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In vitro evaluation of shoot induction and proliferation protocol for olive cultivars by assessing morpho-physiologic effects of pre-cooling and growth regulators

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Abstract

To set a definite micro-propagation protocol for olive has always remained a challenging task owe to its woody nature. This study was consist of different olive cultivars (Arbosana, Arbequina, Koroneiki and Sorany) to devise a comprehensive shoot induction and proliferation protocol of olive media supplemented with various combinations of growth regulators (zeatin and BAP) and pre-cooling treatments (0, 24 and 48 hours). The *in vitro* performance of all the cultivars was assessed using a range of parameters including, morphological (length of primary shoot, percentage of induced shoots, number of leaves shoot^{-1}, number of shoots explant^{-1}), and physiological (chlorophyll a, chlorophyll b, carotenoids, CO_2 absorption. All cultivars indicated encouraging results for pre-cooling, and increasing concentration of hormones, zeatin and BAP. However, performance of 48 hrs pre-cooling, Arbosana and Arbequina cultivars, and zeatin hormone was outstanding. Furthermore, the interaction of 48 hrs pre-cooling with 2.5 mgL^{-1} zeatin exceed to BAP as revealed by the parameters included in the study. Likewise, SEM micrographs endorsed the higher affectivity of zeatin compared to BAP in accelerating the uptake of nutrients from olive medium. Findings of the study present more reliable results for olive shoot induction and proliferation as it basis on wide range of parameters.

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Introduction

Olive (*Olea europaea* L.) production industry is growing in many individual countries as well as on a world scale. Consequently, there is a foremost demand for increasing olive trees which is not possible to accomplish with conventional propagation techniques of cuttings and grafting. For this reason, micro-propagation is preferred as it provides opportunity to produce a large number of plants that can be stored for longer in a small area. Nevertheless, to date several micro-propagation protocols have been optimized for different plants, however development of definite micro-propagation protocol for olive has always remained a challenge due to explant oxidation (Fabbri *et al.,* 2009). In the past decade limited success has been achieved for shoot induction and proliferation from axillary buds (Lambardi *et al.*, 2006; Fabbri *et al.*, 2009). The previous studies show that in vitro propagation of olive is highly dependent on the effectiveness of culture medium as well as supplementation of growth regulators for obtaining the reliable results. Zeatin as a growth regulator in olive medium is considered as an important supplement for the induction of desirable shoot proliferation (Rugini, 1984; Micheli *et al.,* 2009). Similarly, Revilla *et al.* (1996) reported successful micro-propagation of mature olive cultivars using Kuniyuki walnut medium (Driver and Kuniyuki, 1984) provided with sucrose as carbohydrate source, while benzylaminopurine (BAP) and indole butyric acid (IBA) as growth regulators. Peixe *et al.* (2007), has proposed that BAP and coconut water can be used instead of zeatin in olive micropropagation. Many researchers' confirm the importance of phytohormones because of their key role in control of growth and development (Zuccherelli and Zuccherelli 2002; Peixe *et al.*, 2007; Mazri *et al.*, 2011; Chaari-Rkhis *et al.*, 2011; Rabelsi *et al.*, 2011).Hormones regulate wide range of physiological processes in plants that plays pivotal role in signal transduction, metabolite production and activation of genes (Gururani *et al.*, 2015**;** Aslam *et al.*, 2016; Shah *et al.*, 2016). However, they are required in balanced amount because interaction between phytohormones determines growth and development and other processes of plant (Pazurkiewicz-Kocot *et al.*, 2008). Along with adjustment of tissue culture media, explants pre-cooling treatments prior to culture manifests higher percentage of shoot induction due to breakage of dormancy, as pre-cooling activate the enzymes responsible for the initiation of preliminary physiological activities (Williams *et al.*, 2003; Xu *et al.*, 2011; Sardoei *et al.*, 2014).

Untill now there is no comprehensive study available to signify that how pre-cooling and growth regulators play their roles in mediating physiological processes and developmental activities during *in vitro* propagation especially in olive plant.

The main objective of this study is to evaluate shoot induction and proliferation protocol for different olive cultivars by assessing morphological and physiological of different levels of pre-cooling and growth regulators. These comprehensive findings are expected to be helpful in the optimization of definite protocol for olive shoot induction and proliferation.

Materials and methods

The current study was performed on plant material taken from same aged mature plants of four olive cultivars, Arbosana, Arbequina, Koroneiki and Sorany cultivated in the field area of Aljouf Company (*Al Jouf*, Skaka, Kingdom of Saudi Arabia). Excised nodal segments from healthy soft lateral branches were exposed to pre-cooling treatments (0, 24 and 48 hours) at 4 to 8^{o}C. After that nodal segments were grown on olive media (Rugini, 1984) with different concentrations (0, 0.5, 1.5 and 2.5 mgL^{-1}) of cytokinins, BAP and Zeatin for shoot induction and proliferation. The experiment was run two times in tri-replicate using 3 level factorial design, with pre-cooling treatments as factor A , varieties as factor B and hormones as factor C.

Assessment of morphological parameters

The nodal segments collected from healthy, soft and lateral branches of olive cultivars were prepared,

sterilized and used for *in vitro* culture on olive media according to methods described by Binet *et al.* (2007) and Chaari-Rkhis *et al.* (2011). The response of 50 to 60 days old olive cultivars was assessed on the basis of % induced shoots, length of primary shoots (cm), number of leaves per shoot and number of shoots per explant.

Assessment of physiological parameters

Chlorophyll a, b and Carotenoids: Chlorophyll and carotenoid contents were extracted from the olive leaves following method of Mahmood *et al.* (2016). Then absorbance was recorded at 645, 663 and 710 nm using UV-VIS spectrophotometer, and subsequently chl a, chl b and total carotenoids were determined using the equations of Lichtenthaler and Buschmann (2001).

Assimilation of CO_2: Assimilation rate of CO_2 was determined by using the method of Boussadia *et al.,* 2010.

Statistical analysis

The data obtained were analyzed using SAS software and the means were compared using LSD ($P \leq 0.05$; Steel *et al.*, 1997).

Scanning electron microscopy

SEM micrographs of leaves from representative plantlets were generated to compare the effect of BAP and zeatin on the uptake of nutrient elements from the medium.

Results

Growth parameters

Length of primary shoot: It was observed that pre-cooling, varieties and hormones significantly ($P \leq 0.05$) improved the length of primary shoot (cm) (Table 1).

Table 1. Effects of pre-cooling treatments, varieties and hormones levels on various growth parameters of olive explants.

Treatments	Length of primary shoot (cm)	Percentage of induced shoots	Number of leaves shoot^{-1}	Number of shoots explant^{-1}
Pre-Cooling Treatment (P)				
P_0 (0 Hour)	1.98±0.12^{b}	52.03±2.01^{b}	3.72±0.16^{c}	2.048±0.12^{c}
P_1 (24 Hour)	2.28±0.14^{ab}	59.06±1.88^{ab}	3.94±0.18^{b}	2.57±0.15^{b}
P_2 (48 Hour)	2.56±0.16^{a}	65.062.63±^{a}	4.29±0.21^{a}	2.79±0.21^{a}
LSD	0.36	12.97	0.14	0.15
Variety (V)				
V_1 (Sorany)	1.99±0.16^{c}	53.04±2.24^{d}	3.59±0.19^{d}	2.06±0.14^{d}
V_2 (Koroneiki)	2.27±0.15^{b}	58.83±2.50^{c}	3.89±0.20^{c}	2.44±0.19^{c}
V_3 (Arbequina)	2.35±0.16^{b}	59.83±2.75^{b}	4.10±0.21^{b}	2.60±0.21^{b}
V_4 (Arbosana)	2.48±0.18^{a}	63.17±3.14^{a}	4.35±0.24^{a}	2.78±0.22^{a}
LSD	0.12	0.72	0.085	0.082
Hormones (H)				
Zeatin (H_Z) (mgL^{-1})				
H_0 (0 mgL^{-1})	1.06±0.035^{d}	41.08±0.74^{d}	2.49±0.046^{d}	1.16±0.022^{d}
H_1 (0.5 mgL^{-1})	2.45±0.101^{c}	59.29±1.88^{c}	3.85±0.11^{c}	2.43±0.10^{c}
H_2 (1.5 mgL^{-1})	2.68±0.075^{b}	65.04±1.89^{b}	4.58±0.10^{b}	2.95±0.12^{b}
H_3 (2.5 mgL^{-1})	2.90±0.079^{a}	69.46±1.75^{a}	5.01±0.98^{a}	3.33±0.14^{a}
LSD	0.094	1.28	0.075	0.068
BAP (H_B) (mgL^{-1})				
H_0 (0 mgL^{-1})	1.06±0.035^{d}	41.08±0.074^{d}	2.49±0.046^{d}	1.16±0.022^{d}
H_1 (0.5 mgL^{-1})	2.04±0.083^{c}	55.42±0.81^{c}	3.41±0.10^{c}	2.11±0.094^{c}
H_2 (1.5 mgL^{-1})	2.31±0.089^{b}	61.75±1.94^{b}	4.20±0.11^{b}	2.55±0.11^{b}
H_3 (2.5 mgL^{-1})	2.56±0.084^{a}	66.13±1.88^{a}	4.59±0.99^{a}	2.99±0.15^{a}
LSD	0.082	1.31	0.1139	0.084
Significance				
P	*	ns	**	*
V	**	**	**	**
H	**	**	**	**
P x V	ns	**	*	**
P x H	**	**	**	**
V x H	ns	**	**	**
P x V x H	ns	ns	ns	ns

Means followed by the same letter (s) in each column and treatment showed no significant difference

*, ** indicate significant differences at 0.05, 0.01 probability levels respectively while 'ns' indicate non-significant difference.

Mean comparison between different levels of pre-cooling revealed that improvement in length of primary shoot by P_2 (2.56 cm) was statistically significant compared to P_0 (1.98). Among all cultivars, Arbosana revealed maximum mean value of 2.48 cm for shoot length which was significantly different from the means of other varieties. A dramatic increase in length was noticed for both zeatin and BAP, at 2.5 mg L^{-1} as compared to other levels; however zeatin depicted higher mean value (2.9 cm) as compared to BAP (2.55 cm).

Table 2. Effect of pre-cooling treatments, varieties and hormonal levels on various physiological parameters of olive leaves.

Treatments	Chlorophyll a (µg cm^{-2})	Chlorophyll b (µg cm^{-2})	Carotenoids (µg cm^{-2})	CO_2 Absorption (µmol $m^{-2}s^{-1}$)
Pre-cooling treatment (P)				
P_0 (0 Hour)	42.84±2.53^b	30.16±1.94^b	4.54±0.21^c	6.94±0.38^c
P_1 (24 Hour)	48.97±3.08^a	31.84±2.08^b	5.048±0.26^b	7.48±0.39^b
P_2 (48 Hour)	52.56±3.46^a	34.56±2.31^a	5.53±0.31^a	8.10±0.43^a
LSD	5.15	1.87	0.39	0.33
Variety (V)				
V_1 (Sorany)	44± 3^c	29.96±2.23^c	4.82±0.29^c	6.90±0.41^c
V_2 (Koroneiki)	47.58±3.38^b	32.29±2.47^b	5.30±0.36^a	7.42±0.45^b
V_3 (Arbequina)	49.54±3.77^{ab}	32.88±2.55^b	5.10±0.31^b	7.77±0.49^{ab}
V_4 (Arbosana)	51.38±4.12^a	33.63±2.60^a	4.94±0.29^c	7.94±0.51^a
LSD	2.75	0.61	0.13	0.38
Hormones (H)				
Zeatin (H_Z) (mgL^{-1})				
H_0 (0 mgL^{-1})	20.71±0.27^d	12.46±0.13^d	2.72±0.051^d	3.83±0.11^d
H_1 (0.5 mgL^{-1})	52.00±1.38^c	35.33±0.73^c	5.18±0.13^c	8.27±0.17^c
H_2 (1.5 mgL^{-1})	57.58±1.60^b	38.96±0.58^b	5.85±0.12^b	8.76±0.16^b
H_3 (2.5 mgL^{-1})	62.21±1.62^a	42.00±0.63^a	6.41±0.14^a	9.16±0.20^a
LSD	1.34	0.37	0.12	0.19
BAP (H_B) (mgL^{-1})				
H_0 (0 mgL^{-1})	20.71±0.27^d	12.46±0.13^d	2.72±0.051^d	3.83±0.11^d
H_1 (0.5 mgL^{-1})	28.63±0.71^c	20.33±0.69^c	4.18±0.13^c	5.83±0.13^c
H_2 (1.5 mgL^{-1})	33.54±0.84^b	24.79±0.84^b	4.85±0.12^b	6.41±0.13^b
H_3 (2.5 mgL^{-1})	38.67±0.78^a	28.46±0.84^a	5.45±0.14^a	6.96±0.13^a
LSD	0.6005	0.6181	0.1351	0.1669
Significance				
P	*	*	*	**
V	**	**	**	**
H	**	**	**	**
P × V	*	ns	ns	ns
P × H	*	*	**	ns
V × H	**	**	**	**
P × V × H	ns	ns	ns	ns

Means followed by the same letter (s) in each column and treatment showed no significant difference
*, ** indicate significant differences at 0.05, 0.01 probability levels respectively while 'ns' indicate non-significant difference.

Besides individual treatments, Table1 indicates significant effect of interactions between pre-cooling treatments, varieties and hormones on length of primary shoot (cm). The values for the interaction are given in Table 3, where interaction between pre-cooling and zeatin (P × H_Z) affected length significantly (P≤ 0.01), while no significant (P≤ 0.05) affect was observed for variety and zeatin interaction (V × H_Z). Moreover, significant interactions (P≤ 0.01) affect was reported for pre-cooling × BAP (P × H_B), and variety × BAP (V × H_B). The mean values of P × H interactions (Table 3) in both phases indicated that the highest values for shoot length in case of both pre-cooling treatments were at 2.5 mg L^{-1}

hormonal concentration. However, maximum mean value (3. 25 cm) of zeatin and pre-cooling interaction was higher than the maximum value (2.9 cm) of BAP and pre-cooling interaction which explicated the promising interaction of zeatin with cooling as compared to BAP. Moreover, least values of shoot lengths were observed for the interaction of Sorany cultivar with BAP while the highest results were shown by the interaction of Arbosana and BAP (Table 4). On the other hand interaction between pre-cooling and cultivar (P × V) did not affect the length of primary shoot significantly. Moreover, no three way interaction was observed.

Table 3. Effect of interaction between pre-cooling treatments and hormones (P x H) on growth and physiological parameters of olive.

		Growth parameters				Physiological parameters			
Pre-cooling treatments (P) (Hours)	Zeatin (H_Z) (mgL^{-1})	Length of primary shoot (cm)	Percentage of induced shoots	Number of leaves $shoot^{-1}$	Number of $shoots\ explant^{-1}$	Chl a ($\mu g\ cm^{-2}$)	Chl b ($\mu g\ cm^{-2}$)	TCar. ($\mu g\ cm^{-2}$)	A_{CO_2} ($\mu mol\ m^{-2}s^{-1}$)
0	0	1.00±0.09	37.7±1.42	2.46±0.09	1.07±0.04	19.6±0.38	12.3±0.25	2.63±0.08	
	0.5	2.04±0.17	50.6±2.58	3.61±0.16	1.96±0.12	46.9±1.86	32.0±0.73	4.61±0.12	
	1.5	2.36±0.10	57.5±2.95	4.16±0.15	2.40±0.10	49.9±1.44	36.9±0.74	5.22±0.10	
	2.5	2.53±0.08	62.3±2.40	4.64±0.14	2.76±0.11	55.0±0.80	39.5±0.86	5.71±0.06	
24	0	1.05±0.03	43.1±0.74	2.50±0.06	1.22±0.03	20.6±0.32	12.4±0.26	2.83±0.07	
	0.5	2.44±0.01	59.8±1.81	3.59±0.07	2.63±0.07	52.3±1.35	35.0±0.60	5.04±0.11	ns
	1.5	2.70±0.08	64.5±1.72	4.64±0.12	3.05±0.07	59.8±1.37	38.1±0.64	5.95±0.13	
	2.5	2.93±0.06	68.9±1.14	5.05±0.14	3.38±0.09	63.3±1.67	41.9±0.77	6.41±0.10	
48	0	1.14±0.04	42.4±0.60	2.51±0.1	1.21±0.10	21.9±0.30	12.8±0.16	2.72±0.11	
	0.5	2.88±0.11	67.5±2.11	4.36±0.18	2.69±0.02	56.9±2.5	39.0±0.89	5.88±0.14	
	1.5	2.99±0.10	73.1±2.49	4.94±0.12	3.40±0.24	63.1±2.78	41.9±0.61	6.37±0.14	
	2.5	3.25±0.12	77.3±2.62	5.34±0.14	3.86±0.30	68.4±3.07	44.6±0.82	7.13±0.18	
	LSD	0.1606	2.1818	0.1281	0.0409	2.2836	0.6236	0.2093	
	BAP (H_B) (mgL^{-1})								
0	0	1.00±0.09	37.8±1.42	2.46±0.09	1.07±0.04	19.6±0.38	12.2±0.25	2.63±0.08	
	0.5	1.70±0.13	47.0±2.33	3.16±0.18	1.75±0.11	24.9±0.40	17.2±0.59	3.61±0.12	
	1.5	1.95±0.1	53.5±2.95	3.80±0.15	2.13±0.12	28.6±0.42	21.1±0.40	4.23±0.10	
	2.5	2.20±0.08	58.1±2.78	4.23±0.16	2.41±0.13	34.6±0.53	24.9±0.48	4.82±0.15	
24	0	1.05±0.03	43.1±0.74	2.50±0.06	1.22±0.03	20.6±0.32	12.3±0.26	2.79±0.07	
	0.5	2.05±0.07	56.1±1.89	3.26±0.09	2.24±0.13	28.5±0.32	19.5±0.50	4.04±0.11	
	1.5	2.30±0.1	62.3±1.89	4.26±0.17	2.58±0.13	34.4±0.56	23.8±0.62	4.95±0.13	ns
	2.5	2.58±0.08	66.4±1.55	4.71±0.17	2.99±0.14	38.5±0.68	27.0±0.46	5.41±0.10	
48	0	1.14±0.04	42.4±0.60	2.51±0.1	1.21±0.02	21.9±0.30	12.8±0.16	2.73±0.11	
	0.5	2.38±0.12	63.1±2.16	3.80±0.17	2.34±0.18	32.5±0.73	24.2±0.70	4.88±0.14	
	1.5	2.68±0.14	69.5±2.52	4.54±0.13	2.94±0.21	37.6±0.68	29.5±1.05	5.38±0.14	
	2.5	2.90±0.15	73.9±2.59	4.84±0.12	3.58±0.29	42.9±0.64	33.5±0.93	6.13±0.18	
	LSD	0.1394	2.2446	0.1945	0.1433	1.0255	1.0558	0.2307	

Means having difference greater than LSD are significant at $P \leq 0.05$.

Percentage of induced shoots: It was found that cultivars and hormonal levels significantly (P≤ 0.01) increased the shoot induction percentage of explants, while no significant affect was observed for pre-cooling (Table 1). Comparison of means revealed that all cultivars were statistically different in their ability of shoot induction. Among cultivars, Arbosana exhibited significantly high shooting percentage (63.2) as compared to other cultivars. An abrupt increase in percentage was noticed for both zeatin and BAP, at 2.5 mg L^{-1} as compared to other concentrations; however zeatin showed higher mean value (69.5) as compared to BAP (66.1). Furthermore, apart from individual treatments significant (P≤ 0.01) effect of interactions between pre-cooling, cultivars and hormones were noticed on shoot induction.

The mean values of P x H interactions (Table 3) in both phases indicated that the highest values for shoot induction in case of both pre-cooling treatments were observed at 2.5 mg L^{-1} hormonal concentration. However, the value (77.3) of zeatin and pre-cooling interaction was greater than the value (73.9) of BAP and pre-cooling interaction which ratified the strong interacting tendency of zeatin with cooling as compared to BAP. Similarly, the mean values of V × H interactions (Table 4)

presented higher values for all cultivars at 2.5 mgL^{-1} hormonal level. Nevertheless, the value (75) of zeatin × Arbosana (V × H_Z) interaction was highest compared to value (72) of BAP × Arbosana (V × H_B) interaction. Moreover, comparative analysis showed that all cultivars depicted to some extent better interaction with zeatin corresponding to BAP as confirmed by slighter higher values of interaction V × H_Z (Table 4). Furthermore, the mean values of P x V interactions (Table 4) indicated that the maximum values for shoot induction in case of all pre-cooling treatments were observed for Arbosana cultivar. However, the highest value (71.8) of pre-cooling and variety interaction was observed for Arbosana cultivar at 48h cooling treatment. No three way interaction was observed.

Table 4. Effect of interaction between varieties and hormones (V x H) on growth and physiological parameters of olive.

		Growth parameters				Physiological parameters			
Varieties (V)	Zeatin (H_Z) (mgL^{-1})	Length of primary shoot (cm)	Percentage of induced shoots	Number of leaves $shoot^{-1}$	Number of shoots $explant^{-1}$	Chl a ($\mu g\ cm^{-2}$)	Chl b ($\mu g\ cm^{-2}$)	TCar. ($\mu g\ cm^{-2}$)	A_{CO_2} ($\mu mol\ m^{-2}s^{-1}$)
Sorany	0		40.0±2.17	2.28±0.08	1.13±0.05	20.3±0.61	12.1±0.31	2.65±0.11	3.67±0.17
	0.5		51.7±3.33	3.37±0.13	1.97±0.19	47.7±1.87	32.3±1.23	4.88±0.24	7.58±0.27
	1.5		57.5±3.09	4.13±0.17	2.45±0.15	52.3±2.12	36.5±1	5.63±0.2	8.05±0.25
	2.5		63.0±2.94	4.57±0.16	2.70±0.13	55.7±1.02	38.8±0.79	6.11±0.2	8.30±0.32
Koroneiki	0		40.5±1.28	2.50±0.05	1.18±0.05	21.0±0.58	12.7±0.21	2.88±0.05	3.83±0.31
	0.5		60.0±2.23	3.73±0.15	2.35±0.15	51.2±2.07	35.3±1.2	4.98±0.26	8.08±0.15
	1.5		65.5±2.29	4.48±0.15	2.85±0.23	56.7±1.96	38.8±1.24	5.63±0.25	8.67±0.21
	2.5		69.3±2.27	4.85±0.12	3.37±0.25	61.5±1.80	42.3±1.58	6.80±0.35	9.08±0.24
Arbequina	0	ns	41.5±1.43	2.60±0.04	1.17±0.03	21.0±0.37	12.3±0.33	2.71±0.08	3.83±0.17
	0.5		60.5±3.90	4.03±0.22	2.58±0.15	52.2±2.02	36.5±1.34	5.35±0.21	8.67±0.21
	1.5		66.8±3.75	4.68±0.15	3.15±0.21	59.8±3.32	39.7±0.88	5.90±0.18	9.00±0.26
	2.5		70.5±2.75	5.08±0.11	3.52±0.26	65.7±3.53	43.0±0.93	6.45±0.30	9.58±0.35
Arbosana	0		42.3±0.95	2.58±0.12	1.20±0.04	20.5±0.62	12.7±0.21	2.63±0.14	4.00±0.22
	0.5		65.0±3.87	4.28±0.21	2.80±0.18	57.0±3.74	37.2±1.49	5.51±0.30	8.75±0.44
	1.5		70.3±4.41	5.02±0.14	3.35±0.25	61.5±4.24	40.8±0.87	6.25±0.29	9.33±0.42
	2.5		75.0±4.47	5.53±0.15	3.75±0.25	66.5±4.17	43.8±0.60	6.28±0.24	9.67±0.46
	LSD		2.5192	0.1479	0.1335	2.6369	0.7201	0.2418	0.3705
	BAP (H_B) (mgL^{-1})								
Sorany	0	0.88±0.08	40.0±2.17	2.28±0.08	1.12±0.05		12.2±0.31	2.65±0.11	
	0.5	1.68±0.16	48.0±2.96	2.92±0.16	1.65±0.13		19.2±1.19	3.88±0.24	
	1.5	1.93±0.13	54.2±3.09	3.67±0.15	1.95±0.1		23.5±1.38	4.63±0.2	
	2.5	2.22±0.11	58.3±3.37	4.08±0.16	2.27±0.12		26.7±1.45	5.11±0.2	
Koroneiki	0	1.10±0.04	40.5±1.28	2.50±0.05	1.18±0.05		12.7±0.21	2.88±0.05	
	0.5	1.98±0.14	55.7±2.42	3.23±0.09	2.02±0.15		19.8±1.40	3.98±0.26	
	1.5	2.23±0.12	61.3±2.50	4.12±0.19	2.47±0.2		24.2±1.58	4.63±0.25	
	2.5	2.52±0.11	66.0±2.41	4.50±0.15	3.03±0.3	ns	28.0±1.53	5.28±0.24	ns
Arbequina	0	1.13±0.05	41.5±1.43	2.60±0.04	1.17±0.03		12.3±0.33	2.70±0.08	
	0.5	2.20±0.13	57.2±3.41	3.62±0.17	2.25±0.12		20.7±1.54	4.35±0.21	
	1.5	2.43±0.17	63.5±3.62	4.33±0.12	2.77±0.17		25.2±1.92	4.90±0.18	
	2.5	2.63±0.17	68.0±2.72	4.82±0.15	3.24±0.28		29.3±1.74	5.80±0.26	
Arbosana	0	1.13±0.05	42.3±0.95	2.58±0.12	1.20±0.04		12.7±0.21	2.63±0.14	
	0.5	2.30±0.15	60.8±4.07	3.87±0.16	2.52±0.16		21.7±1.54	4.51±0.3	
	1.5	2.63±0.17	68.0±4.49	4.68±0.14	3.00±0.18		26.3±1.96	5.25±0.3	
	2.5	2.87±0.18	72.2±4.46	4.97±0.15	3.43±0.27		29.8±2.12	5.62±0.35	
	LSD	0.1612	2.599	0.2245	0.1654		1.2190	0.2664	

Mean values having difference greater than LSD are significantly different at $P \leq 0.05$.

Number of leaves per shoot: All treatments considerably enhanced the number of leaves per shoot (Table 1).

Comparison of means for all levels of pre-cooling treatments demonstrated significant increase in leaves number by P_2 (4.29) as compared to control, P_0 (3.72). Arbosana showed highest mean of value (4.35) as compared to all other cultivars. An increase in leaf number was noticed for both zeatin and BAP at 2.5 mg L^{-1} compared to other concentrations; however zeatin depicted higher mean value (5.01) as compared to BAP (4.60). Moreover, number of leaves in each explants was significantly ($P \leq 0.01$) improved by the interactions between pre-cooling, varieties and hormones.

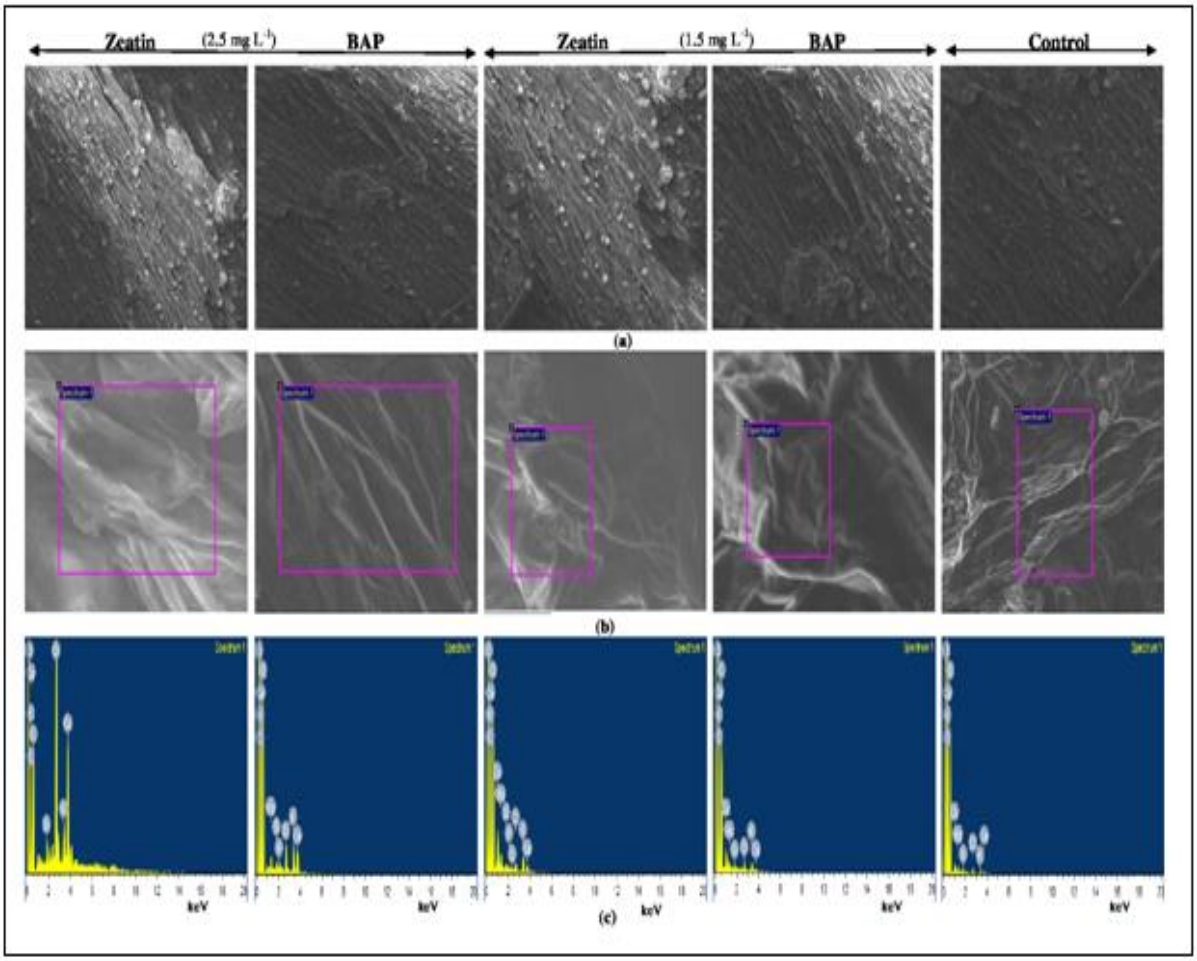

Fig. 1. SEM micrographs demonstrating comparative differences in elements level in the leaves of olive's explants propagated on olive media in the presence of growth regulators Zeatinand BAP. (a) SEM image of leave showing deposition of minerals. (b) Targeted areas for spectra study (c) spectra whose area of peaks exhibits difference in elemental levels.

The mean values of P × H interactions (Table 3) showed that the highest leaves number in case of both pre-cooling treatments was recorded at 2.5 mg L^{-1} hormonal level. Nevertheless, the mean value (5.34) of zeatin and pre-cooling interaction was greater than the mean value (4.84) of BAP and pre-cooling interaction which may validate the strong tendency of zeatin with pre-cooling as compared to BAP. Likewise, the mean values of V × H interactions (Table 4) revealed that the maximum leaf numbers for all cultivars were recorded at 2.5 mgL^{-1} hormonal concentration. Here the highest value (5.53) was recorded for zeatin x Arbosana (V x H_Z) interaction compared to the value (4.97) of BAP × Arbosana (V x H_B) interaction. Moreover, comparative analysis showed that all cultivars depict slightly better interaction with zeatin compared to BAP as confirmed by slighter higher values of interaction V × H_Z (Table 4). Besides this, the performance of cultivars cultured on the OM supplemented with zeatin was reasonably better than the media supplemented with BAP. On the other hand pre-cooling x cultivar interaction (P × V) significantly improved the number of leaves in explants. Although for both pre-cooling treatments, cultivar Arbosana depicted the higher mean values (Table 4). However, at 48 h pre-cooling treatment it showed the highest mean (4.61) followed by Arbequina (4.46). No significant effect of three way interaction was reported.

Number of shoots per explant: Significant ($P \leq 0.01$) increase in the number of shoots per explants was noticed for all treatments (Table 1). Comparison of

means for different levels of pre-cooling treatments revealed significant increase in shoot number by P_2 (2.52) as compared to other pre-cooling treatments. Among all cultivars, explants of Arbosana showed significantly high shoot number (2.78). Maximum increase in number of shoots was noticed for both zeatin and BAP, at 2.5 mg L^{-1} as compared to other levels; however zeatin depicted higher mean value (3.33) as compared to BAP (2.99). In addition to individual treatments, significant effect of interaction between pre-cooling, varieties and hormones was reported on shoot number. In case of P x H interaction, the maximum number of shoot was reported for both pre-cooling treatment at 2.5 mgL^{-1} hormonal concentration (Table 3).

Moreover, the mean value (3.86) of zeatin x pre-cooling interaction was higher than the mean value (3.58) of BAP × pre-cooling interaction which proved the strong affinity of zeatin with pre-cooling as compared to BAP. Accordingly, the mean values of V × H interactions (Table 4) revealed that the maximum shoot numbers for all explants were reported at 2.5 mgL^{-1} concentration. Furthermore, the mean (3.75) of zeatin x Arbosana interaction was greater than the mean (3.43) of BAP × Arbosana interaction. Moreover, relative investigation showed that all cultivars depicted somewhat better interaction with zeatin compared to BAP as indicated by little higher values of V × H_Z interaction (Table 4). Likewise, the number of shoots in cultivars cultured on the OM supplemented with zeatin was reasonably higher than the media supplemented with BAP. Furthermore, interaction of pre-cooling × variety (P × V) significantly ($P \leq 0.01$) increased the number of shoots. For both pre-cooling treatments Arbosana revealed maximum tendency of shoot induction as indicated in Table 4. However, the maximum mean, 3.23 was observed for Arbosana at 48h pre-cooling. No three way interaction effect of treatment was reported for this parameter.

Performance of physiological parameters

Chl a: All treatments significantly affected ($P \leq 0.01$) the formation of Chl a in the leaves of olive (Table 2). Mean comparison between different durations of pre-cooling showed that the plantlets originating from the 48h pre-cooled explants exhibited the highest level (52.6 µg cm^{-2}) of pigment. Among cultivars, statistically high chl a (51.4 µg cm^{-2}) content was observed in Arbosana. A dramatic increase in chl a was noticed for both zeatin and BAP, at 2.5 mg L^{-1} as compared to other concentrations; but zeatin illustrated higher mean (62.2 µg cm^{-2}) as compared to BAP (38.7 µg cm^{-2}). Interactions between pre-cooling and zeatin (P × H_Z), and variety and zeatin affected pigment amount significantly ($P \leq 0.01$). Moreover, significant ($P \leq 0.01$) increase in chl a was seen for pre-cooling × BAP (P × H_B), while no significant affect was observed for variety × BAP (V × H_B). The amount of chlorophyll was higher for the plants cultured on medium supplemented with 2.5mgL^{-1} hormone, after following pre-cooling treatments of 48 hour (Table 3). It elucidates that initial pre-cooling treatments and higher hormonal levels positively trigger high chl a in the leaves of olive plantlets. Besides, the mean value (68. 4 µg cm^{-2}) of P × H_Z interaction was significantly higher than the maximum mean value (42. 875 µg cm^{-2}) of P × H_B interaction. Comparative evaluations of Table 3, differentiates better interacting tendency of zeatin with pre-cooling as compared to BAP. On the other hand all cultivars depicted significantly high concentration of chl a, at 2.5 mgL^{-1} zeatin concentration as shown in Table 4. Furthermore, the chl a content was significantly ($P \leq 0.01$) high for the interaction between pre-cooling and cultivar (P × V). Though for both pre-cooling treatments, Arbosana showed the highest chl a contents (Table 4). However, the maximum mean (59.6 µg cm^{-2}) value for Arbosana was observed at 48h pre-cooling interval. No significant effect of three way interaction was observed for this parameter.

Chl b: It was observed that all treatments significantly (Table 2) improved the amount of chl b in olive leaves. Comparison of means revealed that extended duration of pre-cooling increased the amount of pigment significantly, as 48h (34.6 µg cm-

^{2}) was the most effective one. Moreover, cultivars and varying hormonal levels made statistically significant increase in pigment content, where variety Arbosana (33.6 µg cm^{-2}) and 2.5mgL^{-1} hormonal concentrations being the most effective ones (Table 2). In addition to individual treatment, significant effect of interactions between pre-cooling, cultivars and hormones was observed on chl b. The mean values of P × H interactions (Table 3) in both hormones indicated that the highest means of chl b in case of both pre-cooling treatments were at 2.5 mg L^{-1} hormonal concentration. However, the mean value (44.6 µg cm^{-2}) of zeatin and pre-cooling interaction was higher than the maximum mean value (33.5 µg cm^{-2}) of BAP and pre-cooling interaction which may provide evidence of strong interaction between zeatin with pre-cooling (Table 3). Similarly, mean values of V × H interaction (Table 4) illustrated that the higher means of chl b for all cultivars were at 2.5 mgL^{-1} hormonal level. However, the value (43.8 µg cm^{-2}) of zeatin x Arbosana interaction was larger than the value (29.8 µg cm^{-2}) of BAP x Arbosana interaction. Thus, the relative comparison of means of both interactions indicates that zeatin has strong interacting tendency with cultivars compared to BAP. The interaction between pre-cooling and cultivar (P × V) did not affect Chl b amount significantly. Moreover, no three way interaction was observed among treatments.

Total carotenoids: It was observed that all treatments significantly (P≤ 0.01) increased the total carotenoids (Table 2). Prolonged duration of pre-cooling proved more effective in increasing carotenoids content, as the highest value (5.53 µg cm^{-2}) was recorded at 48h. Moreover, cultivars and different hormonal concentrations significantly affected carotenoids, cultivars Koroneiki (5.30 µg cm^{-2}) and Arbequina (5.10 µg cm^{-2}), and hormonal concentration 2.5 mgL^{-1} being the most effective ones. Significant (P≤ 0.01) effect of interactions, P × H and V × H, were recorded for carotenoids during the study. The highest levels of total carotenoids, 7.12 and 6.12 µg cm^{-2} were observed for interactions P x H_Z and P x H_B respectively, at 48h pre-cooling and 2.5mgL^{-1} hormonal concentrations (Table 3). Higher concentrations of carotenoids were found in all cultivars at hormonal concentrations of 2.5 mgL^{-1} with Koroneiki and Arbequina were the leading ones (Table 4). Moreover, comprehensive look at Table 4 revealed that for same concentrations, cultivars supplemented with zeatin showed high carotenoids as compared to BAP. Besides no significant effect of interaction between pre-cooling and cultivar (P × V) was observed on total carotenoids. Also, no three way interaction of treatments was noticed.

CO_2 absorption: The rate of CO_2 absorption was affected significantly (P≤ 0.01) by all treatments (Table 2). Comparison of means revealed that extended duration of pre-cooling (P_2) made statistically significant increase in the rate of CO_2 absorption compared to control (P_0). Likewise, different cultivar and hormonal levels significantly affected the rate of CO_2 fixation, where cultivars Arbosana and Arbequina, and hormonal concentration 2.5 mgL^{-1} being the most effective ones. The assimilation of CO_2 was significant (P≤ 0.05) for interaction of V × H while non-significant for P × H. Comparison of means showed that all cultivars illustrated statistically significant rise in CO_2 assimilation with increasing doses of zeatin. However, more dramatic increase was noticed for Arbosana (9.67 µmol m^{-2}s^{-1}) and Arbequina (9.58 µmol m^{-2}s^{-1}) at zeatin concentration of 2.5 mgL^{-1} (Table 4). No significant affect was noticed for three way interaction.

SEM micrographs: Scanning electron micrographs were generated for representative sample of 48h pre-cooled Arbosana cultivar as it performed best at all hormonal concentrations of 0, 1.5 and 2.5mgL^{-1}. Clear differences were seen in the deposition of nutrient elements in the leaves of cultivar on varying concentrations of hormones (Fig. 1a). However, the SEM micrographs of targeted area depicted high uptake of mineral elements only at 2.5 mgL^{-1} concentration of zeatin as indicated by the broader peak area of the spectrum (Fig. 1b and 1c). Besides this other concentration did not show notable

difference in their peak area as compared to control.

Discussion

It is a recognized fact that establishment of an optimized *in vitro* micropropagation protocol for woody plants has always remained an aspect of potential concern (Fabbri *et al.* 2009). Despite of continuous efforts to set specific culture medium composition for micropropagation of olive, it seems that medium formulations vary from cultivar to cultivar **(**Peixe *et al.* 2007). Present study aimed to develop definite protocol for shoot induction in different cultivars of olive by using various pre-cooling and growth regulators combinations. With regards to growth regulators, the outcomes of current study depicted that for the induction of proliferation activity at least 0.5 mg L^{-1} of hormones was necessary. In past many studies have illustrated zeatin as a pivotal cytokinin needed for the induction of auxiliary buds in olive (Zuccherelli and Zuccherelli, 2002; Chaari-Rkhis *et al.*, 2003; Micheli *et al.*, 2009; Chaari-Rkhis *et al.*, 2011). However, the novelty of the current study is supplementation of this hormone in olive media at higher concentration which showed comparatively better results as supported by Chaari -Rkhis *et al.* (2011).

In various studies utilization of synthetic cytokinins like BAP and kinetin did not reveal satisfactory results in proliferation of olive explants (Zacchini and De-Agazio, 2004; Ali *et al.*, 2009; Chaari-Rkhis *et al.*, 2011). Contrary to this, our study revealed significant induction and proliferation results when varying concentrations of BAP from 0.5 to 2.5mgL^{-1} were used with pre-cooling treatments of explants. Explants cultured after pre-cooling treatments of 24 and 48h not only showed better growth, proliferation and shooting, but also demonstrated high level of physiological and metabolic activities as evident by the higher concentrations of pigments and metabolites in the study as compared to control. In fact, cold temperature triggers physiological and metabolic changes inside the explants and break the dormancy as supported by several previous researches (Kamenetsky and Okubo, 2013; Khodorova and Boitel-Conti, 2013).

Supplementation of growth regulators to OM had not only accelerated the growth of explants, but also significantly increased the percentage shoot induction, number of leaves and number of shoots per explants that is supported by previous as well (Vengadesan *et al.*, 2003; Peixe *et al.,* 2007). Analogous, results were found in this study, where both hormones, addition of Zeatin and BAP enhanced the fore mentioned parameters with their increasing concentrations in olive media. Based on previous study it was presumed that both Zeatin and BAP may improve the in vitro growth of the explants of woody species (Chaari-Rkhis *et al.*, 2011; Sardoei *et al.,* 2014). However, the current study along with pre-cooling revealed that all cultivars depicted better optimization with all levels of zeatin as compared to similar levels of BAP. Plant hormones possess unrelated group of small molecules that are product of different vital metabolic pathways.

It has been illustrated by many studies that hormonal signaling interact at numerous points during growth and development processes. Growth regulators such as cytokinins have potent signaling functions during morphogenesis and organogenesis processes (Ibrahim *et al.*, 2014; Ng *et al.*, 2016). Santoro *et al.* (2013) support our findings that supplementation of zeatin and BAP to micropropagated plants induced notable increase in shoot number, shoot length and leaf number.

In present study the photosynthetic competence of plantlets was judged by the comparative estimation of their pigment contents. Chlrophylls a and b, and carotenoids are vital photosynthetic pigments that required for growth and developmental activities by enhancing the rate of photosynthesis (Bollivar, 2006; Sakakibara, 2006). With succession of days a dynamic increase was recorded in both chlorophylls and carotenoids contents for higher doses of cytokinins which indicated the adaptation of plants with *in vitro* environment as supported by Tantos *et al.* (2001). Chlorophyll contents are positively

correlated with photosynthesis, therefore more CO_2 assimilation results in higher level of carbohydrates (Wang *et al.*, 2008). In present study dynamic increase in pigments concentration as well as CO_2 fixation was attributed to the accelerated photosynthesis; however this increase was more dramatic for zeatin. Increase in the uptake of micro and macro elements from the nutrient medium was observed due to physiological linkage between hormones and ions as endorsed by previous studies (Tanaka and Tanaka, 2006; Pazurkiewicz-Kocot *et al.* 2008). In present study comparative analysis of SEM micrographs of best performing Arbosana cultivar at hormonal concentration of 2.5 mgL^{-1}, depicted the higher peak area for zeatin as compared to BAP which authenticated higher tendency of zeatin to promote the uptake of nutrient elements from olive medium (Fig. 1). Moreover, metabolic activities in plants are implicated with growth regulators in several ways, specifically chlorophyll biosynthesis. Reduced chlorophyll synthesis is an indicator of reduced photosynthesis as revealed by the decreased rate of CO_2 assimilation in control. Furthermore, photosynthesis is an important physiological process directly associated with plant metabolism to determine growth and development. Zeatin has comparatively high tendency to penetrate into chloroplast where it triggers photochemical activities as supported by findings of Aslam *et al.* (2016). As a whole on olive media, during optimization process all cultivars depicted significant variation in their response to the treatments, where the performance of Arbosana and Arbequina was exceptionally notable at morphological, physiological and molecular level.

This study has optimized shoot induction and proliferation protocol of olive with varying concentrations of two different cytokinins. Overall, those cultivars whose explants were treated at 48h pre-cooling depicted noteworthy performance at hormonal concentration of 2.5mgL^{-1} in both cases. However, the interaction of zeatin with cultivars and pre-cooling was more promising at all levels of evaluation. Conclusively, these findings are hopes to be useful and comprehensive for current optimization and a useful guide for future research on three different dimensions i.e morphological, physiological and biochemical.

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