Evaluation of Genetic Stability in Olive Callus-induced and Meristem-induced Shoots using Flow Cytometry and Amplified Fragment Length Polymorphism Techniques

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Abstract

Background

In vitro culture of olive, as an economically valuable tree, has fundamentally genotype-dependant low micropropagation rate which need to be improved in already established cultivars, while needing to be struggled in firstly considered cultivars. Various plant tissue culture media, planting systems and growth factors were evaluated in two promising Iranian olive cultivars 'Amin' and 'Meshkat' plus a commercial Spanish cultivar 'Arbequina'.

Results

The results showed that in 'Amin' cultivar MS enriched with zeatin (4 mg/L), in 'Arbequina' cultivar OM enriched with zeatin (1 mg/L) and in 'Meshkat' cultivar OM and MS enriched with zeatin (2 mg/L) produced reasonably healthier *in vitro* plantlets. The results indicated that using Periodical Mini Bioreactor (PMB) system in the presence of VS medium in 'Amin' cultivar resulted in growth indices amelioration compared to semi-solid media. Regarding carbon sources and light intensities, mannitol and 2000 cd·sr·m⁻² greatly enhanced 'Arbequina' growth indices. The genetic stability of callus induced shoots (CIS) was compared with meristem induced shoots (MIS) using Amplified Fragment Length Polymorphism (AFLP) marker and flow cytometry (FCM). The results revealed that 2C DNA value assessed by partec FCM was mostly depended on the cultivars rather than the origin of generation -callus or meristem-. The AFLP results also showed that the cultivars were classified regardless of the micropropagation origin (CIS or MIS), except for 'Arbequina'. However, 'Arbequina' CIS and MIS still had 62- 80% of genetic similarity with three other categories and the highest resemblance (97%) was seen with MIS of 'Amin' cultivar.

Conclusions

Briefly,olive *in vitro* propagation optimization co-produced large volume and percent of calli resulting in more undesired indirect organogenesis. True to typeness of CIS was assessed showing that no changes in DNA amount occurred. Nonetheless, in phylogenetic analysis cultivars were clustered separately even some discrepancies were seen in 'Arbequina' MIS and CIS, however, 'Amin' and 'Meshkat' cultivars were more stable in clustering.

Background

Olive (*Olea europaea* L.) is the only edible species belongs to oleaceae family which was probably domesticated from subspecies *sylvestris* (López-Escudero and Mercado-Blanco, 2011), and it is amongst the earliest fruit trees in the Middle East (Bandelj et al., 2004). The first olive was cultivated in Asia before 1000 B.C. (Zohary and Hopf, 1994). The genus *Olea* has 30 species all around the world (Rugini and Caricato, 1995) and olive is an outcrossing, diploid species with vast genetic diversity (Bartolini et al.,

1998). Due to its multiple applications including oil, wood and fruit, olive is considered a valuable economic tree (Bandelj et al., 2004; Grati-Kamoun et al., 2006; Loureiro et al., 2007; Owen et al., 2005).

The *in vitro* culture of olive is restricted due to the limited sampling season (Sadder, 2002), tough cultivar-dependent proliferation (Rugini, 1984), slow lateral bud outgrowth (Benelli and De Carlo, 2018), mucus accumulation in the intercellular spaces called hyperhydricity (Grigoriadou et al., 2005), inhibitor phenolic compounds (Sadder, 2002), costly protocols (Benelli and De Carlo, 2018) and high losses of plantlets (Sadder, 2002).

Cultivar, media and PGR combinations are the most critical factors in olive proliferation (Ali et al., 2009; Rugini et al., 2016). The olive growth quality is highly affected by olive media (OM) (Moradnezhad et al., 2017; Rugini, 1984) and zeatin is the most well-known effective PGR in olive's shoot regeneration and plays an important role for salvaging the endangered diploid and triploid 'Laperrine' olive (Haddad et al., 2018). Culture system also influences olive growth indices (Benelli and De Carlo, 2018; Grigoriadou et al., 2005; Rugini et al., 1997; Sadder, 2002). Some species are sensitive to be immersed in liquid continuously which may cause severe vitrification (Benelli and De Carlo, 2018). Temporary Immersion System (TIS) resolve such problematic issues in tropical crops and is promising for woody plants (Grigoriadou et al., 2005). It overcomes gas accumulation, reduces the mass production costs (Benelli and De Carlo, 2018) and causes the least hyperhydricity (Grigoriadou et al., 2005). Survival rates and plantlet quality in TIS is higher than normal solidified conditions. To economize plant production, zeatin can be reduced to 50% in olive tissue culture as reported in 'Canino' cultivar in shoot length (Benelli and De Carlo, 2018).

Mannitol, a sugar alcohol consisting of 30% of total carbohydrates in leaves and branches is the main photosynthesis product in olive (Bustan et al., 2011) and in combination with OM, influences shoots and node numbers per shoot significantly (Moradnezhad et al., 2017). It also uniformes olive's secondary axillary shoots (Leva et al., 2013). The application of mannitol for seven subcultures resulted in total survival in comparison with the application of sucrose with 54% survival (Leva et al., 2013). It is also reported that light type is a determinative environmental factor in olive growth (Moradnezhad et al., 2017).

Tissue culture of olive could encounter some physiological abnormalities such as callusing (Leva et al., 2013). OM normally causes callus in the bottom of petioles and basal part of plantlet, but some of the olive cultivars are only able to multiply on OM (Brito et al., 2010; Sadder, 2002). Callus formation mostly depends on cultivars, media and PGRs (Rugini, 1984; Sadder, 2002; Oulbi, Toufik, & Belkoura, 2014). Since somaclonal variation is considered an approach to breed plants with the restricted genetic base, but elite genotypes selection necessitates fidelity during *in vitro* propagation. Therefore, genetic uniformity detection in *in vitro*-raised plants at an early stage is desirable (Krishna et al., 2016). In ploidy level stability assessment in olive cultivar 'sylvestris' multiplied on OM medium with zeatin after a PGR shock followed by transferring to the same medium without PGRs, the DNA content was reduced to 2.5 to 9% in the micropropagated plants (Brito et al., 2010). It is reported that intraclonal variations in quantitative traits of 'Picual' architecture, which were prominently affected by genotype and continual period of *in*

vitro culturing, occur in somatically propagated plantlets (Bradai et al., 2016). Moreover, nuclear DNA content in highly reproducible histograms in olive species was significantly different between donor plants and acclimatized plants (Brito et al., 2010).

Molecular markers give the chance of early progenies stability check (De la Rosa et al., 2003). AFLP is a tedious and time-consuming method resulting in high reproducibility and efficiency for detecting polymorphism (Bandelj et al., 2004 and Grati-Kamoun et al., 2006). Because of high discriminating capacity of AFLP, it is a reliable method to investigate genetic diversity (Owen et al., 2005). AFLP is normally used for map construction due to the huge number of polymorphism and scorable loci (De la Rosa et al., 2003). For revealing numerous bands per reaction and valuable number of indices, AFLP is considered the most efficient marker for olive in comparison with SSR and RAPD (Belaj et al., 2003) because it is distributed homogeneously in olive linkage groups (De la Rosa et al., 2003; Owen et al., 2005). In addition, not only the efficiency index (number of loci, discriminating power and number of pattern per assay) was reported the most, but also the average confusion probability was the least (Belaj et al., 2003).

‘Amin’ and ‘Meshkat’ (63% and 66% oil, 14 and 21 Kg/tree yield, respectively) are two commercial Iranian cultivars with both table and oil consumption which have similar specifications to the international commercial cultivars like ‘Arbequina’ (65% oil and 25 Kg/tree yield in Iran) and so, their mass propagation optimization guaranteeing ascertained genetic trueness is a serious demand. In this regard, different plant tissue culture media such as OM, MS and VS, planting systems including PMB and SSM and growth requirements like light intensity and carbon sources were investigated to obtain the most optimized olive propagation protocol.

Results

Effects of zeatin and cultivar on growth indices at proliferation step

MS containing 4 mg/L zeatin in ‘Amin’ cultivar produced the highest number of axillary shoot, main shoot length (cm), and growth quality (Table 1). In this study, there were no negative signs in ‘Amin’ cultivar in high PGR MS. Different PGR concentrations had no impact on ‘Arbequina’ and there was no special trend by increasing the zeatin concentration, (Table 1). ‘Meshkat’ plantlets were depressed by high zeatin concentration in both media enriched with 4 mg/L zeatin which with increment of zeatin only callus production as a negative factor increased (Tables 1 and 3). In ‘Meshkat’ cultivar, OM and MS containing 2 mg/L zeatin produced well-grown plantlets (Table 1).

Table 1

Media and zeatin interaction on growth indices of olive plantlets cv. *‘Amin’*, *‘Arbequina’* and *‘Meshkat’*.

Cultivar	Media	Zeatin (mg/L)	No. of axillary shoot	Main shoot length (cm)	No. of leaves in main shoot	Growth quality
<i>‘Amin’</i>	OM	0	1.33 ab	1.40 def	4.16 d	2.61 fg
		1	0.08 e	0.56 g	5.64 bcd	1.09 g
		2	0.32 de	0.51 g	4.81 cd	1.89 gh
		4	0.33 de	1.34 def	5.39 cd	2.89 efg
	MS	0	0.48 cde	1.03 fg	7.11 abc	3.36 cdef
		1	0.75 bcd	1.48 cdef	8.47 ab	3.48 cdef
		2	0.78 bcd	1.48 cdef	8.96 a	3.12 def
		4	1.50 a	2.23 a	9.43 a	4.08 abc
<i>‘Arbequina’</i>	OM	0	0.17 de	1.83 abcd	8.57 ab	4.23 abc
		1	1.03 abc	2.11 ab	8.71 ab	4.55 ab
		2	0.75 bcd	1.62 bcde	9.20 a	4.67 ab
		4	0.77 bcd	2.00 abc	9.26 a	4.80 a
	MS	0	0.41 cde	1.50 cdef	7.36 abc	3.49 cdef
		1	0.11 e	1.20 ef	6.61 abcd	2.80 efg
		2	0.22 de	1.41 cde	8.39 ab	3.73 bcde
		4	0.13 e	1.51 cdef	6.90 abcd	3.05 def
<i>‘Meshkat’</i>	OM	0	0.48 cde	1.05 fg	6.10 bcd	4.85 a
		1	0.75 bcd	1.35 def	6.62 abcd	4.82 a
		2	1.48 a	1.43 cde	7.32 abc	3.74 bcde
		4	0.14 e	1.06 fg	3.27 e	2.80 efg
	MS	0	0.33 de	1.22 ef	6.15 bcd	2.88 efg
		1	0.49 cd	1.64 bcde	6.17 bcd	4.84 a
		2	1.45 ab	1.84 abcd	6.93 abcd	4.90 a

Cultivar	Media	Zeatin (mg/L)	No. of axillary shoot	Main shoot length (cm)	No. of leaves in main shoot	Growth quality
		4	0.25 de	1.60 bcde	3.87 e	3.05 def

Interactive effect of culturing system and genotype at proliferation stage

The results showed that the most growth indices were higher in the periodical mini bioreactor (PMB) compared to semi-solid media (SSM) in 'Amin' cultivar (Fig. 1). In all three culture media OM, MS and VS (Fig. 3) PMB system prominently showed the superiority, but growth quality in SSM and PMB of OM did not show any significant differences (Fig. 1).

The results showed that among growth indices in 'Arbequina' cultivar only the number of axillary shoots was significantly higher in PMB compared to SSM. Suffocated plantlets resulted in a reduction in growth quality of plantlets in PMB were compared to SSM (Fig. 2).

Using PMB for OM in 'Amin' cultivar improved main shoot length, but in 'Arbequina' SSM showed superiority in growth quality (Figs. 1 and 2). Regarding MS, main shoot length (cm) plus growth quality in 'Amin' cultivar were superior in PMB, but in 'Arbequina' the number of axillary shoot were significantly differences between PMB and SSM. Regarding VS, although in 'Amin' cultivar it revealed absolute superiority in main shoot length, number of axillary shoot and growth quality using PMB, in 'Arbequina' cultivar it did not follow a specific trend (Figs. 1, 2 and 3).

Carbon and light source

The number of axillary shoot, main shoot length and growth quality were significantly higher in medium containing mannitol compared to sucrose-enriched medium in 'Arbequina' (Table 2). The results indicated that mannitol and sucrose induced 100% and 81.3% callus initiation at the base of explants, respectively (Table 3). In this study, at 2000 cd·sr·m⁻² light intensity, plantlets' main shoot length, the number of axillary shoots and growth quality were enhanced, whereas number of main shoot leaves were not significantly different (Table 2). At 2000 cd·sr·m⁻², callus formation (87.5%) was more than at 5000 cd·sr·m⁻² treatment (75%) (Table 3).

Table 2
The simple effect of carbon source and light intensity on 'Arbequina' growth indices

Variable	No. of axillary	Main shoot length (cm)	Main shoot leaves	Growth quality
Mannitol	0.9 ± 0.2^a	3.5 ± 0.1^a	10.3 ± 0.4^a	5 ± 0.04^a
Sucrose	0.7 ± 0.1^b	2.2 ± 0.19^b	9 ± 0.6^b	4.2 ± 0.2^b
2000 cd·sr·m ⁻²	0.5 ± 0.04^a	3 ± 0.04^a	9.3 ± 0.21^a	4.8 ± 0.01^a
5000 cd·sr·m ⁻²	0.4 ± 0.1^b	2.1 ± 0.16^b	9.2 ± 0.5^a	3.6 ± 0.2^b

Calli obtained from different media and genotype had different morphological characteristics like color and texture. In some cases, calli were green in color and strict solid in texture resulting in indirect organogenesis and their callus-differentiated shoots were morphologically healthy, but some of them were crystallized, spongy and vitrified which never gave rise to indirect shoot formation (Fig. 4).

Callus formation at proliferation stage

In 'Arbequina' and 'Meshkat' cultivars, there was a direct correlation between zeatin concentration and callus formation, but in 'Amin' there was no correlation in callus production (Table 3). In both 'Arbequina' and 'Amin' cultivars, PMB produced significantly higher callus induction rates and volume than SSM. Regarding carbon source and light intensity in 'Arbequina' cultivar, mannitol resulted in 100% callus formation and 2000 cd·sr·m⁻² produced more callus rather than 5000 cd·sr·m⁻² (Table 3).

Table 3
The effects of treatment and genotype on callus percent and callus volume

Cultivar	Medium	Treatment	Callus production (%)	Callus volume (cm ³)
Amin	OM	Zeatin 0 (mg/L)	59.4	1.26 ± 0.12
		Zeatin 1 (mg/L)	57.15	1.52 ± 0.13
		Zeatin 2 (mg/L)	59.4	1.94 ± 0.16
		Zeatin 4 (mg/L)	60.25	2.53 ± 0.21
	MS	Zeatin 0 (mg/L)	87.5	1.89 ± 0.16
		Zeatin 1 (mg/L)	62.5	2.34 ± 0.21
		Zeatin 2 (mg/L)	68.8	2.51 ± 0.17
		Zeatin 4 (mg/L)	30	1.94 ± 0.23
Arbequina	OM	Zeatin 0 (mg/L)	68.75	1.86 ± 0.3
		Zeatin 1 (mg/L)	74.15	2.01 ± 0.26
		Zeatin 2 (mg/L)	75.05	2.09 ± 0.26
		Zeatin 4 (mg/L)	84.4	2.17 ± 0.03
	MS	Zeatin 0 (mg/L)	50	1.83 ± 0.15
		Zeatin 1 (mg/L)	43.8	1.91 ± 0.13
		Zeatin 2 (mg/L)	56.3	2.18 ± 0.12
		Zeatin 4 (mg/L)	68.8	2.56 ± 0.02
Meshkat	OM	Zeatin 0 (mg/L)	15	0.74 ± 0.21
Molecular assessment of CIS and MIS				

Cultivar	Medium	Treatment	Callus production (%)	Callus volume (cm ³)
	MS	Zeatin 1 (mg/L)	27.5	1.45 ± 0.34
		Zeatin 2 (mg/L)	35	1.53 ± 0.13
		Zeatin 4 (mg/L)	67.5	2.76 ± 0.41
		Zeatin 0 (mg/L)	10	0.80 ± 0.14
		Zeatin 1 (mg/L)	30	0.80 ± 0.11
		Zeatin 2 (mg/L)	35	0.82 ± 0.07
		Zeatin 4 (mg/L)	65	0.85 ± 0.27
Amin	OM + 2 mg/L zeatin	PMB	100	2.25 ± 0.40
		SSM	70	2.03 ± 0.14
	MS + 2 mg/L zeatin	PMB	80	1.44 ± 0.20
		SSM	20	0.12 ± 0.14
	VS + 2 mg/L zeatin	PMB	80	1.19 ± 0.17
		SSM	60	0.9 ± 0.14
Arbequina	OM + 2 mg/L zeatin	PMB	70	2.0 ± 0.26
		SSM	70	1.98 ± 0.24
	MS + 2 mg/L zeatin	PMB	70	3.50 ± 0.11
		SSM	10	3.30 ± 0.23
	VS + 2 mg/L zeatin	PMB	60	3.70 ± 0.17
		SSM	40	1.10 ± 0.12
	OM + 1 mg/L zeatin	Mannitol	100	3.25 ± 0.3
		Sucrose	81.3	1.78 ± 0.28
	OM + 1 mg/L zeatin	2000 cd·sr·m ⁻²	87.5	1.88 ± 0.06
	Molecular assessment of CIS and MIS			

Cultivar	Medium	Treatment	Callus production (%)	Callus volume (cm ³)
		5000 cd·sr·m ⁻²	75	1.69 ± 0.24
Molecular assessment of CIS and MIS				

From 12 primer combinations tested, 259 bands (50 bp to 1000 bp) were analyzed in all tested cultivars consisting of MIS and CIS with 238 bands were polymorphic. Polymorphism rate varied from 39.02% for EAGG- MGC to 100% for EAGC- MCAA. Each primer pair resulted in 28 to 55 markers with an average of 42 amplified fragments (Table 4).

Table 4
Primer combinations and polymorphism rate.

Primer combination	Primer sequence (5' to 3')	Total number of bands	Number of polymorphic bands	Polymorphism rate (%)
EAGG- MGT	GACTGCGTACCAATTCAGG GATGAGTCCTGAGTAAGT	37	28	75.67
ECT- MGAG	GACTGCGTACCAATTCCT GATGAGTCCTGAGTAAGAG	45	42	93.33
ECT- MGC	GACTGCGTACCAATTCCT GATGAGTCCTGAGTAAGC	37	32	86.48
EAGC- MCAA	GACTGCGTACCAATTCAGC GATGAGTCCTGAGTAACAA	50	50	100
MGT- ETA	GATGAGTCCTGAGTAAGT GACTGCGTACCAATTCTA	33	27	81.81
MGC- ETA	GATGAGTCCTGAGTAAGC GACTGCGTACCAATTCTA	55	25	45.45
EGA- MCAT	GACTGCGTACCAATTCGA GATGAGTCCTGAGTAACAT	39	34	87.17
MCT- EAGG	GATGAGTCCTGAGTAACT GACTGCGTACCAATTCAGG	28	21	75
MCAG- EAT	GATGAGTCCTGAGTAAGA GACTGCGTACCAATTCAT	71	63	88.73
EAGG- MCAG	GACTGCGTACCAATTCAGG GATGAGTCCTGAGTAAGA	28	25	89.28
EAGG- MTTT	GACTGCGTACCAATTCAGG GATGAGTCCTGAGTAATTT	35	16	45.71
EAGG- MGC	GACTGCGTACCAATTCAGG GATGAGTCCTGAGTAAGC	41	16	39.02

Genetic similarity

Primer combination	Primer sequence (5' to 3')	Total number of bands	Number of polymorphic bands	Polymorphism rate (%)
Total		499	379	75.95
Genetic similarity				

Clustering the CIS and MIS in 'Meshkat', 'Amin' and 'Arbequina' cultivars was conducted according to the genetic similarity SAHN, method UPGMA and tie WARN. The phylogenetic analysis splitted the samples into 8 distinct groups indicating that callus-regenerated plants had highly monomorphic amplicons similar to those of the meristem-regenerated plants (Fig. 5). Cophenetic correlation coefficient was ($r = 0.80$) and based on Jaccard's coefficient, similarity matrix and the pair-wise value between the CIS and MIS showed similarity levels at 62–98%. Marker analysis showed that genotype was the most prominent factor in clustering the plantlets rather than the origin of propagation (Fig. 6). In 'Meshkat' cultivar no matter the origin of regeneration (meristem or callus), each sample had the most resemblance with other samples of its cultivar according to the similarity coefficient factor, Jaccard and Dice (Fig. 6).

In 'Amin' cultivar, MIS and CIS were 98% similar in the third group. Also in 'Amin' MIS and 'Arbequina' MIS more than 97% resemblance was seen. This group was the only group that consisted of both 'Amin' and 'Arbequina' cultivars in both CIS and MIS type (Fig. 6).

One of the callus induced samples in 'Arbequina' cultivar appeared in a separate branch of phylogenetic tree which was similar to all groups consisting 3 cultivars with 63% genetic similarity and one of its MIS samples appeared in a separate category with 79% resemblance with both CIS and MIS of 'Meshkat'. It is worth mentioning that even in MIS samples in 'Amin' and 'Arbequina' cultivars, more than 97% genetic similarity was obtained in the third category. In the present study, 'Arbequina' in both CIS and MIS showed the most dispersal in classification, but both Iranian cultivars showed more stability (Fig. 6).

FCM assessment of CIS and MIS

Simultaneous analysis of nuclei isolated from Parsley (*Petroselinum crispum*) (2C DNA amount = 4.46 pg, as internal standard) and olive cv. 'Arbequina', 'Meshkat', 'Amin' in CIS and MIS resulted in highly reproducible histograms with P1/P2 ratio and low CV value which approved the priority of cultivar rather than the origin of propagation in genetic similarity, consequently there were no differences between the studied cultivars (Table 5). Moreover, there was no discrepancy between CIS and MIS in total DNA amount (2.87–3.12 pg) (Table 5). Figure 7 illustrates a representative of FCM histograms for CIS and MIS in 'Meshkat' cultivar which indicate the similarity between the peak $_1$ /peak $_2$ ratios (mode $P_1/P_2 = 0.67$) in both CIS and MIS.

Cultivar	Type of origin	P1/P2	DNA amount (pg)
Amin	CIS	0.67 a	2.99 ± 0.01
	MIS	0.68 a	3.02 ± 0.01
Meshkat	CIS	0.67 a	3.01 ± 0.01
	MIS	0.64 a	2.87 ± 0.05
Arbequina	CIS	0.70 a	3.12 ± 0.02
	MIS	0.66 a	2.95 ± 0.03

Effects of zeatin concentration and cultivar on growth indices at proliferation stage

It is shown that combined cytokinins in olive increased the proliferation rate (Moradnezhad et al., 2017), zeatin in combination with other cytokinins like BAP, a fixed compound of media in this study was used to have synergistic effect on proliferation improvement as well. In *Moraiolo* regeneration improved to have cytokinins like BAP growth depression occur in cultivars between microshoot formation (Ali et al., 2009). In other subspecies of olive (Laperrine olive), regeneration was to a high extent related to media and PGRs and only in case of not applying PGRs, microshoot formation did not develop (Haddad et al., 2018). Zeatin plus carbon source might have an effect on complex plant signaling network (Leva et al., 2013).

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Apical dominance and oxidized tannins from phenolic compounds in semi-solid media inhibited the normal regeneration in olive (Rugini, 1984; Sadler, 2002). Using bioreactor is advantageous because of lower production cost due to reducing the labor, zeatin concentration and increased efficiency of proliferation rate plus removing gelling agent (Benelli and De Carlo, 2018). A novel temporary immersion system was reported to be a suitable method in olive micropropagation in comparison with other liquid systems such as Erlenmeyer flasks, filter paper bridges in test tubes and LifeReactor (Grigoriadou et al., 2005). The advantage of liquid system is due to lowering the toxic compound aggregation, well aerated plantlets (Benelli and De Carlo, 2018; Sadler, 2002) and horizontal position of plantlets. This method allows reduction in carbon dioxide, ethylene gases and relative humidity in the vessel's headspace and thus reduces morphogenesis abnormality (Benelli and De Carlo, 2018). They showed using plantform™ bioreactor, desired zeatin concentration in olive was reduced to 25–50%. In the present study, PMB functioned the way that TIS did. Sadler, 2002 reported that in olive cultivar 'Nabali' suffocation was also reported in liquid OM (Sadler, 2002).

Analysis of 4–5 cm of olive shoot tip by Rugini (1984) resulted in introducing the best medium for olive micropropagation called OM which is high in Ca, Mg, S, Cu and Zn. Mg which is triple higher in OM compared to MS and VS (Duchefa, NL) increased shoot elongation by affecting enzymes positively, especially those involved in phosphate transfer as modulator of shoot initiation and growth promoter (Shaul, 2002). Nevertheless, it has as twice Boron as MS and VS have which evidently plays important roles in nucleic acid synthesis, cell differentiation and elongation (Läuchli, 2002).

VS medium contains macro elements as described by Murashige and Skoog (1962) and contains chelated Fe form EDDHA instead of EDTA as described by Van der Salm et al. (1994) which provides a better iron supply at higher pH and phosphate content since it is much stronger than Fe-EDTA (Duchefa, NL). It is showed that OM in olive cv. 'Arbequina' was more effective rather than WPM and MS in producing total number of shoots and healthy plantlets (Moradnezahad et al., 2017). Different fundamental formulations are required for olive's genotypes (Moradnezahad et al., 2017; Rugini, 1984), as minerals may sensitize cells to PGRs and nutrient mobilization occurs in PGRs existence by creating new source sink relationship. High calcium (CaCl_2 and $\text{Ca}(\text{NO}_3)_2$) in OM plays a role in cytokinin signal transduction in cells which leads to better cell division (Ali et al., 2009). Folic acid co-enzymes are involved in carbon transfer, which are vital for methionine, serine, deoxythimidylic acid and purines synthesize that are necessary for cell differentiation (Ali et al., 2009).

Carbon and light sources

Leva et al. (2013) showed more undifferentiated parenchyma-like callus growth in medium containing sucrose rather than medium enriched with mannitol. Haddad et al. (2018) reported mannitol resulted in greener foliage and morphologically healthy plantlets similar to their mother plants. It is proved that shoot development pattern, plantlet survival, growth quality and secondary shoot formation in olive are affected by carbon sources (Leva et al., 2013). Starch, mannitol and sucrose are the most carbohydrates

in olive and soluble sugar mannitol is predominant in leaves and branches twice as abundance as sucrose (Bustan et al., 2011) which contains 30% of olive total carbohydrates (Flora and Madore, 1993) and varies according to the year of bearing (on or off year), season as mannitol peaks is related to high temperature in summer, olive's organs and even fruit harvesting time (Bustan et al., 2011). In olive, subsequent sub-culturing (seven times) in media containing mannitol increased the survival rate approximately twice compared with sucrose (Leva et al., 2013). Mannitol promoted bud sprouting and secondary shoot uniformity rather than sucrose regardless of its concentration (Leva et al., 2013). Mannitol may directly and indirectly influence endogenous hormonal status protecting against detrimental compounds in media (Leva et al., 2013) in contraction with sucrose in higher concentrations. As mannitol is involved in activating osmotic effect, plants with high amount of mannitol or well-acclimatized in mannitol enriched media have negligible osmotic stresses (Moradnezhad et al., 2017). Moreover, mannitol transfer in intracellular metabolism aids olive against abiotic stresses by acting as a supportive agent against olive cell suspension dehydration (Leva et al., 2013). In catabolism of mannitol in sink cells, hexose phosphate generates 2 ATP molecules, while initial generation of hexose phosphate in catabolism of sucrose spends ATP, so that energetic advantages of mannitol might be used for exponential growth (Leva et al., 2013; Moradnezhad et al., 2017).

In stevia, shoot induction occurred more frequently in 6000 $\text{cd}\cdot\text{sr}\cdot\text{m}^{-2}$ intensity rather than 2000 and 4000 $\text{cd}\cdot\text{sr}\cdot\text{m}^{-2}$ under the controlled situation. Light intensity affected stevia organogenesis as well and 6000 $\text{cd}\cdot\text{sr}\cdot\text{m}^{-2}$ was suitable for axillary shoot formation (Roshandel et al., 2013). In *Ternstroemia gymnanthera* three light intensities (500, 1000, 2000 $\text{cd}\cdot\text{sr}\cdot\text{m}^{-2}$) were compared and the results showed 1000–2000 $\text{cd}\cdot\text{sr}\cdot\text{m}^{-2}$ were more acceptable in growth quality and in case of LED light and fluorescent lamp the most proper luminous intensity was 2000 $\text{cd}\cdot\text{sr}\cdot\text{m}^{-2}$ (Ahn and Choi, 2016) similar to this study findings.

Callus is normally regenerated due to zeatin application in the proximal end of plantlets and at the end of petioles which is a common reaction to *in vitro* stresses. It was reported that OM induced callus in olive without additional PGRs (Sadder, 2002) however, callus organogenesis only occurred in special olive cultivars as it happened often in 'Amin', 'Meshkat' and 'Arbequina' cultivars in comparison with two Iranian cultivars 'Dire' and 'Tokhmekabki' (Mirzaei et al., 2020 under submission data) and two commercial cultivars 'Conserveolea' and 'Koroneiki'.

Callus formation at proliferation stage

Krishna et al. (2016) reported that making wound in cutting samples, auxin and auxin analogs, sterilization and PGRs can cause mutagenesis and also, salts, temperature and light changes can result in oxidative stresses. Consequently, superoxide, hydrogen peroxide, hydroxyl, peroxy and alkoxy compounds can cause hyper/hypo methylation of DNA, DNA base deletion and substitution, chromosomal rearrangement and number change, which in turn resulting in mutation and somaclonal variation in *in vitro* culture (Bradaï et al., 2016; Krishna et al., 2016).

Molecular assessment of CIS and MIS in three cultivars

In order to further assess the olive genome coverage, several primers should be tested to increase the number of loci. It was previously stated that phenotypical, cytological and biochemical methods can be used in this purpose, though molecular assessment repetition in another growth phase is desirable (Bradaï et al., 2019) because somatical genetic stability of olive scarcely was studied so far. SSR was not informative in olive genetic variation assessment since replication slippage was more frequent that might ignore single nucleotide mutation (Bradaï et al., 2019), so that AFLP was chosen because its discriminating capacity was proved in analyzing olive cultivars (Belaj et al., 2003).

Nearly 97% resemblance between 'Amin' MIS and 'Arbequina' MIS was seen proving that the resemblance between these genotypes for stem cutting *in vitro* culture was considered stable (Brito et al., 2010), consequently they were genetically close to each other (Bradaï et al., 2019 and 2016).

Embryogenic culture-induced olives from the same cotyledon maintained in *in vitro* conditions for 3 years showed genetic instability due to the long term maintenance (Bradaï et al., 2019). Although variation in olive was reported slightly higher in aged plants *in vitro* subcultured continually, it was highly genotype-dependent and biometric analysis of quantitative traits also showed intraclonal variation. Haddad et al. (2018) reported all amplicons in ISSR marker between regenerated diploid and triploid endangered olive in comparison with donor plants were monomorph, so that all the regenerants in high PGRs and their mother plants were grouped in the same category (Haddad et al., 2018). Nevertheless, as the stability of *in vitro*-raised olives in OM and zeatin enriched media was proved (Haddad et al., 2018), in this study MIS was considered as donor plants and CIS was compared to MIS. On the other hand, Guillaume et al., (2009) reported that meristem mutation position was the first important factor in chimerism maintenance and mutation in apical dome normally resulted in permanent chimera (Besnard and Baali-Cherif, 2009). In marker investigations, DNA is usually extracted from leaves and so, the mutation level in diploids might be underestimated (Besnard and Baali-Cherif, 2009). Nevertheless sampling after 6 subcultures could resolve such a problem as the results of this study showed.

No correlation between genetically somaclonal varied plants with phenotypic changes was found as phenotypical characteristics are controlled by several genes (Bradaï et al., 2019), moreover special part of this genuine variation might be due to the differences in non-coding portion of genome, which might not be expressed and revealed in olive appearance and performance. To reduce such controversial issues, complementary phenotypic analysis is recommended (Bradaï et al., 2016). Provoked changes in cells might also result in developmental reprogramming in plants (Belaj et al., 2003; Bradaï et al., 2019). The problem of undesired callus induction at the basal end and petiole end part in olive propagation was reported frequently. Callus phase is a prerequisite of variations. Studies on genetic stability in somaclonal varied olives are scarce (Bradaï et al., 2019), therefore this is the first study that compared plantlets originated from MIS and CIS as genetic stability and ploidy level fidelity in olive. Belaj et al. (2003) stated that the combination of two different genetic change assessments in olive is another approach and so, evaluation of ploidy level changes by FCM was used.

FCM assessment CIS and MIS

In this study DNA amounts were similar to olives measured previously (2.90–3.07 pg) (Loureiro et al., 2007). There was no significant nuclear DNA content difference among cultivars. Nonetheless, in the previous studies DNA amount fluctuation might be attributed to the cytosolic compounds like phenols causing tannic acid effect as a result of interference with DNA-PI staining since there is an abundant amount of such materials in woody plants (Loureiro et al., 2007). These interfering phenolic compounds are genotype-dependent and normally micropropagated plants accumulate higher amounts of cytosolic compounds which do not allow proper PI staining resulting in lower DNA amount (Loureiro et al., 2007). Even though olive is extremely high in such mentioned interferer compounds and its discrepancy in DNA 2C value should be considered cautiously, fortunately in the present study interferer materials were not problematic. PVP was added to nuclei isolation buffer to solve such problematic issues in secondary metabolites (Loureiro et al., 2007). Not severely chopped leaves by razor blade can also equilibrate interferer material (Loureiro et al., 2007).

Loureiro et al. (2007) reported that 2C DNA content of *O. europaea* ssp. *europaea* var. *europaea* ranged between 2.90 ± 0.020 pg/ 2C and 3.07 ± 0.018 pg/ 2C and for wild olive, it was 3.19 ± 0.047 pg/ 2C. In this study, 84% of samples had lower DNA amounts. Ploidy level changes are prominent processes in plant evolution. Due to reduction in inbreeding depression effect, triploids are much more vigorous than diploids especially in extreme situations and as 'Amin' was highly resistance to salinity stress and other abiotic stresses (data are not shown), it was suspected to be a polyploid which was not proven in this study as previously reported for Iranian olive cultivars and checking other genetic variation disorder in this genotype is recommended.

Conclusion

It was proved that the most optimized protocols (PMB, mannitol carbon source and $2000 \text{ cd}\cdot\text{sr}\cdot\text{m}^{-2}$ light intensity) normally co-produced larger calli both in percent and volume: resulting in more undesired indirect organogenesis. To verify true to typeness of CIS, genetic stability was assessed using AFLP and FCM. The results showed that no DNA amount change happened. Nonetheless, in marker analysis cultivars were clustered separately however some discrepancies were seen in 'Arbequina' MIS and CIS.

Methods

Plant materials and general procedures

Three commercial cultivars including one Spanish cultivar ('Arbequina') and two native cultivars ('Amin' and 'Meshkat') were selected. The huge number of semi-hard cuttings with apical and lateral buds consisting of axillary buds were collected in July from mature trees grown in olive collection at Tarom Olive Research Station (Tarom, Zanjan) in Iran. Explants with at least 2 opposite nodes (2 cm) were surface sterilized in 70% ethanol (v/v) for 60–75 seconds followed by agitating in 2.5% sodium

hypochlorite containing 1% Tween- 20 for 13 min, then three times washing in sterilized distilled water each time for 5 min.

At establishment stage, a large number of explants were cultivated on OM (Olive medium, Duchefa, NL) supplemented with 4 mg/L zeatin (Duchefa, NL) plus 0.5 mg/L 2ip (6- γ γ -Dimethylallylaminopurine) (Duchefa, NL) in order to have enough source for experiments.

All media including solid and liquid were enriched with 3% (w/v) sucrose (unless otherwise stated) and 0.5 mg/L 2ip + 0.23 mg/L BAP + 0.04 mg/L IBA + 540 mg/L calcium gluconate + 50 mg/L FeEDDHA + 4 mg/L H₃Bo₃ and pH was adjusted to 5.8. Then were solidified with 0.7% (w/v) plant agar (Duchefa, NL). All media and glassware were autoclaved for 15 min at 121 °C and 1.37 bar. Phytotrons with high pressure metal halide lamps (2609 cd sr m⁻²) and 16/8 hour light/dark cycle and temperature of 24 $\pm$ 1 °C were used to maintain the cultures. Olive growth indices the most important of which axillary shoot number, leaf number, shoot length and callusing were measured and recorded in all experiments. At least 25 repetitions of each treatment were averaged.

Interactive effects of cultivar, culture media and zeatin concentration at proliferation stage

Three different olive cultivars (‘Arbequina’, ‘Amin’ and ‘Meshkat’), two culture media (OM and MS) (Murashigue and Skoog, 1962) and various zeatin concentrations (0, 1, 2 and 4 mg/L) were added to basal studied media (Plant materials and general procedures).

Application of Periodical Mini Bioreactor (PMB) at proliferation stage

Two cultivars (‘Amin’ and ‘Arbequina’) under different culture media (OM, MS and VS) (van Der Salm, 1994) containing 2 mg/L zeatin beside the previously mentioned additives (Plant materials and general procedures) in two forms of semi-solid media (SSM) and liquid were investigated. VS was included in this experiment, because in another study of mine, olive cv. Amin was reacted well to it (Mirzaei et al., 2019).

Autoclaved periodical mini bioreactors (BPM; bioreactor D 345, AZMAYESH ABZAR DI 2413, IR) were filled with 300 mL of sterilized liquid medium (Fig. 8). Ten explants with three replications were placed in the inner jars and periodically immersed in the liquid medium. The compressed air passed through two 0.2 μ m micropore filters providing additional protection. The nutrition program in 24 h included three times immersion (each time 10 min) followed by three times drainage and aeration (each time 10 min) and stationary stage (each time 7 h and 40 min).

Effect of carbon source and light intensity at proliferation stage

To optimize proliferation stage more affordable with less amount of costly zeatin for *Arbequina*, two carbon sources (sucrose and mannitol) and two different light intensities (2000 and 5000 $\text{cd}\cdot\text{sr}\cdot\text{m}^{-2}$) in OM containing 1 mg/L zeatin plus previously mentioned additives (Plant materials and general procedures) for 25 sample repetition (5 glass jars and 5 sample per each), to separate experiments were carried out.

Genetic stability in callus induced shoots (CIS) and meristem induced shoots (MIS)

Since large masses of calli at the basal part of shoot explants in most olive proliferation experiments were created, therefore, callus formation percent and callus volume (cm^3) were recorded. For volume measurement, sphere volume ($\frac{4}{3} \pi r^3$) for approximately spherical calli and immersion in graduated cylinder half filled with water for multi-dimensional calli were used. To confirm the similarity of microshoots originated from calli (CIS) and shoots originated from meristems (MIS), Amplified Fragment Length Polymorphism (AFLP) and Flow cytometry (FCM) were used. The shoots were cultured on their optimized culture media (Table 1) for 6 months while sub-cultured every 6 weeks. During this experiment, CIS samples equal to their counterpart MIS were marked and subcultured separately, in order to check their similarity. Repetitions are mentioned in each experiment.

Amplified Fragments Length Polymorphism (AFLP)

Total genomic DNA was extracted from 200 mg of fresh tissue of *in vitro* plant leaves of both MIS and CIS following the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987). DNA concentration and quality were evaluated by 1% agarose gel electrophoresis and NanoDrop (Implen, DE). The AFLP technique was carried out as described by Vos et al., (1995) with the following modifications: 500 ng of genomic DNA of 17 samples with 4 replications were double digested using 5 units of EcoRI and MseI enzymes, and adaptors were ligated to the obtained fragments. The ligation mixture was diluted 1:4 with nuclease-free water, and 3.75 μL of it was used for PCR pre-amplification in a 25 μL volume with 10 picomol of each pre-amplification primer carrying three selective nucleotides. After 2 min at 72 °C, 20 cycles were carried out at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s in Thermocycler (Applied Biosystems, USA). A total of 12 primer combinations of M and E primers with selective nucleotides were screened among those previously tested in the lab because they provided the highest number of heterozygous bands in the literatures as well. E primers were end-labeled radioactively with IRD700 and IRD800. The amplified products were separated by denaturing for 6 min at 96 °C on 6% polyacrylamide gel done at 1500 V, 60 mA, and 15 W for 60–90 min at 55 °C by DNA analyzer 2400 (Bioscience, USA). Amplified bands were scored as present (1) or absent (0), smeared and weak bands

were discarded. Only reproducible bands were used to construct the original binary data matrix. Genetic similarities among accessions were evaluated by estimating the Dice and also Jaccard methods (Dice, 1945; Jaccard, 1908). The generated dendrogram (NTSYS-PC ver. 2.02 package) was compared by computing the cophenetic correlation. Genetic similarity (GS) between two cultivars i and j was calculated based on Jaccard's similarity coefficient (Jaccard, 1908), $GS_{ij} = a / (n - d)$, where a is the number of fragments in common between the two cultivars, d is the number of fragments absent in both cultivars and n is the total number of fragments scored.

Flow cytometry (FCM)

Young leaf segments (around 1–2 cm²) from 21 samples of the actively growing *in vitro* olive shoots and the same size of fresh young leaf of Parsley (*Petroselinum crispum*) ($2n = 2x = 22$; 2C DNA amount = 4.46 pg, as an internal standard), were chopped with a razor blade in 400 µL nuclei isolation buffer (Cystain UV Precise P, Partec, DE) on a plastic petri dish according to (Yokoya et al., 2000). To reduce the interaction between secondary compounds, 10 g/L polyvinyl pyrrolidone (PVP) was added to the nuclei isolation buffer. The lysate was filtered through a 50 µm Cell Trics and brought up to 2 mL with staining buffer (saline solution containing fetal bovine serum and sodium azide (0.09%) as a preservative including 12 µL Propidium Iodide (PI) and 6 µL ribonuclease (RNase)). In order to eliminate cell debris, the nuclear suspension was then filtered through a 30 µm Cell Trics. After 5 min incubation period on ice, around 10,000 nuclei were analyzed by FCM (Partec, DE). Fluorescence intensity – 540 and 615 nm-excitation and emission respectively were measured by means of laser beam. Estimates of the ratio of fluorescence intensities of each olive to parsley sample ratio (P1/P2) were based on the means of at least 4 repetitions, giving peaks with a coefficient of variation of less than 5%. The nuclear genome size of each type of plant material was estimated according to Yokoya et al., (2000).

Experimental design and statistical analysis

A flow diagram of the experimental design is illustrated in (Fig. 9). It shows the processes of sampling, formation of CIS and MIS and leaf sampling for assessment of genetic diversity. The diagram simply shows the materials and methods highlighting undesired callus formation at the base of plantlets to distinguish shoots originated from callus and those generated from meristem (normal *in vitro* proliferation), and necessitate the investigation of genetic stability of CIS and MIS.

The micropropagation experiments were conducted under a completely randomized design with at least 25 replications. In all proliferation experiments, growth indices including number of axillary shoots, main shoot length (cm), number of leaves on main shoot and visual growth quality (1–5 scoring where 1 and 5 are the worst and the best qualities, respectively) were recorded after 6 weeks. The percent of callus production at the base of *in vitro* shoots and callus volume (cm³) were also recorded. Data were evaluated by the analysis of variance (ANOVA) according to the general linear model (GLM) procedure

using statistical software SAS (Institute Inc., Cary, NC, USA). Mean comparisons among treatments were conducted using LSD at the $P \leq 0.01$ level of probability.

Abbreviations

AFLP, amplified fragments length polymorphism; BAP, 6-benzylaminopurine; CIS, callus-induced shoots; FCM, flow cytometry; MIS, meristem-induced shoots; MS, Murashige and Skoog; OM, olive media (ROM, Rugini olive media); PGRs, plant growth regulators; PMB, periodical mini bioreactor; PVP, polyvinyl pyrrolidone; VS, Van der salm; 2iP, 6- γ -Dimethylallylaminopurine.

Declarations

Ethics approval and consent to participate

'Not applicable'

Consent for publication

'Not applicable'

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files or the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

"The authors declare that they have no competing interests"

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Authors' contributions

AY and MJK conceived and designed research. LM conducted experiments. MN, AAZ, MF and DD contributed new reagents or analytical tools. LM and MF analyzed data. LM wrote the manuscript. All

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Figures

Figure 1

Interaction effects of media and culture system on olive cv “Amin” growth indices.

Figure 2

Interaction effects of media and culture system on olive cv. *Arbequina* growth indices.

Figure 3

Growth quality, number of axillary shoot, total length and leaves comparison in (A) *Arbequina* in PMB with VS, (B) *Arbequina* in SSM with VS, (C) *Amin* in PMB with VS, (D) *Amin* in SSM with VS (scale bar 1.5 cm).

Figure 4

In vitro-raised (A) Meshkat, (B) Arbequina and (C) Amin cultivars in optimized media (Table 1) after 6 weeks, (D) different kinds of calli formed in Meshkat, Arbequina and Amin cultivars: 1) spongy, 2) dead crystalized, 3) puffy, 4) dense vitrified, 5) spongy bicolored, 6) green solid, 7) green dense, 8) greenish spongy and 9) strict tricolored.

Figure 5

Highly monomorphic bands in MIS and CIS in 1) Meshkat4MIS, 2) Meshkat4CIS, 3) Meshkat2CIS, 4) Meshkat1MIS, 5) Arbequina2MIS, 6) Arbequina2CIS, 7) Arbequina3MIS, 8) Arbequina3CIS, 9) Amin3MIS, 10) Amin3CIS, 11) Amin4MIS and 12) Amin1CIS in application of EAGG and MGC primer pair (50 bp ladder, Fermentas, USA).

Figure 6

Dendrogram (NTSYS-PC ver. 2.02 package) of three cultivars with MIS and CIS.

Figure 7

Typical histograms of “Meshkat” cultivar for (A) CIS and (B) MIS with more than 10,000 nuclei analyzed with 4 repetitions.

Figure 8

Periodical mini bioreactor (PMB) system: (1) complete equipment including (A) controller, (B) jars with two glass layers which inner layer contained the plant material and outer layer contained the culture medium, (C) and (D) 0.2 µm cellulose nitrate filters for entrance of sterilized air to appliance (where (C) was for vacuuming air, and (D) was for importing air and vice versa) (2) Immersion step.

Figure 9

Schematic flow diagram of olive (*O. europaea* L.) 'Amin', 'Arbequina' and 'Meshkat' micropropagation: Well-grown single node cuttings were surface-sterilized and established in modified OM (OM supplemented with 4 mg/L zeatin plus 0.5 mg/L 2ip). During proliferation stage, regarding the media with unintentionally callus formation (high zeatin media, mannitol enriched media and lower light intensity condition), CIS and MIS were proliferated separately. In vitro plantlets were sub-cultured for at least 126 days. Then leaf materials were tested for genetic stability assessment by amplified fragments length polymorphism (AFLP) and flow cytometry (FCM).