaluated by relative water content and membrane stability index. The use of NO increased the resistance of *Pinus eldarica* to salinity and improved its growth characteristics (Zamani *et al.,* 2014). Uchida *et al.* (2002) reported that H_2O_2 and NO are the important signaling molecules in rice for resistance to salinity stress.

Tomato is one of the most important vegetable crop in the world. In Iran, the tomato also holds the number one position among vegetables, with almost 6.4 million metric tons of production (FAO, 2014). The cultivated tomato has been classified as moderately sensitive to salinity. Salinity affects tomato plant growth at various stages including seed germination, root and shoot development and fruit production (Cuartero and Fernandez-Munoz, 1999).

This research was undertaken to assess changes in plant growth, water relations, cell membrane stability and photosynthesis parameters in salt-treated tomato plants and to examine neutralizing effects of NO and H_2O_2 to exposure to salt.

2. Materials and Methods

Plant material

Tomato seeds, cv. Falat were surface-sterilized in 2.5% sodium hypochlorite for 10 min, followed by four washes with distilled water. Seeds were sown in the plastic tray filled with a silica sand in the greenhouse under controlled conditions (photoperiod of 16/8 h day/night, 60-65% humidity and 25-30°C temperature). Seeds were irrigated with tap water daily. Seedlings with 2 true leaves were transplanted to 25×25 cm pots (one plant per pot) maintained under similar conditions as the tray containing developing seedlings and fertilized alternate days with half-strength Hoagland solution (Hoagland and Arnon, 1950) until solution drainage occurred at the bottom of the pot at each fertigation.

Treatment and experimental design

Seven days after transplanting, uniform seedlings of tomato cultivars were sprayed to run off with distilled water, 10 mM H_2O_2 or 0.1 mM SNP in 0.025% Tween 20 (as a surfactant) at 6:30 am and then the sprays were repeated at 7 and 14 days later. The concentrations of H_2O_2 and SNP and the number and timing of sprays were based on results from a preliminary experiment (data not shown). After the last spraying, irrigation was done with half strength Hoagland solution supplemented with 0, 25, 50 and 75 mM of NaCl solution. The experimental procedures were completely randomized in 3 × 5 factorial design, with three foliar spray (sodium nitroprusside [SNP], H_2O_2 and distilled water) and four salt concentrations (0, 25, 50, and 100 mM NaCl in nutrient solution), performed in triplicate. The number of plants were six in each replicate. Plants were sampled at 90 day after seeding. Three samples were analyzed for each replication (9 samples in each treatment). The fully-expanded leaves were used for the determination of all experimental parameters.

Determination of plant growth traits

Ninety-day-old tomato plants were carefully removed from each pot and the leaves, stems and roots of plants were weighed to record their fresh

weights and then placed in an oven at 70°C till the constant weight to record their dry weights.

Determination of relative water content (RWC)

RWC was estimated using 2-cm-diameter fully-expanded leaf discs, excluding midrib according to the method of Hayat *et al*. (2013). The discs were weighted for fresh mass (FM) and immediately floated on double-distilled water in Petri dishes for 24 h, in the dark, to saturate them with water. Water adhering to discs was blotted and the turgid mass (TM) was measured. The dry mass (DM) of discs was recorded after dehydrating them at 70°C until the constant weight. The RWC was then calculated using the formula:

$$RWC = [(FM - DM)/(TM - DM)] \times 100.$$

Determination of proline content

Free proline content was determined according to the method of Bates *et al.* (1973). Samples (0.5 g) were homogenized in 5 ml 3% sulfosalicylic acid and extracts were centrifuged at 8000 x *g* for 15 min. The amount of 1 ml filtrate was mixed with equal volumes of acetic acid and ninhydrin reagent (1.25 g ninhydrin, 30 ml of glacial acetic acid, 20 ml 6 M H_3PO_4) and incubated for 1 h at 100°C. The reaction was stopped by placing the test tubes in ice cold water. The samples were vigorously mixed with 3 ml toluene. After 50 min, the light absorption of the toluene phase was estimated at 520 nm on a UV-VIS spectrophotometer. The proline concentration was determined using a standard curve. Free L- proline content was expressed as µg/g dry weight.

Determination of total soluble sugar content

Total soluble sugar content was determined by phenol-sulfuric acid according to the method of Dubois *et al.* (1956). Dry leaves sample (0.1 g) were extracted with 5 ml of 80% ethanol, by boiling the samples in glass tubes in a 95°C-water bath for 10 min. After extraction, the tubes were centrifuged at 500 x *g* for 5 min, and the supernatants of the extractions were used for sugar analysis. One hundred ml of sample was added to 900 ml of distilled water then the mixture was vortexed. One ml of 5% phenol and 5 ml of H_2SO_4 were added to 1 ml of sample and the mixture was stirred. After cooling under room temperature for 15 min, the absorbance of the sample was recorded at 490 nm.

Determination of membrane stability index (MSI)

The MSI was determined according to methods of Sairam and Srivastava (2002). Leaf disc (0.2 g) were thoroughly washed in double distilled water and thereafter placed in a test tube containing 10 ml of double distilled water in two sets. One set was heated at 40°C in a water bath for 30 min and the electrical conductivity (EC1) of the solution was recorded using an electrical conductivity meter. Another set was boiled at 100°C for 10 min and their electrical conductivity was recorded as above (EC2). The MSI was calculated as:

$$MSI = [1-(EC1/EC2)] \times 100$$

Determination of leaf photosynthetic pigments

Chlorophyll a, b and total chlorophyll were extracted and determined (in mg/ g FW) following the procedure is given by Lichtenthaler and Buschmann (2001). Fresh leaf samples (0.2 g) were homogenized in 50 ml acetone (80%) and then centrifuged at 10,000 × *g* for 10 min. The absorbance of the acetone extract was measured at 663, 645 and 470 nm using a UV-visible spectrometer (Shimadzu, Kyoto, Japan).

Determination of leaf photosynthetic attributes

Photosynthetic attributes (stomatal conductance [gs], internal CO_2 concentration [Ci], transpiration rate [E], and net photosynthetic rate [Pn]) in intact leaves were measured by a infrared gas analyzer (CI-340, Photosynthesis system, CID Bio-Science, USA) between 10:00 and 12:00 h under a clear sky. Photosynthetic Pigments and Attributes were measured on three samples of leaves in each pot and three pot in each replication.

Statistical analysis

The experimental design was a completely randomized factorial, four salinity levels (0, 25, 50 and 100 mM NaCl) and two levels of H_2O_2 and SNP (10 and 0.1 mM respectively). All measurements were carried out in three replicates and data were subjected to one-way analysis of variance using SAS program (SAS 9.1; SAS Institute Inc., Cary, NC). Significant differences between means were determined by Tukey's tests. P values less than 0.05 were considered statistically significant.

3. Results

Growth parameters

Salinity markedly decreased fresh weight and dry weight of root, leaf and shoot (Fig. 1, A-H). However, the H_2O_2 and SNP spraying were able to reduce the

adverse effects of salt stress. Moreover, the fresh weight and dry weight of root, leaf and shoot from H_2O_2 and SNP-sprayed plants were higher than the ones stressed plants (Fig. 1).

Relative water content (RWC)

When salinity was absent, RWC was not significantly altered by H_2O_2 and SNP pretreated plants (Fig. 2, I). Under salinity condition, plants sprayed with SNP or H_2O_2 displayed higher RWC when compared to water sprayed ones. Plant pretreated with H_2O_2 was not significantly affected by salinity of 25 mM NaCl. Salinity did not promote any significant alteration in SNP pretreated plants in 25 and 50 mM NaCl stress. Under 100 mM NaCl stress conditions, the RWC was reduced in all evaluations (Fig. 2, I).

Proline content

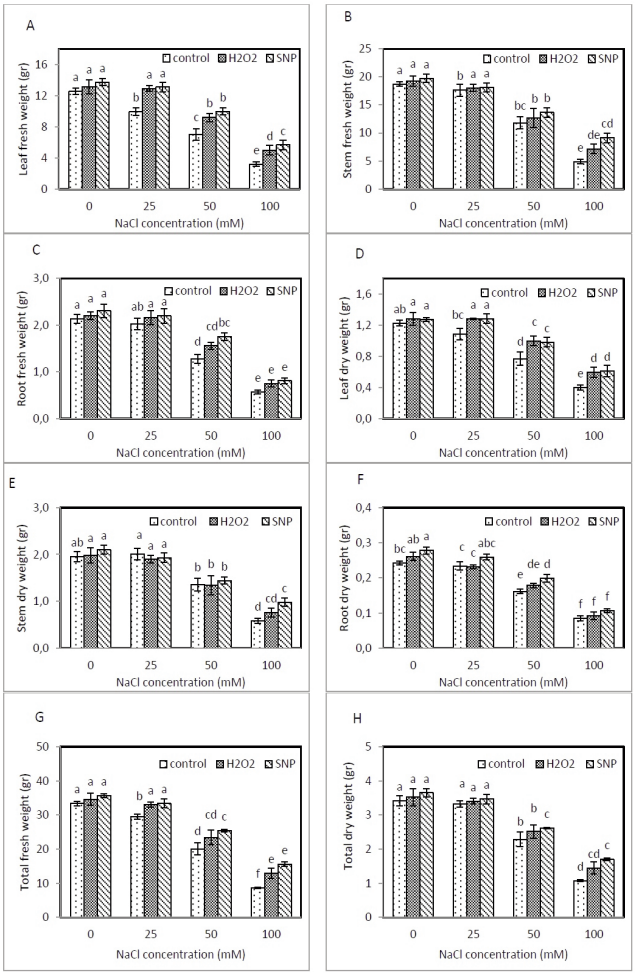

Fig. 1 - Effect of salt treatment and application of exogenous H_2O_2 and SNP on growth parameter of tomato plants. Leaf fresh weight (A), stem fresh weight (B), root fresh weight (C), leaf dry weight (D), stem dry weight (E), root dry-weight (F), total fresh weight (G), and total dry weight (H). Data shown are the mean (±SE) of three independent experiments. Significant differences among treatments were determined by Tukey's Test (P<0.05).

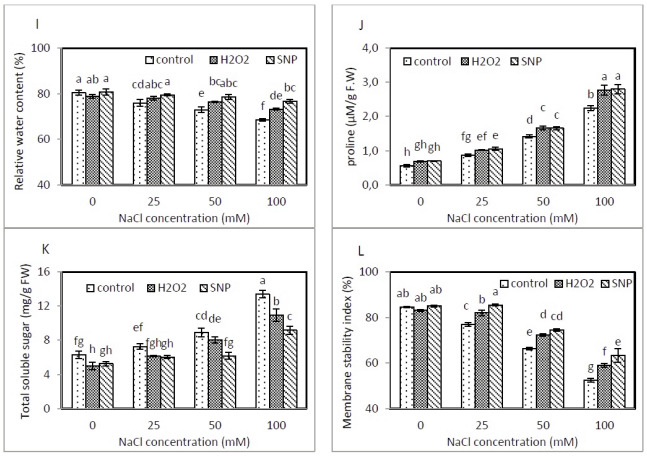

Fig. 2 - Effect of salt treatment and application of exogenous H_2O_2 and SNP on relative water content (I), proline (J), total soluble sugar (K) and membrane stability index (L). Three plants were analyzed for each treatment. Data shown are the mean (±SE) of three independent experiments. Significant differences among treatments were determined by Tukey's Test (P<0.05).

The proline-specific increase in plants exposed to NaCl (Fig. 2, J). Pretreatment to either H_2O_2 or SNP resulted in an increase in proline levels of plants under salinity stress. Interestingly, among unstressed plants, treatment of H_2O_2 and SNP also increased the proline levels (Fig. 2, J).

Total soluble sugar content (TSSC)

Salinity stress significantly increased the TSSC (Fig. 2, K). Pretreatment of H_2O_2 or SNP significantly decreased the TSSC compared to water sprayed plants. The highest amount of TSSC was observed with 100 mM NaCl without H_2O_2 or SNP, while the lowest amount of TSSC was observed with SNP and H_2O_2 application without salinity (Fig. 2, K).

Membrane stability index (MSI)

Under non saline conditions, the MSI were not affected by H_2O_2 and SNP spraying (Fig. 2, L). Although salinity had decreased the leaf MSI, it did not promote any significant alteration in H_2O_2 and SNP sprayed plants in 25 mM NaCl stress compared to non saline conditions. The MSI was significantly decreased by 50 and 100 mM NaCl stress, however, plants treated with H_2O_2 and SNP were less affected by salinity stress compared to water sprayed plants. The SNP-sprayed plants showed values of MSI higher than the stressed plants sprayed with water and H_2O_2 (Fig. 2, L).

Leaf photosynthetic pigments

There were significant decreases in the chlorophyll a, b and total chlorophyll contents in salt-stressed plants. Plants treated with H_2O_2 and SNP

spray had higher values compare to the water-sprayed plants (Fig. 3, M-O). The chlorophyll content (a, and total chlorophyll) in the water-sprayed plants and plants receiving H_2O_2 and SNP were similar in 0 and 25 mM NaCl treatment (Fig. 3, M and O).

Leaf photosynthetic attributes

The H_2O_2 and SNP-sprayed plants showed higher Net photosynthesis (Pn), Stomatal conductance (gs) and intercellular CO_2 concentration (Ci) than the water-sprayed plants under non saline conditions (Fig. 1A-1C). Although the Pn, E, gs and Ci were strongly decreased by salinity stress, plants sprayed with H_2O_2 and SNP were less affected than the water-sprayed ones. The Pn in the plants receiving H_2O_2 and SNP were higher at 25 mM NaCl treatment compared with the water-sprayed ones. Plants receiving 100 mM NaCl had lower Pn, gs, Ci and E than other treatment.

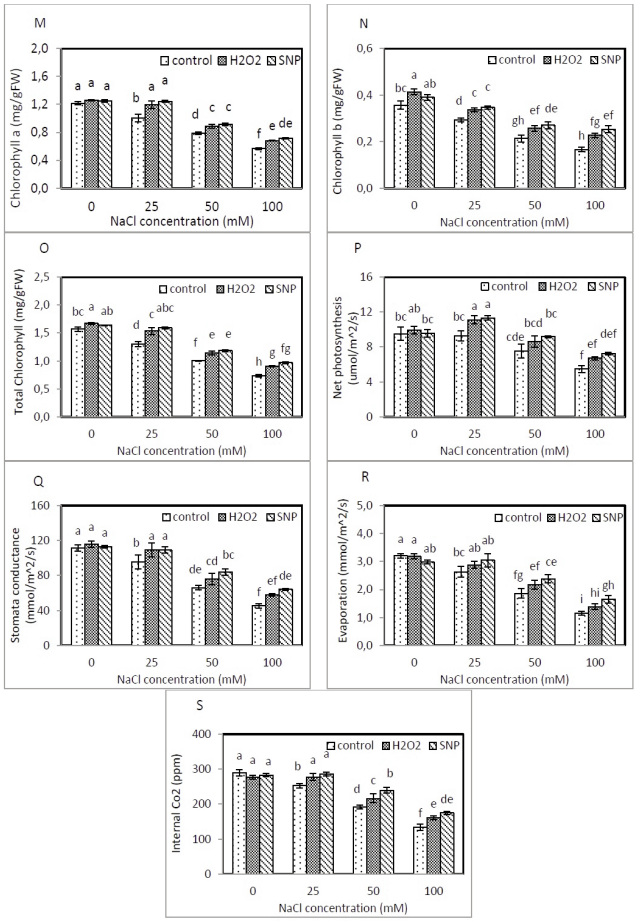

Fig. 3 - Effect of salt treatment and application of exogenous H_2O_2 and SNP on photosynthesis pigment and attributes of tomato plants. Chlorophyll a (M), chlorophyll b (N), total chlorophyll (O), Photosynthetic rate (P), stomatal conductance (Q), transpiration (R) and intercellular CO_2 concentration (S). Data shown are the mean (±SE) of three independent experiments. Significant differences among treatments were determined by Tukey's Test ($P<0.05$).

4. Discussion and Conclusions

Salt stress is a major abiotic stress that imposes osmotic and toxicity stress to plants and consequently induces a reduction in plant photosynthesis and growth (Acosta-Motos *et al.,* 2017). In this study, our results confirmed that salinity at the tested concentrations inhibited the growth of tomato plants (Fig. 1, A-H). H_2O_2 and NO are bioactive molecules that are known as important signals not only in plant disease resistance, but also in the process of growth, development, and responses against abiotic stress (Mazid *et al.,* 2011; Niu and Liao, 2016). Salinity stress is known detrimental effect on the overall growth and productivity of plants (Ashraf, 2004) and may inhibit plant growth due to reduction of water uptake by plants (Kaya *et al.*, 2003). Several studies have shown the beneficial effects of H_2O_2 and SNP pretreatment on salt tolerance in plants (Uchida *et al*., 2002; Azevedo-Neto *et al.*, 2005; Wahid *et al.*, 2007). Potikha *et al.* (1999) suggested that H_2O_2 increases cell division and is involved in the differentiation of the cell wall. Our results are in agreement with those previously reported for maze (Azevedo-Neto *et al.*, 2005; Gondim *et al.,* 2013), rice (Sathiyaraj *et al.,* 2014), cotton, cowpea and sorghum (Freitas *et al.*, 2011). Terasaki *et al.* (2001) noted that SNP possibly enhances exo- and endo-β-D-glucanase activities in cell walls, where the glycosidic linkage between glucose units within cell walls is broken by these enzymes (Zhang *et al.,* 2003), and growth enhance by increasing internal turgor pressure and water content. Similarly to our results, growth stimulation by exogenous NO was demonstrated in tomato (Wu *et al.,* 2010; Hayat *et al.,* 2013).

In this study H_2O_2 or NO resulted in higher increase of relative water content, proline and membrane stability index in leaf of tomato plant and decrease of total soluble sugar content (Fig. 2, I-L) which could promote plant growth under salt stress (Duan *et al.,* 2007) and non saline conditions (Zhang *et al.,* 2005), indicating that H_2O_2 and NO are involved in the intrinsic mechanism of growth under different conditions. The higher relative water content in H_2O_2 and SNP-sprayed stressed plants (Fig. 2, I) appears to be the result of H_2O_2 and NO-induced increased levels of compatible solutes under salt-induced osmotic stress (Tan *et al.,* 2008; Hayat *et al.,* 2013), which resulted in better growth of stressed plants. Proline accumulation is an essential indicator for plant response to salinity stress (Sathiyaraj *et al.,* 2014). The H_2O_2 and SNP-pretreated plants showed a signifi-

cantly higher amount of proline than the salt-stressed ones (Fig. 2, J). stress-induced proline accumulation in plants help in osmotic adjustment (Sathiyaraj *et al.,* 2014). In addition to the role as a compatible osmolyte, proline can also increase membrane stability, confer enzyme protection and help in non-enzymatic free radical detoxifications (Khan *et al.,* 2002; Sathiyaraj *et al.,* 2014). Thus, the increase of proline may trigger tolerance to salt stress in tomato plants.

The salt stressed plant showed an increase in Total soluble sugar content, but H_2O_2 and SNP sprayed plants showed a significantly decreased of TSSC compared with the water-sprayed plants (Fig. 2, K). The reduction in TSSC by H_2O_2 and NO application in this experiment (Fig. 2, K) may be attributed to the crucial role of H_2O_2 and NO in mitigating the negative effect of salinity stress (Semida, 2016). Similarly, TSSC reduction by exogenous H_2O_2 was demonstrated in onion (Semida, 2016).

H_2O_2 and SNP sprayed plants showed a significantly increased of Membrane Stability Index compared with the water-sprayed ones (Fig. 2, L). The salt stressed plant showed an decreas in MSI, and the decrease in MSI reflects the extent of lipid peroxidation caused by active oxygen species. The rate of lipid peroxidation has been widely used as an indicator of oxidative damage (Sathiyaraj *et al.,* 2014). Result showed exogenous H_2O_2 and SNP treatment are able to prevent lipid peroxidation and thus protect the cells from the damage of salinity stress.

H_2O_2 and NO are known to enhance chlorophyll content in plant (Gondim *et al.,* 2013; Hayat *et al.,* 2013). In this experiment, the chlorophyll content was negatively affected by salinity (Fig. 3, M- O). Singh and Dubey (1995) showed that the loss of chlorophyll content could be related to photoinhibition or oxidative damages that acts as a cellular marker of salinity stress. Therefore, the pretreatment with H_2O_2 and SNP was effective to reduce the detrimental effects of salinity in chlorophyll content (Fig. 1A).

Salinity stress is also known to reduce photosynthesis, due to an increase in reactive oxygen species formation, water status alteration, and a decrease in chlorophyll content and CO_2 diffusion through stomatal guard cells (Chaves and Oliveira, 2004; Munns and Tester, 2008). Silva *et al.* (2011) reported that reduction in photosynthesis by stomatal closure occurs during early exposure to salinity stress, while biochemical limitations concern due to long-term NaCl exposure. Thus, the reduction of photosynthesis in plants was caused by reduction in stomatal conductance, decreasing the intercellular CO_2 concentration for Rubisco activity (Shahbaz *et al.*, 2010). Some studies reported the maintenance of gas exchange correlate with salt tolerance in plants (James *et al.*, 2006; Munns and Tester, 2008). In this experiment, results showed all gas exchange parameters were less affected by salinity in plants previously treated with H_2O_2 and SNP (Fig. 3, P-S). Therefore, our data indicate that H_2O_2 and NO-pretreatment increased stomatal conductance, which enabled high net photosynthetic rate and
improved growth parameters. In addition, the higher leaf MSI induced by the H_2O_2 and SNP pretreatment in NaCl stressed plants is an evidence that plants were able to control oxidative damages caused by ROS in the photosynthetic apparatus and maintain leaf gas exchange (Fig. 2, L). Similarly, it is observed that that the H_2O_2 and SNP-pretreatment caused increases in net photosynthetic rate, transpiration rate, stomatal conductance and intercellular CO_2 concentration in plants subjected to salinity when compared to non-treated seedlings (Wahid *et al.*, 2007; Gondim *et al.,* 2013; Hayat *et al.,* 2013).

As a whole, exogenous H_2O_2 and NO are able to improve plant growth of tomato and 0.1 mM SNP produces the most effective improvement. Exogenous H_2O_2 and NO greatly alleviated the oxidative stress induced by salt stress in tomato plant. Therefore, exogenous H_2O_2 and NO treatment on tomato seedling may be an option to improve photosynthesis and growth under saline conditions. Foliar application of H_2O_2 and SNP provides an easy, low cost, and effective strategy to overcome environmental stress problems. Exogenous H_2O_2 and SNP application is a convenient and effective approach to increase salt tolerance of crops and eventually improving crop growth and, productivity under salinity condition.

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Climate change effect on the bud break and flowering dates of the apple trees in mountainous and plain regions of Algeria

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Abstract: Global warming is a strongly felt reality in recent years in Algeria. The fruit trees crop is particularly exposed to the impact of this warming, especially apple trees. A comparative study has been realized between a chronological daily temperature series from 1980 to 2016, and phenological data series (budburst and flowering) from 2000 to 2016, regarding the apple tree variety of *Golden Delicious* in two zones of Northern Algeria, Sidi Lakhdar (town of Ain Defla, in an altitude of 211 m) and Benchicao (town of Médéa, in an altitude of 1133 m). Some contrasting tendencies according to sites and periods have been demonstrated: very significant warming at Sidi Lakhdar site in autumn and spring, in particular in October and April, disturbing thus the entrance of the buds in the endodormancy and ecodormancy. The result is a late action of the cold until February, which proved to be insufficient. However, no average warming has been demonstrated at the Benchicao site, where the temperatures between November and January were cold enough to satisfy the need of cold units and raise the endodormancy. It seems that the failure to fulfill the need of cold units at Sidi Lakhdar site has strongly affected the goodness of fit of the classic phenological models, confirming indirectly the existence of more complex physiological processes (not taken in consideration by models), which manifest themselves in limited zones such as Sidi Lakhdar site.

1. Introduction

According to the experts of the Intergovernmental Group of the Climate Evolution (IGCE), from now on to the end of the 21st century, the average temperature will be raising from 2 to 6°C in Europe following the regions, the climatic models and the socio-economic scenario. The summer droughts will be more intense as well (Giannakopoulos *et al.,* 2005;

Gleizer *et al.*, 2007).

According to Legave (2009), a worrying acceleration of the global warming has appeared during the 1990 decade and much more during 2000 decade. In occidental Europe and the Mediterranean Basin, on a recent period of 30 years (1973-2002), we can estimate the average increase of the annual temperature at about 1°C since the end of 1980's. Regional differences are noticed though, with a warming relatively marked in the region of Meknes in Morocco (2.3°C) (Balaghi, 2017), definitely higher than the one marked in the South-West of France (+1.3°C in Nimes)(Legave, 2009).

The global warming will affect, and has already notably done, a wide range of physical/biological systems and human activity sectors, among which agriculture (including livestock) and its principal function of producing and nutrition (Seguin, 2010). For Algeria, the 21st century shall be characterized by temperatures increase, in the order of 1.0 to 1.5°C at the horizon of 2020 (Fourth report of the IGCE in Bourchef, 2013) and precipitations decrease in the order of 15 to 20%. Extreme climatic phenomena are already affecting the region, like the rain and thunderstorms of November 2001 in Algiers and October 2008 in Ghardaia, and the cold waves in January 2005 and February 2001 in all Algeria. All these events can be qualified as historic at least regionally. Some simulations realized for two types of agricultural years in terms of pluviometry (normal and dry), show at the horizon of 2020 a decrease in the yield of winter cereals from 6% to 14% according to the geographical regions and the year type in Algeria (Tabet-Aoul, 2000; Tabet-Aoul and Bessaoud, 2009).

Phenology is the study of the occurrence of periodic events in animal and plant life in relation with the climate variations. Those are characters that interpret the organisms' adaptation to the climatic variation (Chuine, 2005). The task of plant-phenology is to observe and record the periodically recurring growth stages. Leaf unfolding, flowering of plants in spring, fruit ripening, colour changing and leaf fall in autumn are all examples of phenological events (Koch *et al.*, 2006).

It has been designated as a key point to evaluate the global warming impact on the agricultural cultivations (Moriondo and Bindi, 2008). Many studies have pointed an agreement which many species advanced the spring phenology events (budburst and blooming dates) particularly (Doi and Katano, 2008; Gordo and Sanz, 2010; Malagi, 2014). Since distinct phenological stages were defined decades ago (Baggiolini, 1952; Lichou *et al.,* 1990; Meier *et al.,* 1994), a comparison of available definitions of phenological stages in cherry used independently throughout Europe showed overlaps and shortcomings; hence, harmonisation was reached in this respect in the COST Cherry FA 1104 working group 2 (cherry phenology and climate change) based largely on the acceptance of the BBCH scale and agreed standard cultivars for phenology monitoring. Cultivars were selected on the basis of early, medium and late flowering and most widely grown throughout Europe. This contribution presents the agreed phenology stages in both visual and wording evidence. Similarly, this contribution presents the agreed cultivars to be monitored in future for phenology and climate change effects for harmonization (Wenden *et al.*, 2017).

In this context, the fruit arboriculture seems relatively vulnerable from the fact of some of its characteristics, rather biological (eg: the fruit trees sustainability and their need to many years of growth before fruiting) than economic. Compared to other productions (annual cultivations), the fruit arboriculture is particularly exposed to unfavorable climatic impacts from the fact of multiannual consequences (alternation of production after ceasing) and accumulative (repeated impacts on the tree architecture). On the socio-economic level, strong links have been woven during the time between the product and its production place (eg: Provence almonds, Roussillon apricots…etc). This characteristic developed in France, for commercial valorization reasons, in a regulatory form of origin and quality naming (eg: Agen prunes, Lorraine plums…etc.). From this fact, the substitution of varieties and much more species for long-term climatic adaptation reasons seems relatively difficult to be implemented, probably risking to encounter regulatory and human obstacles (Legave, 2009).

These characteristics constitute an obstacle to the fast changes, not only to the variety range but also to the cultivation systems to cope with rising temperatures or other constraints from climate change. This climate vulnerability has already been expressed in the 2000s by strong production irregularities. Unprecedented accumulations of unfavorable climatic conditions (frost, high temperatures, excessive rainfall) have been observed during key phases of the annual cycle of trees, from flowering to fruiting. Thus, in southern France, very significant production losses were provoked, especially in 2007 for cherry trees following stormy episodes in May and June, which strongly penalized the French production, and in 2008 for apricot following episodes of excessive

heat as blooming approached. Sensitive varieties have had abortion rates that strongly penalize the national production.

Phenological notes on flower buds of fruit trees, collected under contrasting temperature (time and place) conditions in Europe, showed a significant advance of the different phenological stages, especially the flowering dates, for all the places. Modeling work on spring phenology strongly suggests that warming has two opposite effects: (1) in autumn and early winter, a slowdown in the satisfaction of cold unit needs, delaying endodormancy; (2) at the end of winter and in spring, an acceleration of the satisfaction of the heat needs during the ecodormancy phase. The more pronounced intensity of this latter effect, consistent with the more pronounced increases in temperature at the end of winter-early spring than in autumn, largely explains the advances in flowering (Legave *et al.,* 2009).

The analysis of flowering dates over long periods in Western Europe for the *Golden Delicious* apple variety reveals more significant progress in the North of the continent (10 days) than in the oceanic west (6-7 days) and a shortening of flowering time in continental regions (Legave *et al.,* 2012). These regional differences across Western Europe led to a decrease in spatial variability, that is to say, smaller differences between the flowering dates in the contrasting regions (decrease of 8-10 days for complete flowering between the Mediterranean and continental regions). Modeling studies, based in particular on the correlations between the average temperature of the period of ecodormancy and the observed flowering dates, confirm the notion that flowering advances and shortenings are mainly due to a faster satisfaction of the demand for heat units (Legave *et al.,* 2015).

However, delayed endodormancy has also been noted in the oceanic and Mediterranean regions, which may explain the shorter advances in these areas despite similar or greater warming and ultimately lead to delayed flowering. The joint statistical analysis of flowering date series for the *Golden Delicious* variety and temperature dynamics reveals a geographical diversity of responses to warming from autumn to spring. Temperate climates in Europe are characterized by flowering progress, while soft climates are characterized by flowering progress or stationary flowering dates (eg. Morocco and Brazil), (Legave *et al.,* 2015). At the same time, Legave *et al.* (2015) and El Yaacoubi *et al.* (2016) have shown in mild winter conditions, a longer flowering time associated with the high average temperature of the endodormancy period.

In the same context, a comparison of dormancy dynamics of vegetative and floral buds of apple and almond trees was recently conducted between southern France, southern Brazil, and northern Morocco. Differences in dormancy intensity and kinetics have been identified in relation to regional differences in the satisfaction of cold needs and different levels of requirements of the genotypes studied. The observed diversity of dormancy patterns suggests that genotypes adapted to mild climates (eg, almond trees, apple trees with low cold needs) are characterized by the ability of vegetative buds to remain in a state of low dormancy and ability of flower blanks to grow rapidly, guaranteeing the absence of phenological anomalies subsequent to foliage and flowering (El Yaacoubi *et al.,* 2015).

The apple tree is currently an important fruit species in Algeria. Production is the most important fruit production, but it does not sufficiently cover the demand. The central region (Medea - Blida - Ain Defla) totals about 7400 ha or about a quarter of the total area. Apple cultivation has grown considerably, from 30,000 ha in 2003 to 41,000 ha in 2013, with a production reaching 400,000 tons (F.A.O 2013, mentioned by Meradi, 2015). Due to the levels of yield and quality obtained, the *Golden Delicious* variety is one of the three varieties that dominate the Algerian market, particularly in the region of Medea (*Golden Delicious* 70%, *Starkrimson* 20% and *Granny Smith* 5%) (Hadj Sahraoui, 2014). The apple "hanna", of its real name "anna", is a new variety of apple trees introduced in Algeria. It is planted in less cold areas, in the center of Algeria on the perimeter of high chellif in Ain Defla, in the west on the Sebaou valley of Telemcen and in the east to Khenchela and M'sila. They are among the varieties less demanding in cold and generally give apples of lesser quality, hardly storable (Hamdani *et al.,* 2016).

However, apart from regionalized studies aimed at predicting climate change through time series of temperature and rainfall and estimating its impact on crops through the increase of yields in all regions of Algeria including Constantine region in the east of the country (Kherief Nacereddine and Alatou, 2004; Tabet, 2008; Zekri *et al.,* 2009) and Oran region in the west (Benabadji and Bouazza, 2000; Labani *et al.,* 2006), no study on phenological development as a key element to characterize the impact of climate change has been undertaken. We therefore wanted to begin to fill this gap with this study aiming at first,

the characterization of climate change via temperature series and the search for a possible impact on the phenology of the apple tree and, in a second step, the determination of the critical periods with regard to the accumulation of cold units and units of heat, by the implementation of the classical phenological models. For this, we analyzed the time series of phenological data of the *Golden Delicious* variety in two contrasting zones from the climatic point of view: a zone of plain with a rather warm climate, Sidi Lakhdar (town of Ain Defla) and a cooler zone in altitude, Benchicao (town of Medea).

2. Materials and Methods

Sites and climatic data

The temperature data recorded for each site are shown in Table 1. The two zones selected in this study are: Sidi Lakhdar (town of Ain Defla, latitude: 36° 15 '50'' N, longitude: 2° 09' 39'' E, altitude 211 m, located in the center of Algeria 145 km south-west of Algiers) known for its semi-arid climate with a mild winter and a very hot summer, and Benchicao (town of Médea, latitude: 36° 11 '59'' N, longitude: 2° 50' 55'' E, altitude 1133 m, located 80 km south-west of Algiers) in a mountainous area with a warm temperate climate.

Daily maximum and minimum temperature data obtained over a period of 37 years (1980 to 2016) were collected at weather stations near selected sites belonging to the National Office of meteorology. Average temperatures were calculated using maximum and minimum temperatures. Missing daily data were estimated using two methods:

A linear interpolation for some values over 1 to 3 days (means to fill the missing values of Tmax and Tmin were carried out): Correlations with another site for the longest periods namely; between the station of Sidi Lakhdar and the station of Chlef (latitude 36° 10' N, longitude 1° 20' E, altitude 116 m) for the month of May of the year 2005, and between the station of Benchicao (Médéa) and the station of Bordj Bou Arreridj (latitude 36° 04 '23'' N, longitude 4° 45' 39'' E, altitude 930 m) for the months of January, February, March and April of the year 1980 and the month of May of the year 2001.

Similarly, a correlation was made between the Médéa site and the Sétif meteorological station (latitude 36° 11 '28'' N, longitude 5° 24' 49'' E, altitude 1038 m) for the months of September, October and November of the year 1981, and February and December of the year 1990.

Phenological data

Data collected from 2000 to 2012 were provided by specialized state agencies. These are average dates that represent all the orchards visited. Those from 2013 to 2016 were collected directly from the same orchards, which were among the most apple orchards planted at both sites. Phenological monitoring 3 to 4 times a week was carried out on adult trees, the number of which sufficiently covered the total area of a given orchard (50%), respecting the two orientations (North-South and West-East). These orchards have not undergone any chemical treatment to break endodormancy or accelerate flowering. Phenological stages were described according to the BBCH scale (Meier *et al.,* 1997, 2001). The phenological stages of bud break (bud burst, Baggiolini stage C and stage 51 of the BBCH scale) and early flowering (10% open flowers, Baggiolini F1 stage and 61 BBCH scale) were observed from 2000 to 2016 on the two apple orchards maintained according to conventional horticultural practices. Both stages were reported affected when 60% of the trees in the orchard had reached the given stage.

Table 1 - Phenological and temperature data collected in climate-contrasting sites for 'Golden delicious' apple trees

Site	Benchicao (MD)	Sidi Lakhdar (SD)
Region (town)	Medea	Ain Defla
Latitude/longitude	36°11' 59'' N / 2°50' 55'' E	36°15' 50'' N / 2°09' 39'' E
Altitude (m)	1133	211
Climatic area	sub-humid	semi-arid
Period recorded of temperature	1980-2016	1980-2016
Bud burst stage and observation period	a BBCH 51 / 2000-2016	BBCH 51 /2000-2016
Flowering stage and observation period	a BBCH 61 / 2000-2016	BBCH 61 /2000-2016

a: BBCH 51, 61; stages in phenological code BBCH (Meier, 1997), are respectively swelling buds of inflorescences and 10% of flowers open.

Modeling and data analyses

To better explain the phenological behavior of the *Golden Delicious* variety in the two sites studied and to highlight the effect of the temperatures on the latter in terms of satisfaction in cold units and heat units, statistical analyses were carried out under R (R Development Core Team 2008), concerning regression curves between the different temperature components (minimum, average and maximum) and the year as well as the two phenological stages (bud burst and flowering). Similarly, parametric name correlation tests of Spearman were performed between two variables namely annual and monthly temperature (minimum, average and maximum) and year on the one hand and phenological stages on the other hand. Calculation and establishment of cold unit accumulation curves were performed using the Utah model (Richardson *et al.,* 1974).

Utah model

The Utah model was designed by Richardson *et al.* (1974). This model combines the cold units for temperatures between 0 and 16°C and associates a negative value with temperatures higher than 16°C. This model is built to use fixed degree-days (independent of cold units) to predict bud break. The Utah model (Richardson *et al.,* 1974) transforms the hourly temperature into a cold unit from -1 to 1. The cumulative number of Utah cold units at time t is expressed as follows:

$$UCUtot = \sum_{i} Tu$$

With (U= 0 for T≤1.4°C, U= 0.5 for 1.4°C <T≤2.4°C, U= 1 for 2.4°C <T≤9.1°C, U= 0.5 for 9.1°C <T≤ 12.4°C, U= 1 for 12.4°C <T≤15.9°C, U = -0.5 for 15.9°C <T≤18.0°C, U = -1 for T≥18.0°C) (Ricard, 2014).

Phenological modeling platform PMP5.5

The phenological models were adjusted using the Phenology Modeling Platform (PMP5.5) proposed by Chuine *et al.* (2013). PMP5.5 is an environmental-use interface aimed solely at managing the construction of a phenological model, fitting a phenological model to the data and simulating using a phenological model. The best results of the bud burst and flowering date prediction in the two studied sites are obtained by two-phase models (knowing that phase 1 corresponds to the accumulation of cold units and phase 2 corresponds to the accumulation of heat units) quoted below.

Chuine/Wang and Chuine/Sigmoid

The Chuine model has been described in Chuine (2000) and is composed of three parameters, namely A, B and C. The parameter A determines the width of the window on which the function is not zero. The larger the value, the larger the temperature range over which the cold units are wide. Parameter B determines the sharpness of the response curve and its asymmetry. The more B differs from zero, the sharper the image (and more asymmetric). Parameter C determines the value of the average response when B is close to zero and represents a limit to the temperature range over which cold units accumulate, when B is significantly different from zero.

The Wang model was first defined by Wang and Engel (1998). It is characterized by an optimum and is not symmetrical. This concerns the family of the beta function. It is composed of three parameters, namely Tmin, Topt and Tmax (minimum, optimal and maximum temperatures).

The Sigmoid model was introduced by Hänninen (1990). It consists of two parameters, D and E. The D parameter defines the sharpness of the response. Values far from zero induce a sharper response curve. The parameter E is the average response temperature.

Smooth Utah/ Wang and Smooth Utah/ Sigmoid

The Smooth Utah model was introduced by Bonhomme *et al.* (2010) and is a smoothed version of the Utah function proposed by Richardson *et al.* (1974). This function assumes that cooling can occur only over a range of temperatures and has four parameters: Tm1, Topt, Tn2 and min. Negative cooling values can be accumulated on hot days, increasing the amount of cold to reach.

Tm1: This parameter defines the sharpness of the decrease of the cold effect on the endodormancy of the buds. The lower Tm1, the slower is the decrease.

Topt: This parameter corresponds to the optimum average daily temperature, for which a cooling unit is accumulated each day.

Tn2: This parameter defines the intermediate response, i.e. the temperature (above Topt) that has half of Topt's effectiveness for inducing endodormancy.

Min: This parameter defines how much the impact of high temperatures can be negative.
When min = 0, high temperatures do not have a negative impact on endodormancy release. When min = -1, the negative impact of a day that is too hot is equivalent to the positive effect of a day in Topt.

Each model is characterized by efficiency (EFF), an estimated time (t0) and a quadratic error (RMSE: Root mean square error).

3. Results

General climatology. Annual tendencies

The annual average maximum, medium, and minimum temperatures for both sites are shown in figure 1. The linear regression of mean annual temperatures over the 36 available years revealed a significant warming (P = 0.004) at the Sidi Lakhdar (SD) site, with a significant increase in the mean annual minimum temperatures (P = 0.001), and less for the average annual maximum temperatures (P = 0.04). There is rather a cooling tendency at mountain site of Benchicao, although not significant (P = 0.09), concerning the annual average and where the maximum temperatures experienced some significant regression (P = 0.001). The hottest years were 1990, 2010 and 2007 at the site of Sidi Lakhdar and 2016, 1997 and 2000 at Benchicao site.

Tendencies for the autumn-spring period

A seasonal analysis from the 36 years available shows marked differences between the two sites. The monthly average temperature tendencies for the months of October to May (minimum, mean and maximum daily temperatures) measured during the period 1980 to 2016 for the two sites (Sidi Lakhdar and Benchicao) are summarized in Table 2 and figure

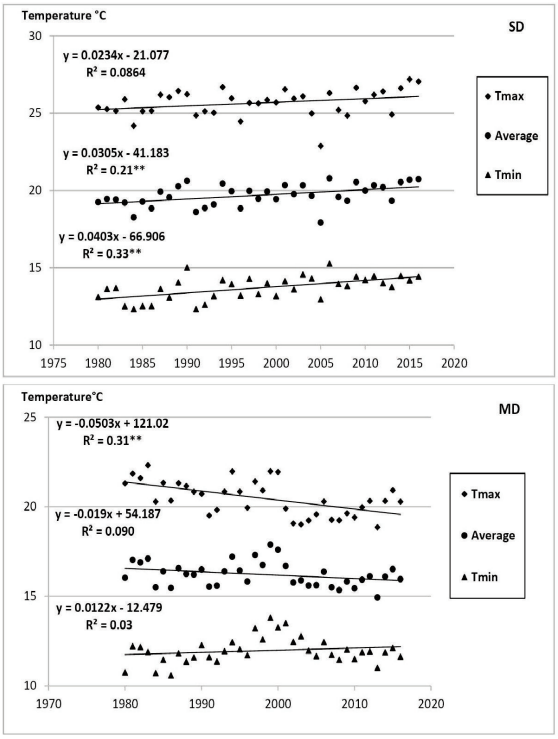

Fig. 1 - Average annual temperatures (minimum, average, maximum) for the two sites, Sidi Lakhdar (SD) and Benchicao (MD) from 1980 to 2016. *, ** indicate the significance level of the correlation for P <0.05 and for P <0.01, respectively. Sidi Lakhdar: (SD) Tmin: y = 0.0403x - 66.906 (R^2 = 0.33**), Taverage : y = 0.0305x - 41.183 (R^2 = 0.21**), Tmax: y = 0.0234x - 21.077 (R^2 = 0.0864). Médéa:(MD) Tmin: y= 0.0122x - 12.479 (R^2 = 0.03), Taverage: y = -0.019x + 54.187 (R^2 = 0.090), Tmax: y = -0.0503x + 121.02 (R^2 = 0.31**).

Table 2 - Temperature data collected characteristics in the two studied sites in Algeria during 36 years

Month	Daily temperature	Mean daily temperature during 36 years in °C		Correlation coefficient of trends and level of significance			
				Sidi Lakhdar (SD)		Benchicao (MD)	
		Sidi Lakhdar	Benchicao	R	p	R	p
October	Average	21.3	17.4	0.40	0.01*	0.07	0.50
	Maximum	27.0	21.6	0.38	0.018*	0.14	0.40
	Minimum	15.6	13.3	0.44	0.006**	0.36	0.028*
November	Average	15.7	11.4	-0.004	0.1	- 0.40	0.007**
	Maximum	20.3	14.7	- 0.03	0.842	0.20	0.008**
	Minimum	10.8	8.1	0.11	0.48	- 0.17	0.30
December	Average	12.0	8.0	0.17	0.28	- 0.16	0.16
	Maximum	16.4	10.9	0.10	0.556	- 0. 17	0.31
	minimum	7.6	5.2	0.19	0.26	0.009	0.966
January	Average	10.9	7.3	0.35	0.03*	- 0.20	0.08
	Maximum	15.3	10.2	0.24	0.143	-0.20	0.23
	minimum	6.4	4.6	0.32	0.051	0.10	0.54
February	Average	11.9	8.1	0.11	0.48	-0.32	0.005**
	Maximum	16.8	11.2	0.047	0.783	-0.47	0.003**
	Minimum	6.8	5.0	0.16	0.34	- 0.22	0.18
March	Average	14.2	10.5	0.01	0.51	-0.23	0.03*
	Maximum	20.0	14.3	0.008	0.961	-0.47	0.030*
	Minimum	8.5	6.8	0.25	0.13	- 0.025	0.88
April	Average	16.6	13.3	0.42	0.008**	-0.07	0.51
	Maximum	23.0	17.5	0.36	0.027*	0.012	0.565
	Minimum	10.4	9.03	0.49	0.002**	0.18	0.28
May	Average	21.0	17.9	0.32	0.04*	-0.1	0.38
	Maximum	27.9	22.7	0.21	0.19	0.027	0.403
	Minimum	14.3	13.0	0.40	0.012*	0.089	0.600

* p<0.05, ** p<0.01.

2. Table 2 generates Spearman parametric name correlation values between maximum, average and minimum monthly temperatures and year, and where p is the level of significance and R is the correlation coefficient.

We focused on the period from October to May, which is the period that most affects the physiological processes associated with the spring phenology of flower buds of fruit trees in our region. Average October temperatures are high and increase significantly at Sidi Lakhdar site. The month of April is also warming significantly on this site (Table 2, Fig. 2). No significant tendency is recorded for the other months. Correlations on monthly temperature tendencies also clearly showed significant summer warming in July at Sidi Lakhdar site (data not shown). At mountain site of Benchicao, on the other hand, as already indicated above, during the 36 years, no significant warming is recorded in average temperature. On the contrary, average temperatures decreased in November and February (P = 0.007 ** and P = 0.005 *, respectively).

Regarding average minimum temperatures, the lowest values were recorded during the month of January for both sites with 6.4 and 4.6°C. The highest minimum temperature value of 12.1°C and the lowest value of 8.2°C were recorded by order in 2006 and 1991 at the site of Sidi Lakhdar for this month of January. At mountain site Benchicao, the highest value of 9.7°C is reported in 2000 compared to a lower value of 6.6°C in 1980. The site of Sidi Lakhdar experienced extreme maximum temperatures during the months of October and April which explains the significance of the increase in average temperatures during these two months (Fig. 2a). Significant regressions of maximum temperatures were recorded at the Benchicao site during the months of November, February and March.

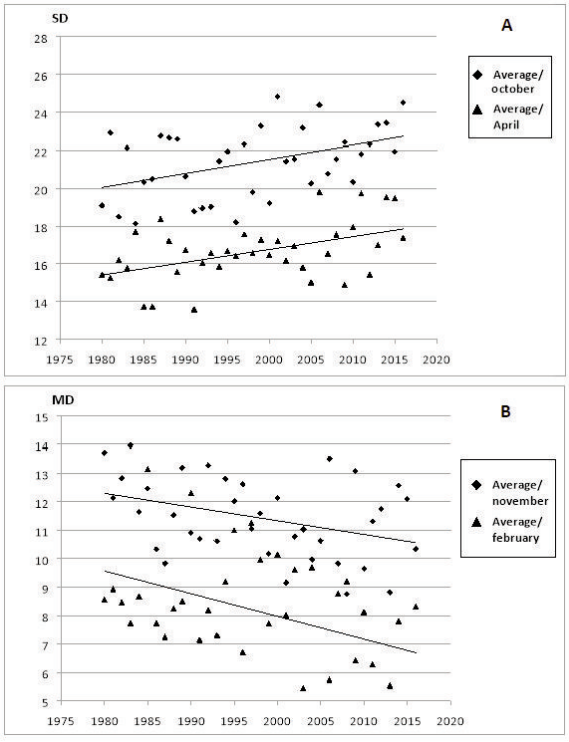

Fig. 2 - Evolution of average monthly temperatures, on both sites, for months where the trend is significant at Sidi Lakhdar (October and April) and at Benchicao (November and February). *, ** indicate the significance level of the correlation for P <0.05 and for P <0.01, respectively. Sidi Lakhdar: (SD) Average April: y = 0.068x - 119.18 (R^2 = 0.2252**), Average October: y = 0.0742x - 126.9 (R^2 = 0.1909**). Médéa:(MD) Average February: y = -0,0799x + 167,72 (R^2 = 0, 1833), Average November: y = -0.0483x + 107.88 (R^2 = 0.1376).

Phenological development

Comparisons of the phenological tendencies of the apple tree (in terms of bud burst and flowering) in the two contrasting environments were made from the 16 years available. For budding dates of flower buds at Sidi Lakhdar site, some variation between years was revealed with marked tardiness during the years 2002, 2007, 2012, 2013 and 2016, when there was a bud break between the end of March and the first days of April and an early fruit maturity in 2000, 2003 and 2006, but the overall tendency for all years is not significant (P = 0.07). The tendency towards the advancement of flowering dates (Fig. 3) is also not significant (P = 0.24), the ear-

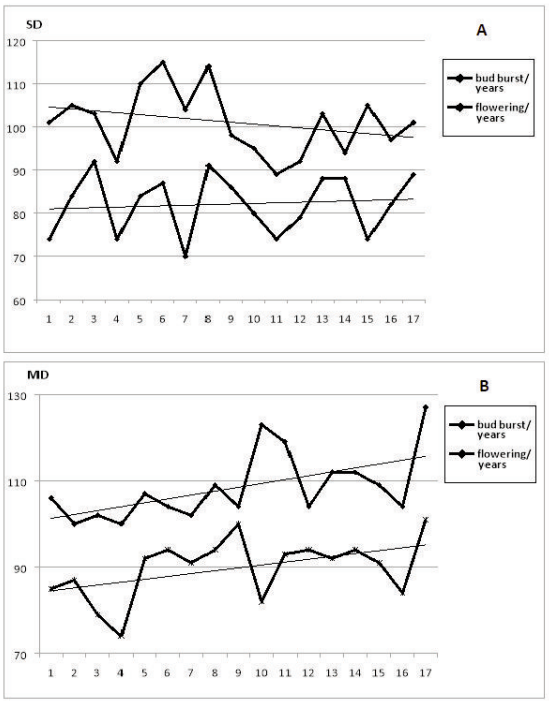

Fig. 3 - Trends in bud burst and flowering dates at the Sidi Lakhdar (SD) and Benchicao (MD) sites. *, ** indicate the significance level of the correlation for P<0.05 and for P<0.01, respectively. Sidi Lakhdar: (SD) Bud burst/years: y = 0,1397x + 80, 86 (R^2 = 0, 0104), Flowering/years: y = -0.4412x + 105.03 (R^2 = 0.0872). Médéa: (MD) Bud burst/years: y = 0.7623x + 83.257 (R^2 = 0.2496), Flowering/years: y = 0.8971x + 100.4 (**R^2 = 0.3263).

liest years being 2003, 2009, 2010 and 2011, and the later years 2001, 2004 and 2005 At Benchicao site, a significant tendency (P = 0.010) at the late flowering dates of the apple tree is to be reported (Fig. 3b). On the other hand, differences in historical trends were shown in the bud break dates, oscillating between advancement during the years from 2000 to 2003, and a delay in the years 2004 to 2008.

Accumulation of chilling units in winter

The Utah cold unit (CU) accumulation curves reveal significant interannual differences at the Sidi Lakhdar site and show that for the years 2001, 2007, 2010 and 2016, this accumulation was insufficient because at below 600 CU, which is well below the estimated needs of the Golden Delicious variety (900 CU) (Fig. 4). On the other hand, at mountain site of Benchicao, the needs are always quickly satisfied.

In order to analyze the influence of the different months in terms of cold units, we calculated the correlations between the dates of bud burst or flowering and the monthly temperatures (minimum, average and maximum) (Table 3). Very schematically, the tendencies can be summed up as follows: At the Benchicao site, the month of January and the whole period from November to January and February have a strong influence on meeting the needs in cold units because the correlation is positive (the warmer it is, the more the budding/flowering is late), whereas it is not the case in November and December (October remains quite neutral with a negative correlation). The impact of January is preponderant because if we look at the influence of the period from November to January, we find a negative effect of high temperatures on the precocity (R^2 = 0.40) while the months of November and December have an inverse effect.

At the site of Sidi Lakhdar, the balance sheet is

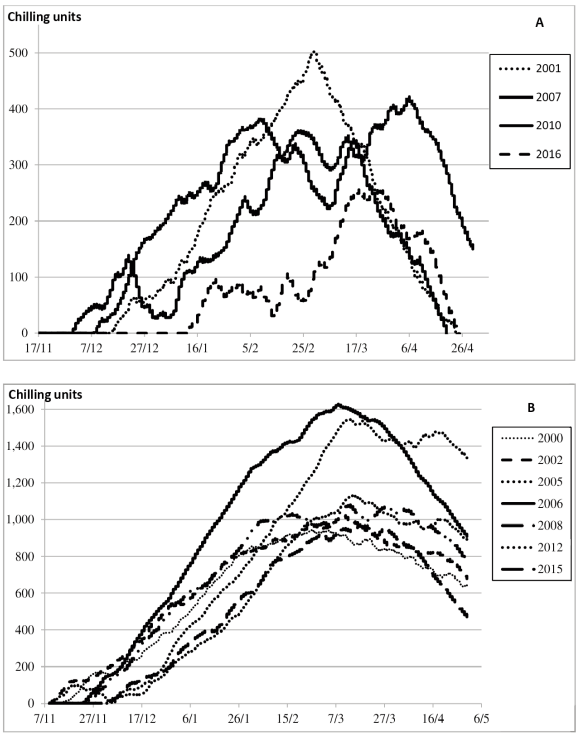

Fig. 4 - Cumulative cold unit according to the Utah model at the Sidi Lakhdar site. A) Years when cumulative cold is less than 500 hrs; B) Years when cumulative cold exceeds 800 hrs.

Table 3 - Spearman's correlation between bud burst and flowering for 'Golden delicious' apple tree and mean temperature from October to April

Variable	Sidi Lakhdar (SD)						Benchicao (MD)					
	Mean Temperature		Minimum Temperature		Maximum Temperature		Mean Temperature		Minimum Temperature		Maximum Temperature	
	Bud burst	Flowering	Bud burst	Flowering	Bud burst	Flowering	Bud burst	Flowering	Bud burst	Flowering	Bud burst	Flowering
October	-0.031	0.11	-0.21	0.13	-0.20	0.033	0.015	0.019	-0.106	0.135	0.081	0.14
November	-0.41 *	-0.12	-0.55 *	-0.21	-0.38	-0.036	-0.54 *	-0.225	-0.57*	-0.232	-0.47*	0.18
December	-0.18	-0.43 *	-0.11	-0.232	-0.2	-0.50 *	-0.40 *	0.054	-0.52*	-0.11	-0.322	0.18
January	0.31 *	-0.002	0.006	-0.24	0.46 *	0.14	0.46 *	0.3	0.232	0.15	0.54*	0.41
February	0.10	0.01	-0.09	0. 12	0.01	0.13	0.063	0.073	0.021	-0.015	0.13	0.15
March	-0.43 *	-0.34 *	-0.27	-0.147	-0.36	-0.36 *	-0.60 *	-0.65 *	-0.60*	-0.64*	-0.55*	-0.56*
April	-0.45 *	-0.30	-0.41 *	-0.145	-0.48 *	-0.33 *	0.08	-0.072	-0.101	-0.30	0.12	0.041
November-January	0.30 *	-0.26	0.14	-0.45 *	0.30*	-0.30*	0.43 *	0.40 *	0.28	0.33	0.52*	0.53*
November-February	0.06	-0.052	0.21	-0.14	0.18	-0.14	0.48 *	0.44*	0.233	0.212	0.30	0.21
November-March	0.041	-0.29	0.01	-0.20	0.132	-0.30	0.184	0.27	0.062	0.104	0.33	0.30
March-April	-0.56 *	-0.45 *	0.08	-0.025	-0.54 *	-0.330	-0.26	-0.44*	-0.304	-0.54*	-0.133	-0.20

* P<0.05, **P<0.01.

globally the same. The correlations between mean and maximum temperatures in January on the one hand, and bud break dates on the other, are significantly positive, indicating the importance of this month's temperatures for the satisfaction of cold unit requirements, the month of October remains little determinant. A hot January is a delay in meeting cold needs and bud break. A negative tendency of the high temperatures of the period from November to January on the precocity (R^2 = 0.30) was recorded whereas the other months go rather in the direction of a gain of precocity. Significant and negative relationships were observed between maximum temperatures in December and flowering, with a correlation coefficient of -0.50.

Accumulation of forcing units in spring

March-April period (-0.44) show the link between the early flowering period and the average temperature It is rather March that plays the main role for both sites. At the Benchicao site, only March temperatures show a significant negative correlation with bud break and flowering dates. Negative correlations obtained between average temperatures of the period, with a very high prevalence of March temperatures. At the site of Sidi Lakhdar, March and April strongly influence the precocity via maximum temperatures and average temperatures.

Phenology modeling

For both sites, the best sequential models selected are given in Table 4. They were chosen on the basis of efficiency (EFF) and RMSE (RMSE), but also taking into account the physiological relevance of the temperature response curves for the cold unit stacking phase and the heat unit stacking phase. RMSE may appear acceptable (~ 5 days) on a flowering date but the efficiencies are less good. The correlation between the observed values and the predicted values confirms this diagnosis (Fig. 5). The efficiency is very slightly improved (reaching a difference of 0.03 to 0.09) by eliminating the years when the cumulative cold units are not satisfactory at Sidi Lakhdar site.

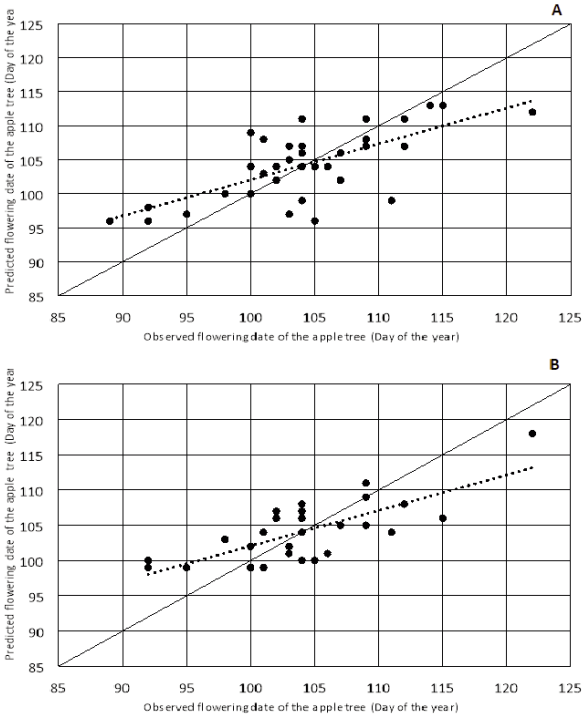

Fig. 5 - Comparison between the observed and the predicted flowering date of apple in two sites. A) for all years by the Smooth Utah/Sigmoid model. y = 0.5286x + 49.199; R^2 = 0.4796; B) without the years where the cumulative cold units are not satisfied by the Smooth Utah/Sigmoid model. y = 0.5041x + 51.647; R^2 = 0.5555.

Table 4 - Modeling bud burst and flowering for 'Golden delicious' apple tree by Chuine/Wang and Smooth Utah/Sigmoid in the two studied sites with all years and without years where chilling does not satisfied

Site	Two sites with all years				Two sites without years where chilling dose not satisfied, 2001, 2007, 2010, 2016			
Phenological stage	Bud burst		Flowering		Bud burst		Flowering	
Model	Chuine / Wang		Smooth Utah / Sigmoid		Chuine / Wang		Smooth Utah / Sigmoid	
T0 starting date	-107.8		-75.3		- 110.6		61.4	
RMSE	5.39		4.86		5.93		4.16	
EFF	0.49		0.47		0.50		0.56	
Settings	Chuine	Wang	Smooth	Sigmoid	Chuine	Wang	Smooth	Sigmoid
	A 0.50	Topt 20.69	Tm1 -37.54	D -40	A 0.68	Topt 17.09	Tm1 9.72	D -2.72
							Topt 18.45	
	B 12.70	Tmin 3.30	Topt 23.52	E 6.40	B -28.90	Tmin 2.40	Tn_2 27.52	E -21.80
							Min -0.86	
	C 27.04	Tmax 43.14	Tn_2 24.40		C -15.36	Tmax 26.36		
			Min -0.99					

By examining separately the two sites, and in particular that of Sidi Lakhdar, which shows years when the accumulation of cold units is not satisfied (Fig. 6a), we see that the withdrawal of these years greatly improves the results of modeling (Fig. 6b) and in particular for flowering. The relevance of the response curve obtained for the cold unit function is also greatly improved (Table 5).

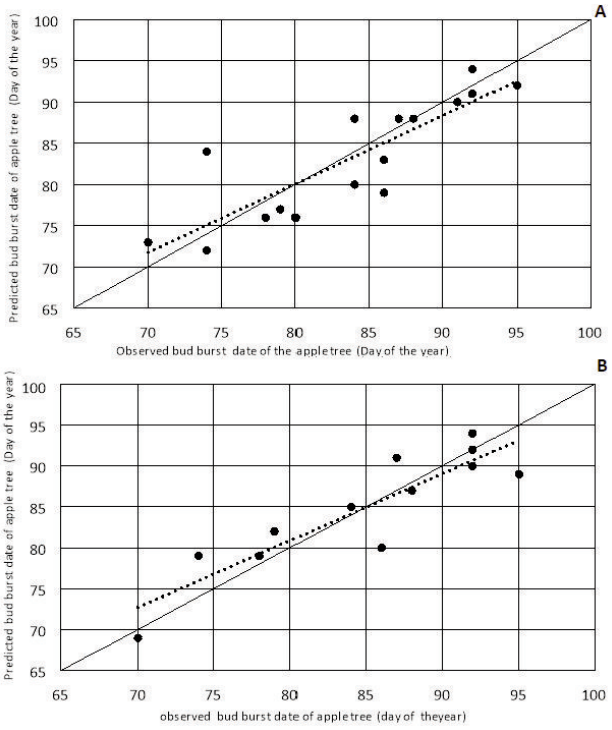

Fig. 6 - Comparison between the observed and the predicted bud burst date of the apple tree at the Sidi Lakhdar site. A) for all years by the Chuine/Sigmoid model. y = 0.8316x + 13.544; R^2 = 0.7207; B) without the years when the cumulative cold units are not satisfied by the Chuine/Sigmoid model. y= 0.8165x + 15.548; R^2 = 0.8066.

At Benchicao site (Table 6), the efficiency of the two-phase model is medium and figure 7 shows that it is difficult to predict the early years. In fact, the two-phase model is not much better than a one-phase model on bud break and not better for flowering. The results of the one-phase model (only the heat-accumulation model: degree-day growth, Parabolic, Richarsdon, Wang, Sigmoid, Threshold and Smooth Utah) yielded non-significant results.

4. Discussion and Conclusions

The sensitivity of phenophases to temperature changes is a good indicator of the long-term biological impacts of climate change and terrestrial ecosystems (Richardson *et al.,* 2013). Several studies have shown that the phenophases most sensitive to temperature variations are those occurring in spring or summer and that there is a relatively linear relationship between the occurrence of these phenophases

Table 6 - Modeling bud burst and flowering for 'Golden delicious' apple tree in Benchicao by Smooth Utah/Wang and Chuine / Sigmoid models with all years

Site	Benchicao with all the years							
Phenological stage	Bud Burst				Flowering			
Model	Smooth Utah / Wang				Chuine / Sigmoid			
T0	-104.9				-78.7			
RMSE	5.43				4.91			
EFF	0.48				0.46			
Settings	Smooth Utah		Wang		Chuine		Sigmoid	
	Tm1	-34.85	Topt	22.31	A	0.45	D	-39.99
	Topt	21.15	Tmin	3.60	B	-10.58	E	6.39
	Tn2	29.42	Tmax	49.9	C	1.03		
	Min	-0.81						

Table 5 - Modeling bud burst and flowering for 'Golden delicious' apple tree in Sidi Lakhdar by Chuine/Sigmoid model with all years and without years where chilling does not satisfied

Site	Sidi Lakhdar with all years				Sidi Lakhdar without years where chilling dose not satisfied, 2001, 2007, 2010, 2016			
Phenological stage	Bud burst		Flowering		Bud burst		Flowering	
Model	Chuine/ Sigmoid		Chuine/ Sigmoid		Chuine/ Sigmoid		Chuine/ Sigmoid	
T0	-102.3		61		-119		-59.7	
RMSE	3.79		5.02		3.30		2.10	
EFF	0.72		0.44		0.80		0.90	
Settings	Chuine	Sigmoid	Chuine	Sigmoid	Chuine	Sigmoid	Chuine	Sigmoid
	A 1.61	D -32.51	A 2.95	D -11.93	A 0.96	D -2087	A 3.21	D -40
	B - 5.67	E -21.66	B -25.47	E 0.50	B -29.7	E -25.47	B 12.47	E 14.93
	C 19.42		C 8.60		C -6.72		C 16.93	

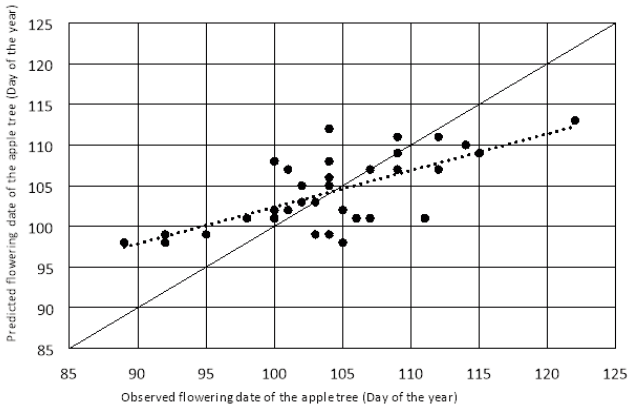

Fig. 7 - Comparison between the observed and the predicted flowering date of the apple tree at the Benchicao site by the Chuine/Sigmoid model. y = 0.4501x + 57.365; R^2 = 0.4632.

and temperature (Gordo and Sanz, 2010; Morin *et al.,* 2010; Beaubien and Hamann, 2011).

Our study is a first attempt to evaluate the impact of global warming on the bud break and flowering of the apple tree in northern Algeria. The study of the chronological series of temperatures (minimum, average and maximum) from 1980 to 2016 (36 years) in the two important apple production sites, namely: Sidi Lakhdar and Benchicao, showed a very significant warming in October and April at the site of Sidi Lakhdar (SD) likely to strongly disrupt the entry into endodormancy and ecodormancy. Strangely, the Benchicao (MD) site does not show global warming and even a cooling trend in November and February, which could lead to an endodormancy and a start of satisfaction in cold units and then an acceleration of the recovery of growth leading to greater precocity.

Establishing the time of endodormancy emergence is important (Guerriero *et al.,* 2002), but is hampered by the complexity of the process, including the fact that, under natural conditions, cold and warm temperatures alternate, causing "Inversions" in the process of endodormancy emergence (Overcash and Campbell, 1955; Couvillon and Erez, 1985; Erez and Couvillon, 1987). Some results suggest that partial dissatisfaction with cold can be compensated by heat unit supplementation for bud break (Dantec, 2014).

To lift the endodormancy, the bud must accumulate enough cold (Campoy *et al.,* 2011). When the plant has reached its cold needs, the endodormancy is lifted and the buds can resume their growth as soon as the conditions become favorable, i.e. under certain (rather high) temperature conditions, soil moisture and nutrients, and for photosensitive species, certain photoperiod conditions (Körner and Basler, 2010; Polgar and Primack, 2011). These favorable conditions must last for a certain period of time for the bursting of the buds to appear.

For our case, the cold needs were always met to lift the endodormancy of the apple tree at the Benchicao site, and this as from the end of December or the first half of January (Fig. 4). Conversely, at the Sidi Lakhdar site, values below the threshold for satisfying cold needs estimated at 900CU for the *Golden Delicious* apple tree were obtained for the years 2001, 2007, 2010 and 2016. When it takes place, the satisfaction of cold needs is later, around mid-February (Fig. 4) and does not really start until the end of November. Everything leads to a dominating importance of the month of January in the course of the endodormancy lifting process and it will be interesting to look at what the future climate scenarios give for this particular period for the choice of future apple varieties.

The months of November and December play a precocious role at the Benchicao site (negative correlation with the date of bud burst or flowering). This can only be understood if the organogenesis in the buds continues during these two months and therefore there is no endodormancy at this time. The observed increase in average November and October minimum temperatures (Table 2) is consistent with this. At the site of Benchicao, the month of March is crucial for the precocity, it is the temperatures of this month which allow the growth after the satisfaction of the needs of cold towards the end of December and the beginning of January all the more so as the temperatures of the February remain rather low (and tend towards a cooling). The tendency to tardiness with the site of Benchicao is thus coherent with the cooling in the month of February. The October warming at Sidi Lakhdar site may explain later entry into endodormancy. Gentle temperatures (12°C) in February can then accelerate bud burst and flowering, at least for years when cold needs are met. Otherwise endodormancy will be greatly delayed or disrupted. According to the study of Legave *et al.* (2015), carried out in three geographically contrasting countries of the Mediterranean region, in Morocco (Meknes), France (Nimes) and Italy (Forlì) over the last 40 years in order to understand the impact of climate change, especially the increase in temperature, on the *Golden Delicious* apple tree, the forcing period is shorter in Meknes. Legave *et al.* (2012) also found a marked trend towards shorter simulated duration of forcing period and late

endodormancy period. The physiological functioning of the *Golden Delicious* apple tree during the dormant and growing season may explain, in part, the regional differences observed in the flowering dates (Heide, 1993). In the same context, Kauffman and Blanke (2018) have reported after a study conducted on three cherry cultivars at different levels of cold needs (minimum, medium and high) that, in *optimum* chill, the *optimum* forcing was ca. 8.000 GDH (>12 °C), irrespective of variety, allowing up scaling of the results to possibly other varieties. Overall, the results have shown that diminishing chilling as a result of climate change can be compensated for, in part up to 50%, by a larger amount of forcing to obtain natural flowering in the orchard. These results may explain the good progress of flowering on the site of Sidi Lakhdar, although the cold needs were not often satisfied. El Yaacoubi *et al.* (2014) also reported that spring temperatures appear to be essential for complete flowering in mild climates. In the latter case, early full flowering dates occurred when the average temperature during the forcing period rapidly exceeded 15°C provided adequate satisfaction of the cold requirements. Phenological models predicting the occurrence of different phenophases as a function of environmental conditions (mainly temperature and photoperiod), predict that the global increase in temperature during the winter will slow or even jeopardize the endodormancy emergence due to lack of cold (Chuine *et al.,* 2016). The one-phase and two-phase models for all years do not give good results at the Sidi Lakhdar site. This is explained by the negative influence of years when cold unit needs have not been met. If these years are removed, the two-phase Chuine/Sigmoid model for bud burst and flowering gives good results. This may mean that in these cases of partial non-fulfillment of cold unit requirements, the physiological processes involved in bud break-up and flowering are different or that "something" in addition occurs. At Benchicao site, the efficiency of the two-phase models is average, since the requirements in cold units are often met; only the forcing period can have an effect on the precocity.

This study aimed to show the effects of the anticipated increase in temperature on two phenological phases of the apple tree (*Golden Delicious*) in two Algerian sites with contrasting climates. We highlighted contrasting trends by site and by period. Warming at Sidi Lakhdar site in autumn and spring, however, the statistical data of temperatures did not raise any average warming at the Benchicao site. Rather surprising and never described before, there has been a tendency to cool down some months at the Benchicao site. Critical periods for cold units were identified, concerning the period between November and January at Benchicao site, but January temperatures were more important in lifting endodormancy. At the site of Sidi Lakhdar, buds enter late into endodormancy and the result is a late action of cold that extends until February without always being sufficient. Forced side, it is the temperatures of the month of March that have a discriminating effect on bud burst and flowering at the site of Benchicao combined with those of April at the site of Sidi Lakhdar. On this site, despite the warming in April, we do not gain in precocity probably because of a satisfaction of cold needs "to the limits" as describe Legave *et al.* (2012) for the Nimes region. We have also highlighted, particularly at the site of Sidi Lakhdar that more complex physiological processes must be at work especially the years of low cumulative cold units. It is not excluded that other factors, not included in this work, could be involved in the budburst process such as photoperiod or precipitation (Vitasse *et al.,* 2009; Grab and Craparo, 2011; El Yaacoubi *et al.,* 2014). Except for the two-phase Chuine/Sigmoid budburst and flowering model, which gave better results at Sidi Lakhdar site after the elimination of the years when the cold unit requirements were not met, all the models give rather weak efficiencies indirectly confirming the non-taking into account of a complexity of factors associated with physiological functioning for sites like Sidi Lakhdar's.

The study of the impact of global warming on the apple tree requires a precise determination of the accumulations in cold units necessary for the emergence of endodormancy and budding in various environments. This involves highlighting these two phases by forcing techniques at the laboratory level and anatomical studies of meristematic bud tissues to see their ability to bud.

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Short note

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Efficient and easy micropropagation of *Morus nigra* and the influence of natural light on its acclimatization

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Abstract: Micropropagation, which employs various plant growth regulators (PGRs) in the culture medium for the induction of multiple shoots as well as adventitious roots, is a widely-used technique for the propagation of the genus *Morus*. The main objective of the present study was to evaluate the effect of PGR-free culture medium on the quantitative and qualitative characteristics of the micropropagation of *Morus nigra* under the growth room and greenhouse environmental conditions. Although a higher rate of multiplication (4.7–5.2 shoots/explant) was obtained from the treatments using Benzyladenine (BA) as the PGR, the PGR-free culture medium also exhibited comparable multiplication rate (4.1 shoots/explant) with a higher quality of shoots and without any symptoms of hyperhydricity. Furthermore, the use of PGR-free culture of *M. nigra* for *in vitro* propagation combined the steps of shoot multiplication and rooting phase using the same culture medium, further simplifying the process. In this study, the micro-shoots were also assessed for their *in-vitro* rooting and acclimatization potential. The incubation of an *in-vitro* culture of nodal explants in a controlled growth room for 28 days exhibited optimum response with about 90% rooting and 100% plantlet survival during the acclimatization phase. These results were better than those incubated under greenhouse conditions.

1. Introduction

Morus nigra is an exotic plant of unknown origin, with wild populations occurring in the Aegean Region of Turkey (Browicz, 2000). Its fruits are a rich source of phenolic compounds such as flavonoids (Arfan *et al.,* 2012).

Its propagation via seeds is limited due to a high degree of heterozygosity and a long period of sexual immaturity to reach fruit production (Vijayan, 2014). Mulberry (*Morus* sp.) is commercially propagated via the rooting of stem cuttings. However, *in vitro* clonal production of plantlets via micropropagation could also be achieved using MS medium supple-

mented with benzyladenine (BA) at concentrations of 0.5 to 2.5 mg L^{-1} for multiple shoot induction and indole butyric acid (IBA) or naphthalene acetic acid (NAA) at concentrations of 0.25 to 2.0 mg L^{-1} for root induction, in different species of this genus (Chitra and Padmaja, 2005; Ahmad *et al.,* 2007; Ji *et al.,* 2008; Chattopadhyay *et al.,* 2011). Some protocols have also used gibberellic acid (GA_3) for the elongation of shoots (Gogoi *et al.,* 2017).

Although plant growth regulators (PGRs) such as BA, IBA, and NAA promote and regulate the *in vitro* development and improve the efficiency of micropropagation, the addition of PGRs to the culture medium may also result in undesirable effects, varying from the loss of quality of generated plantlets and phenotypic variations, to certain undesirable genetic mutations called somaclonal variations (Bairu *et al.,* 2011).

The control of environmental conditions in a growth chamber or culture room for *in vitro* propagation is another requirement of commercial micropropagation, which increases the costs of micropropagated plantlets (Chen, 2016). Cardoso *et al.* (2013) suggested that the use of photoautotrophic micropropagation following pre-acclimatization in a greenhouse using natural light could reduce the costs of this technique, along with enhanced *in vitro* plantlet quality.

This study aimed to develop a simple protocol for the micropropagation of black mulberry (*M. nigra*) without the use of plant growth regulators (PGR-free micropropagation). The study also evaluated the use of *in vitro* pre-acclimatization under greenhouse conditions for the acclimatization of generated *M. nigra* explants.

2. Materials and Methods

Establishment of in vitro cultures of Morus nigra

Apical and axillary buds procured from mature (4-year-old) field-grown plants of *M. nigra* cv. 'Portuguesa' were used for the establishment of *in vitro* cultures. Young shoots of 1 to 2 cm length were immersed in 70% (v/v) ethyl alcohol for 30 sec, followed by surface sterilization in 40% sodium hypochlorite solution (containing 2.0-2.5% of active chlorine) for 15 min. After asepsis, the explants were thoroughly washed with sterile distilled water and trimmed further to a length of 3-5 mm using a scalpel blade, under a laminar airflow hood. The explants were then inoculated in flasks containing 30 mL MS medium (Murashige and Skoog, 1962) with half strength of macronutrients (½ MS) and supplemented with 30 g L^{-1} sucrose, 0.1 mg L^{-1} myo-inositol, 1.0 mg L^{-1} BA, and 0.1 mg L^{-1} NAA. The pH of the culture medium was adjusted to 5.8±0.05, after which 6.4 g L^{-1} agar-agar was added and the medium was autoclaved at 121°C and pressure of 1 kgf/cm² for 20 min. The culture flasks were incubated in a growth room with a light intensity of 2,500 lux (cold fluorescent lamps of 20 watts), 16-h photoperiod, and temperature of 25±2°C.

After *in vitro* establishment, the explants were maintained every 30 days by sub-culturing the nodal segments in ½MS culture medium without the addition of plant growth regulators, until the numbers of segments obtained were sufficient to execute the experiments.

Effect of different PGRs on in vitro shoot multiplication

The basal culture medium described for the *in vitro* establishment (½MS medium supplemented with 30 g L^{-1} sucrose, 0.1 mg L^{-1} myo-inositol) was used throughout the study, with variations in the types and concentrations of the most commonly used PGRs for the genus, *viz.,* PGR-free; BA at 0.25, 0.50, and 1.0 mg L^{-1}; Thidiazuron (TDZ) at 0.10 mg L^{-1}; and GA_3 at 3.0 mg L^{-1} (Vijayan, 2014).

For the experiment, the nodal explants (1.0±0.2 cm) with one axillary bud were obtained from *in vitro* cultures previously grown on PGR-free ½MS medium for 30 days. The growth conditions were identical to those described previously.

The experiments were conducted using a completely randomized design (CRD), with five replicates (flasks) containing four nodal explants in each. After 30 days of incubation of the culture, growth parameters such as shoot length, number, and width of leaves, and multiplication rate (number of new nodal segments obtained from each inoculated explant) were evaluated for each treatment. All the measurements were carried out using a digital caliper.

In vitro rooting and acclimatization

The nodal explants (1.0±0.2 cm) containing one axillary bud were inoculated in flasks containing ½MS basal medium without PGRs. Eight treatments in a 2×4 factorial design, with two environmental conditions, i.e., culture room and greenhouse, and four different durations of *in vitro* culture (7, 14, 21, and 28 days) were used. The culture room conditions were identical to those described in the establishment phase, while the greenhouse was covered with

diffusion agricultural plastic sheets of 150 µm thickness, with a light intensity of 65000±3000 lux, and average temperature ranging from 28.1 to 29.5°C during the period of *in vitro* rooting and acclimatization phase. Each treatment comprised of four flasks containing five nodal segments in each. The *in vitro* assessments were conducted after each period of growth (7, 14, 21, or 28 days). The number of nodal segments with new sprouts and roots was the parameter used for the evaluation. The field-based assessments were also conducted after 30 days of plant growth under greenhouse conditions on a commercial substrate consisting of peat (Pindstrup®, Denmark). The evaluation parameters included the height of plantlets, and the number and width of leaves. All the measurements were performed using a digital caliper.

The obtained data were analyzed using analysis of variance (ANOVA) and the means were subjected to Tukey's comparison post-test at 5% probability. The software ASSISTAT version 7.7 beta was used for the statistical analysis (Silva and Azevedo, 2006).

3. Results and Discussion

Successful establishment of Morus nigra

The development was initiated through the growth of shoots with fresh leaves after 14 days of inoculation, without any contamination or oxidation of explants. The first subculture was carried out after 30 days of inoculation and the shoots were multiplied until sufficient quantity of the explants was obtained for the ensuing micropropagation experiments.

PGR-free culture medium resulted in single-step micropropagation and higher quality of plantlets

The nodal segments cultured on PGR-free ½MS medium showed the best average height of plants (6.4 cm), with a good number (4.4) and width of leaves (1.8 cm). The addition of 0.25 mg L^{-1} and 1.0 mg L^{-1} BA increased the multiplication rate from 4.1 (PGR-free) to 4.7 and 5.24, respectively (Table 1).

However, the addition of BA, even at the lowest concentration used in culture media (0.25 mg L^{-1}), negatively affected the quantitative (height of plants and number and width of leaves) (Table 1) as well as qualitative parameters of regenerated shoots, showing callus formation at the basal cut ends and yellowish leaves (Fig. 1 A-C), compared with PGR-free medium (Fig. 1 F). These symptoms were more intense (yellowish-brown leaves) in case of TDZ, even at a low concentration of 0.1 mg L^{-1} (Fig. 1 D). The addition of GA_3 resulted in a reduction in the number of leaves and axillary buds, leading to a lower multiplication rate (Table 1; Fig. 1 E) than the PGR-free medium.

In most of the protocols related to *in vitro* micropropagation of *Morus* sp., the addition of cytokinins

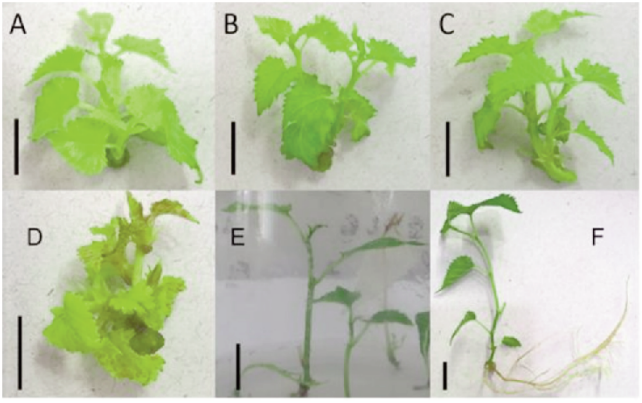

Fig. 1 - Shoots of *Morus nigra* in different types and concentrations of plant growth regulators (PGRs): (A) 0.25 mg L^{-1} BA; (B) 0.50 mg L^{-1} BA; (C) 1.0 mg L^{-1} BA; (D) 0.10 mg L^{-1} TDZ; (E) 3.0 mg L^{-1} GA_3. (F) PGRs-free MS½ medium. Bar = 1 cm.

Table 1 - Effects of culture medium with different PGRs on the *in vitro* development and multiplication of nodal segments of *Morus nigra*

Treatment	Plantlet		Leaves	
	Height (cm)	Multiplication rate*	Number	Width (cm)
PGR-free	6.4 A	4.1 BC	4.5 A	1.8 A
BA 0.25	3.5 B	4.7 AB	3.7 B	1.2 B
BA 0.50	3.5 B	5.0 A	3.7 B	1.1 B
BA 1.00	3.3 B	5.2 A	3.6 B	1.1 B
GA_3	3.6 B	2.2 D	2.4 C	1.2 B
TDZ	2.3 C	3.6 C	3.4 B	1.1 B
F value (PGRs)	18.24	22.66	7.86	16.59
Coefficient of variation (%)	38.3	26.4	30.8	26.6

* Multiplication rate referring to the mean number of nodal segments obtained by each segment inoculated *in vitro*.
Average values followed by the same letter in the column do not differ from each other by Tukey's test at 5% probability.

to the culture medium had shown positive effects on the multiple shoot induction (Chattopadhyay *et al.,* 2011; Lalitha Natarajan *et al.,* 2013). Until now, no report was available on the use of PGR-free culture medium for the *in vitro* multiplication of *Morus* sp. Yadav *et al.* (1990) observed a high shoot multiplication rate from shoot apex (4.2) and nodal explants (11.3) in *M. nigra* using MS medium supplemented with 1.0 mg L^{-1} BA. They also recorded a high shoot multiplication rate (6.3) with nodal explants using kinetin (KIN). Anis *et al.* (2003) recorded *in vitro* multiplication rates ranging from 2.4 to 5.2 using 2.0 mg L^{-1} BA and 0.2 mg L^{-1} NAA in *M. alba*. On the other hand, although Akram and Aftab (2012) did not observe multiple shoot induction in *M. macroura* using either BA or KIN singly in MS medium, the supplementation of BA with IBA in the culture medium resulted in a high multiplication rate of 4.7 shoots/explant.

In the current study, the excision of shoot apical meristem (SAM) of *M. nigra* helped in breaking the dormancy of axillary buds in nodal explants cultured *in vitro* and increased the level of cytokinin. Excision of the SAM also caused a reduction in the endogenous levels of auxins and enhanced the expression of genes encoding Isopentenyl transferase (IPT), a key precursor enzyme for the biosynthesis of endogenous cytokinin. This increased the level of endogenous cytokinin in the axillary buds, resulting in stimulation of cell division and shoot development (Tanaka *et al.,* 2006). The highly resistant nature of BA to the degradation activity of cytokinin oxidases explained the largely improved *in vitro* shoot productivity when this PGR was added to the culture medium (Ashikari *et al.,* 2005).

The use of PGR-free medium resulted in the production of shoots and roots without any supplementation. However, Yadav *et al.* (1990) observed that IBA was more effective in the promotion of rooting in *M. nigra* as compared to IAA and NAA, and increased the number of roots from 1.2 (auxin-free) to 6.1 (0.25 mg L^{-1}). These results are strikingly similar to our findings, as we also obtained only 1-2 roots per stem nodal segment without using auxins (data not shown). Nevertheless, although a lower number of roots were produced, even a single root was enough for the acclimatization of *Morus nigra* plantlets.

The plants obtained from the culture medium containing BA exhibited some morphological alterations compared with those developed in the PGR-free medium. The abnormalities included difficulty in *in vitro* manipulation during subculture due to the breakage of brittle tissues, reduced internodal length, and yellowish leaf appearance. These symptoms of hyperhydricity were observed irrespective of the BA concentration (Fig. 1A-C). The repeated subcultures on BA containing medium led to somaclonal variations in the micropropagated plants (Nasser and Mahmood, 2014). Nanism, hyperhydricity, and chlorosis were some of the abnormalities reported in the *in vitro*-raised plants with continuous use of high concentrations of BA (Israeli *et al.,* 1991; Kumar *et al.,* 1999). In the current study, no morphological abnormality was observed in the obtained plantlets, even after 24 months with mensal subcultures, when PGR-free culture medium and nodal explants of 1.0±0.2 cm were used.

Greenhouse pre-acclimatization did not show optimal development of plantlets

A notable difference was observed in the shoot development from *in vitro* nodal segments of mulberry after seven days of inoculation and 21-28 days of growth in growth room and under greenhouse conditions, with about 100% of nodal explants producing new shoots in the growth room compared to only 80% under greenhouse conditions (Fig. 2 A). However, the highest impact of environmental conditions was observed on the rooting, where only 40% of the nodal segments produced roots in the greenhouse compared to 90% in growth room conditions (Fig. 2 B). Although up to 80% shoot regeneration from the nodal explants was recorded after 14 days of culture in greenhouse conditions, the rate later reduced to 70% and 40% after 21 and 28 days, respectively. This could be attributed to the death of plantlets after cultivation for 14 days in greenhouse conditions, possibly due to excessive ambient light intensity (above 68,000 lux), which caused maximum daytime temperatures reaching between 35.1 to 36.7°C. This temperature was around 10°C higher in comparison to the standard growth room conditions, i.e., 25±2°C temperature at a light intensity of 2,500 lux.

High temperatures may lead to the alteration of cellular processes, such as protein expression (Koini *et al.,* 2009; Stavang *et al.,* 2009) and the hormonal regulation of development by, for example, cytokinin (Macková *et al.,* 2013). The acquisition of thermotolerance (acclimatization) requires the expression of heat shock proteins (HSPs) in order to avoid oxidative stress and involves the synthesis of abscisic acid (ABA) (Penfield, 2008). ABA is a well-known phytohormone, which inhibits the growth of plants under

environmental stress or pathogen attack (Sharp and LeNoble, 2002). Therefore, it could result in the reduced shoot and root formation in the nodal segments of *M. nigra* under the extreme light and temperature in greenhouse conditions. Future experiments should use covers to reduce the light intensity and extreme temperatures in the flasks, similar to those used for the gerbera pre-acclimatization tests (Cardoso *et al.*, 2013).

The *in vitro* culture of micropropagated plantlets during elongation and rooting phase under greenhouse conditions is called as pre-acclimatization. This has been successfully executed in gerbera (*Gerbera jamesonii*) and was reported to promote the growth and acclimatization rate of gerbera plantlets and reduce the costs of micropropagation (Cardoso *et al.,* 2013). The major differences between the previous and present studies lie within the partial control of environmental factors, which in the previous study were maintained as 25±5°C, PPFD 100 µmol m^{-2} s^{-1} and a 12-h photoperiod, i.e., natural growing conditions for gerbera. In contrast, in our study with *M. nigra*, the incidence of high light intensity (900-1800 µmol m^{-2} s^{-1}), a temperature exceeding 30°C, and longer photoperiod (12:30 h) proved detrimental to *in vitro* as well as field-based development of plantlets.

Growth room environment for 28-days of in vitro cultivation provided optimum conditions for in vitro rooting and acclimatization

As shown in Table 2, the previously observed effect of *in vitro* conditions on the development of plantlets was also reflected during the acclimatization stage under greenhouse conditions. The nodal explants maintained *in vitro* under greenhouse conditions also resulted in reduced development of plantlets in the acclimatization stage.

The duration of *in vitro* culture incubation also affected the development of acclimatized plantlets of *M. nigra*. Although a 7-day-incubation period resulted in 96% survival, plantlets were better developed with a 28-day-incubation period before acclimatization (Table 2). The plants maintained for different durations under *in vitro* greenhouse conditions showed improvement only in leaf width (0.57 cm at 7-d versus 1.79 cm at 28-d), with no gain in terms of shoot length and number of leaves (Table 2; Fig. 2C). On the other hand, plants grown under culture room conditions showed better *ex vitro* acclimatization of *M. nigra* plantlets, as the shoots obtained after 28 days of *in vitro* incubation were longer (9.2 cm) and had greater leaf width (3.2 cm) compared to those incubated *in vitro* in the greenhouse (3.8 cm and 1.8 cm, respectively).

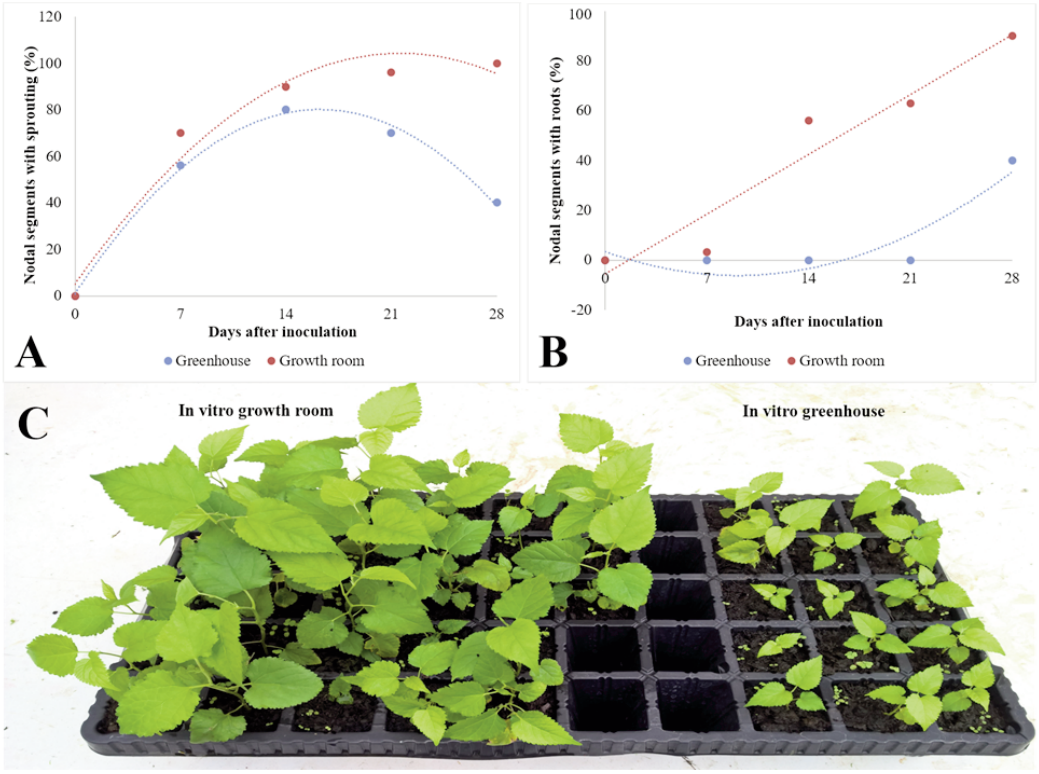

Fig. 2 - Number of nodal segments with new shoots (A) and roots (B) along the period of 28-days of in vitro cultivation. Plants after 30 days from ex vitro acclimatization (C) and previous *in vitro* cultivated under growth room (left) and greenhouse (right).

Table 2 - Influence of the *in vitro* cultivation period (7, 14, 21 and 28-days) and environmental conditions (growth room and greenhouse) on the acclimatization stage of plantlets of *M. nigra*

Environmental conditions	Time of *in vitro* cultivation (days)			
	7	14	21	28
Survival of plants (%)				
Growthroom	96	86	73	86
Greenhouse	73	83	53	50
Length of shoots (cm)				
Growthroom	2.8 bA	4.6 bA	4.3 bA	9.2 aA
Greenhouse	2.2 aA	1.9 aB	2.1 aB	3.8 aB
Number of leaves				
Growthroom	3.6 A	4.5 A	4.5 A	4.8 A
Greenhouse	3.3 A	2.7 B	3.7 B	3.5 B
Leaf width (cm)				
Growthroom	0.78 cA	1.20 bcA	1.42 bA	3.23 aA
Greenhouse	0.57 bA	0.75 bA	0.97 bA	1.79 aB
F test	LS	NL	DL	
Environment	**	*	**	
Period	**	**	**	
Environ x Period	**	NS	**	
Coefficient of variation (%)	30.7	16.9	27.0	

Values with the same letters - upper case between cultivation ambient and lowercase between conduction period - do not differ from one another by Tukey's test. Significant at 1 (**) and 5 (*) of probability. NS - not significant.

The culture room conditions of programmed and controlled temperature with low light intensity were conducive enough for the production of plantlets with healthy shoots and roots, and consequent successful acclimatization (100%) of *Morus nigra*.

4. Conclusions

A single-step protocol using PGR-free culture medium for the *in-vitro* shoot induction, multiplication, and rooting was optimized for efficient large-scale production of *M. nigra* plantlets. The micropropagated shoots of *M. nigra* were maintained in PGR-free culture medium for two years without any symptoms of morphological abnormalities or somaclonal variations. The *in vitro* rooting under culture room conditions after 28 days presented better response during the acclimatization phase, with the production of plantlets of superior quality. Since pre-acclimatization in the greenhouse did not result in enhanced adaptation of plantlets, further extensive experiments with more variations in the controlled conditions of temperature and light intensity should be undertaken in order to optimize a viable and efficient method for the cost-effective micropropagation of *Morus* sp.

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Short note

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Pollen viability and *in vitro* germination of six pistachio (*Pistacia vera* L.) cultivars grown in northern Jordan

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Key words: in vitro germination, Jordan, pistachio, pollen storage, pollen viability.

Abstract: This study was conducted on six pistachio cultivars (Lazaourdi, Nab-El Jamal, Boundiki, Batouri, Marawhi and Aschouri) to investigate the percentage of pollen viability and *in vitro* pollen germination under stored and non-stored (fresh) conditions. The results indicated that there was a significant interaction between pollen viability of pistachio cultivars and storage period. This study showed that the non-stored (fresh) pollen of cultivars Batouri and Lazaourdi had significantly the highest viability (87%) and *in vitro* pollen germination (69.7%), respectively; indicating that such cultivars could be used as best pollinators. On the other hand, cultivar Nab-El Jamal had the lowest viability (43.7%) and *in vitro* pollen germination (40.3%). It was found that pollen viability for all stored pollen cultivars were significantly reduced by 30% when compared with non-stored (fresh) pollen. However, stored pollen germination for one month was zero for all pistachio cultivars. In addition, the results of viability for all fresh pollen cultivars were poorly linearly correlated (r^2=0.149) with the results of *in vitro* germination of fresh pollen. Further research is required to examine both pollen viability and *in vitro* pollen germination under different short and long-term storage periods and methods.

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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

Pistachio (*Pistacia vera* L.) is a member of the family Anacardiaceae (Ak *et al*., 2016). Pistachio trees are dioecious, meaning that the pistillate and staminate flowers are formed on different trees. Pistachio trees are wind pollinated as flowers have no petals to attract insects (Hosseini *et al.,* 2015). One male tree is required for every eight female trees, but this ratio is usually not observed in orchards (Bahramabadi *et al.,* 2018). Therefore, the amount of pollen produced in each cluster and germination rate of pollen must be high in male trees (Ak *et al.*, 2016). Pistachio is mainly cultivated in warm-temperate to subtropical parts of the world for its commercially valuable and edible seeds (Tilkat and Onay, 2009). The center of origin of pistachio species in the Near East includes the Central Asia and Turkey. Jordan ranks number 13 for pistachio production in the world and has a world share of 0.1 %. Production quantity of pistachio in Jordan increased from 10 tons in 1975 to 967 tons in 2016 (FAO, 2016),

with an increase in harvested area up to 301 hectares in 2016.

In vitro pollen germination is a very useful technique because it can unravel the physiological and biochemical conditions required for the successful pollen germination and pollen tube development (Sanjay *et al.*, 2016). In addition, *in vitro* pollen germination is one of the most convenient and reliable methods used to test the viability of fresh or stored pollen. However, pollen grains of *Pistacia vera* L. have been considered to be difficult to germinate *in vitro* (Golan-Goldhirsh *et al.,* 1991). On the other hand, pollen viability usually refers to the ability to distribute functional sperm cells to the embryo sac following compatible pollination (Shivanna and Ram, 1993). The quality of pollen is evaluated on the basis of viability and vigor. Actually, pollen represents a critical stage in the life cycle of plants because viable pollen is essential for effective reproduction of sexual plants.

There has been recurrent interest in developing reliable methods for short and long-term storage of pistachio pollen (Ateyyeh, 2012). Such methods would be useful in storing pollen to be used in pistachio breeding programs (Vithanage and Alexander, 1985) and supplemental pollination programs in pistachio production (Crane and Iwakiri, 1981) which requires collection of sufficient amounts of pollen and its storage for short (hours to weeks) or long (months to years) periods, while maintaining viability (Shivanna and Sawhney, 1997). In addition, storing pollen is very important for cross-pollination, crop breeding, physiology, biotechnology, plant biodiversity and its conservation (Polito and Luza, 1988).

In this study, six pistachio cultivars were grown in Maru Agricultural Research Station, Irbid, Jordan since 1984 (all cultivars were introduced from Syria). The percentages of pollen viability and *in vitro* pollen germination have never been tested in our orchard and these percentages may be varied among these tested cultivars. Thus, the overall aim of this experiment was to investigate *in vitro* germination and pollen viability in six pistachio cultivars under non-stored (fresh) and stored pollen in refrigerator for one month. In addition, this experiment was performed to find a correlation between *in vitro* pollen germination and viability, to check efficiency of stored pollen and to determine which pistachio cultivar could be recommended as best pollinator.

2. Materials and Methods

Plant material and location

This research was carried out on six pistachio cultivars: Lazaourdi, Nab-El Jamal, Boundiki, Batouri, Marawhi and Aschouri from Pistachio orchard at Maru Agricultural Research Station. This station is located in Irbid governorate at 32° 33' mN latitude, 35° 51' E longitude and 589 m above mean sea level (Al-Ghzawi *et al.,* 2018). Maru has typical Mediterranean climate conditions with hot and dry summer and an average annual precipitation of about 380 mm and represents an intermediate drought area. *In vitro* pollen germination and viability tests were carried out at the biotechnology laboratory of the National Agricultural Research Center (NARC), Baqa'a, Jordan.

Pollen collection

Pollen collection took place during the flowering period from 3rd to 6th of April, 2018. Pollen was collected from six pistachio cultivars. Upon anthesis, pistachio clusters of each cultivar were detached and shacked to a glass square. Care was taken through this process to prevent contamination. Afterwards, pollen of each cultivar was placed in a closed vial to be used later on.

Pollen storage

For each pistachio cultivar, samples of pollen were placed in small glass vials and these samples of non-stored (fresh) pollen were immediately taken to laboratory for *in vitro* germination and viability tests. Other samples were stored in refrigerator at 4°C for 1 month to compare *in vitro* pollen germination and viability with those of non-stored pollen.

In vitro *pollen germination test*

The medium used for *in vitro* germination testing as recently described by Ateyyeh (2012) contained 1% agar, 15% sucrose and 100 ppm boric acid (H_3BO_3). Pollen grains were placed on medium and incubated at 24°C for 24 hours. After this time, 100 pollens from each cultivar were counted using light microscope to estimate the percentage of *in vitro* pollen germination. For each cultivar, 3 replicates (petri-dishes) were used. Pollen is considered to be germinated if the developed pollen tube is exceed (2-3 times) of its diameter.

Pollen viability test

Pollen viability was estimated as described by Ateyyeh (2012) by using 1% TTC (2, 3, 5-triphenyl

tetrazolium chloride) and 60% sucrose. TTC-sucrose solution was stored in brown glass bottle in a refrigerator. One drop of solution was placed onto microslide then a small amount of pollen was suspended in the drop and cover glass was placed onto the microslide, wrapped with aluminum foil and incubated in chamber room at 28°C for 60 minutes. After incubation, 100 pollens from each cultivar were counted using light microscope to estimate the percentage of pollen viability. For each cultivar, 3 replicates (microslides) were used. Pollen grains stained orange or bright red color were considered viable.

Experimental design and statistical analysis

The experiment was performed in a factorial design with six pistachio cultivars and two storage treatments (non-stored and stored pollen for 1 month) to investigate pollen viability and *in vitro* pollen germination separately. There were three replicates for each cultivar and storage treatment.

Data were analyzed by factorial ANOVA. When there were significant interactions, one-way ANOVA was used and means were separated using least significant difference (LSD).

3. Results and Discussion

In vitro *pollen germination*

Both proper pollination and pollen vigor are essential for pistachio productivity since the marketable portion is the seed. To obtain a good fruit set, pollination and fertilization are required. Previous studies reported that the yield and quality of nuts were influenced by pollen performance in pistachio (Acar and Kakani, 2010). It has been indicated that the validity of the *in vitro* evaluation of pollen germination is a predictor of *in vivo* behavior (Acar and Kakani, 2010).

The percentages of *in vitro* pollen germination for six pistachio cultivars are summarized in Table 1. The results indicated that the germinability of pistachio pollen varies according to the cultivar under non-stored (fresh) condition (Table 1). In particular, fresh pollen of cultivar Lazaourdi had significantly the highest *in vitro* germination percentage (69.7%), followed by cultivars Aschouri and Boundiki, then cultivars Batouri and Marawhi. However, fresh pollen of cultivar Nab-El Jamal had significantly ($P < 0.01$) the lowest germination percentage (40.3%). In addition, Acar *et al.* (2010) found that under *in vitro* conditions, pollen germination showed that Atli, Uygur and Kaska male pistachio cvs were generally better than their F1 hybrids.

Contrary to the reports of Polito and Luza (1988) who found that pistachio pollen lost its germinability after several days, preliminary tests of Vaknin and Eisikowitch (2000) revealed that fresh pollen lost most of its germinability within several hours. Results of Vaknin and Eisikowitch (2000) indicated that freshly collected pollen showed the highest germination rate (76.7%). In addition, Günver-Dalkılıç and Dayı-Doğru (2011) found that pollen grain germination ratio for pistachio was changed between 78.22% and 63.29% under room conditions at initial day (day 1) in pistachio. However, pollen grain germination ratios were found between 55.83% and 43.26% in pistachio at the 2nd day storage in refrigerator.

After one month of pollen storage in refrigerator, *in vitro* pollen germination was zero for all pollen pistachio cultivars (Table 1). This is supported precisely with findings of Ateyyeh (2012), suggesting that the

Table 1 - *In vitro* pollen germination and pollen viability percentage of six pistachio cultivars grown in Maru Agricultural Research Station under non-stored (fresh) and stored pollen for one month at 4°C

Cultivars	*In vitro* pollen germination (%)		Pollen viability (%)	
	Non-stored pollen (fresh)	Stored pollen (1 month)	Non-stored pollen (fresh)	Stored pollen (1 month)
Lazaourdi	69.7 a	0	66.7 b	43.0 d
Nab-El Jamal	40.3 d	0	43.7 d	34.3 e
Boundiki	60.0 b	0	55.0 c	35.7 e
Batouri	51.0 c	0	87.0 a	53.7 c
Marawhi	47.3 c	0	65.0 b	46.0 d
Aschouri	62.3 b	0	85.0 a	67.0 b
Mean	55	0	67	47
Standard error	1.64	0	1.64	3.24
LSD (0.05)	5.142	0	7.547	7.547

Data with the same letter in each column are not significantly different (least significant difference at P<0.05).

stored pollen in refrigerator for one month is not effective method for *in vitro* pollen germination. Furthermore, the pollen grain germination ratios for pistachio were dramatically decreased and reached to about zero starting from the 4th day of storage under room conditions and at 10th day of storage under refrigerated conditions (Günver-Dalkılıç and Dayı-Doğru, 2011). Pistachio pollen could be stored in refrigerator just for two weeks, which is enough for artificial cross pollination purpose, if the difference in flowering period between males and females didn't exceed two weeks. Vaknin and Eisikowitch (2000) found that germinability of pollen kept in the refrigerator for six days was reduced but it retained about 60%.

Pollen viability

The results showed that the percentages of pollen viability are significantly different in regard to pistachio cultivar under non-stored (fresh) and stored conditions (Table 1). Specifically, non-stored pollen of cultivars Batouri (87%) and cv. Aschouri (85%) had significantly the highest pollen viability percentage, followed by cultivars (Lazaourdi and Marawhi), and then cultivar Boundiki. Nevertheless, cultivar Nab-El Jamal had significantly (P<0.01) the lowest pollen viability percentage (43.7%). On the other hand, stored pollen for one month significantly (P<0.01) reduced pollen viability percentage in all pistachio cultivars when compared with non-stored pollen. For example, the highest and lowest pollen viability were in cultivars Aschouri (67%) and Nab-El Jamal (34.3%); respectively under stored conditions for one month (Table 1). The mean percentage value for fresh pollen viability for all pistachio cultivars was 67%, while for those of stored pollen viability was 47%. Thus, all pistachio cultivars lost approximately 30% of their pollen viability when pollen stored at 4°C in refrigerator. However, the mean value for fresh *in vitro* pollen germination for all pistachio cultivars was 55%. Therefore, the percentage of pollen germination was 18% less than those for pollen viability under non-stored conditions.

Günver-Dalkılıç and Dayı-Doğru (2011) found that the highest and lowest pollen grain viability ratios were obtained as 88.24% (in safranin test) and 70.18% (in TTC test), respectively, in pistachio types (4 male pistachios (*Pistacia vera* L.) grafted on terebinth). Ateyyeh (2012) found that fresh pollen viability of pistachio was 87.4% during 2006/2007 season. Ateyyeh (2012) found that pollen viability of pistachio under refrigerated conditions for 4 weeks was 0% and 38.7% for seasons 2006/2007 and 2007/2008; respectively. In fact, many factors affect pollen viability and longevity such as genetic variation between species, abiotic environmental conditions, temperature, moisture content, oxygen pressure, nutritional and physiological conditions under which the plants are grown, and the methods of pollen collection and storage (Barnabas and Kovacs, 1997). It was suggested that the loss of pollen viability in the course of short-term storage is directly related to changes in the water content of the pollen grains, rather than to a deficiency of essential metabolites (Barnabas and Kovacs, 1997).

Correlation between tests

There was a significant (P<0.05) regression of *in vitro* pollen germination on viability (Fig. 1), meaning that there is a relationship (linear correlation) between *in vitro* germination and viability for the fresh or non-stored pollen but with poor fit (r^2=0.149). Particularly, *in vitro* pollen germination and viability percentages were similar for cultivars (Lazaourdi, Nab-El Jamal and Boundiki), However, for cultivars (Batouri, Marawhi and Aschouri), *in vitro* pollen germination was less than viability percentages. This is why the overall correlation between pollen viability and *in vitro* germination is weak under non-stored pollen condition. On the other hand, there was no correlation between *in vitro* pollen germination and viability when pollen stored in refrigerator at 4°C as pollen germination was zero for all tested pistachio cultivars. Pollen viability has been correlated with *in vitro* pollen germination in Banksia and some other *Proteaceae* Plants (Schori *et al*., 1992). A positive and highly significance correlation between different pollen viability stains and pollen germination test in *Momordica* species

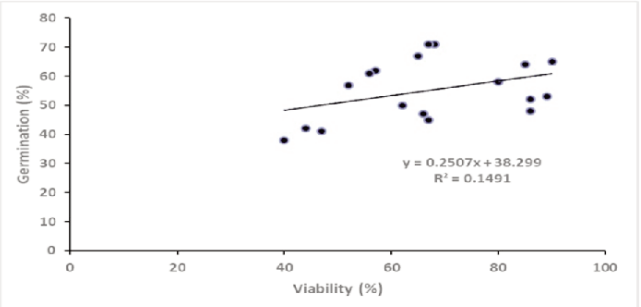

Fig. 1 - Correlation between viability and *in vitro* germination of fresh pollen in six pistachio cultivars grown in Maru Agricultural Research Station. Significant regression at P<0.05. n=3.

(Rathod *et al*., 2018), Even though, very high percent pollen viability was reported but when the germination test was performed the pollen germination was not showed more than 70 %. It has been pointed out that *in vitro* pollen germination rates are considered the best indicator of pollen viability (Shivanna *et al*., 1991). Furthermore, a good correlation was revealed between *in vitro* pollen germination with fruit and seed setting in three ornamental tropical tree species (Sanjay *et al*., 2016).

4. Conclusions

A significant interaction between pollen viability of pistachio cultivars and storage period was found in this experiment. Pollen viability in all pistachio cultivars was reduced under storage conditions. Pistachio cvs. Batouri and Lazaourdi might be used as best pollinators. Based on our results, it is not recommended to store pollen in refrigerator for one month for germination purposes. Further research is necessary to test pollen germination under different conditions and periods of storage.

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