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RESEARCH ARTICLE

MICRO PROPAGATION OF THE MILLENNIUM OLIVE TREES (*OLEA EUROPAEA* L.) IN BSHAALEH LEBANON

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ABSTRACT

In an effort to safeguard the Lebanese genetic resources of olives (*Olea Europea* L.), this study was intended to set up an *in vitro* culture from axillary buds of Bshaaleh millennium trees. A series of treatments preceded the multiplication phase to limit infections in the introduced explants. Best survival rate (56.7%) was recorded when the treatment was based on a mix of fungicides (fosethyl-aluminium (6g/l) and carbendazim (2g/l)), chloroxylenol (2.5%) and NaOCl (15%) as detergents, and oxytetracycline (1mg/l) as antibiotic. Young shoots were used to induce explants *in vitro* on OM, WPM and MS supplemented with BAP (2, 3 and 4mg/l respectively). Autumn season gave the best multiplication rate (1.08), newly developed explants (1.35) and proliferation rate (47%) on MS medium followed respectively by OM and WPM. The longest explants (1.78cm) and the highest number of newly developed leaves (3.58) were obtained during the first subculture on the MS medium added with BAP (3mg/l).All the examined parameters decreased during subcultures. Following the elongation phase, NAA added at a rate of 2mg/l to MS medium produced 22% of rooted explants. During the autumn season, explants where callus developed on the base, were transferred to fresh media that enabled the rooting of 23% where no subculture was needed.

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INTRODUCTION

Olive landraces are disappearing progressively in the Mediterranean Countries of which Lebanon as a result of the abandonment of groves with low olive fertility, urbanization or substitution by new cultivars (Bartolini *et al.,* 1998). The genetic certification of olive and new biotechnology techniques are essential to save the genotypes with distinct characteristics, by providing protection from viruses and using unconventional techniques for their preservation (Ruggini, 2002). The olive tree varieties grown in Lebanon, as in the rest of the world, have significantly decreased in favor of high-reputation varieties. This "genetic erosion" can be limited via genetic screening on strains with characteristics that match the growers' expectations. Such screening may be performed by identifying the trees aged 40 years or more in older plots (Lavee, 1990), with the aim of multiplying them *in vitro* to preserve genetic resources and produce virus-free material (Santos *et al.,* 2003). This procedure also enables the mass production of cultivars and the selection of interesting explants after several subcultures (Ruggini, 2006), in addition to the multiplication of species, that are difficult to breed via conventional means.

In comparison with other species, *in vitro* propagation of olive explants is still at the experimental stage, as success rates between cultivars widely varied (Van der Vossen *et al.,* 2007). During the initiation and multiplication phase, the most used media for olives are based on MS medium (Murashige and Skoog, 1962; Chaari-Rkhi s *et al.,* 1999; Ferreira *et al.,* 2003), Ruggini olive media (ROM) (Ruggini, 1984; Abousalim *et al.,* 2004) and Lloyd and Mc Cown (WPM) (Santos *et al.,* 2003). ROM fortified with Zeatin or BAP (Sakunasingh *et al.,* 2004; Abousalim *et al.,* 2004) at various concentrations, resulted in the best multiplication rates. The rooting difficulty of the adult olive material *in vitro*is mentioned also in different types of fruit trees (Walali and Abousalim, 1993). Previous studies have stated that olive (*Olea Europea* L.) trees in Bshaaleh, located in North Lebanon are among the oldest in Lebanon and they are still productive (BouYazbeck *et al.,* 2018a). AFLP was used to study their diversity (BouYazbeck *et al.,* 2018b), morphological and biochemical characterizations were also undertaken (BouYazbeck *et al.,* 2019). After all these studies, it has become urgent to reduce the loss of their genetic authenticity and to preserve their local genetic resources and rejuvenate it.

Therefore, this work aimed to conserve the eldest trees in Lebanon from Bshaaleh region, by establishing *in vitro* techniques as the cultivation of micro cuttings and the stimulation of axillary buds and their proliferation is the most commonly used in micro propagation of woody plants (Walali, 1993);

MATERIALS AND METHODS

The trees olives of Bshaaleh examined in this study are around1400 years old (BouYazbeck *et al.,* 2018a). The young shoots were collected over spring, summer and autumn in 2009 - 2010. The explants were first soaked for 3hours in a solution of methalaxyl+mancozeb (6g/l) and propamocarb-HCl (2g/l); leaves were then removed and twigs were separated into uninodal portions of 3 cm, disinfected in a fosethyl-aluminium and carbendazim (5g/l) solution, shaken for 30minutes, then rinsed in water for 5 minutes, ethanol (70%) for 10 seconds, and then were incubated in a solution of NaOCl (110g /l) and Chloroxylenol (2.5%)for 5, 10 and 15minutes. Finally, sterile distilled water was used to rinse cuttings three times under laminar flow cabinet for 5 minutes. MS (Murashige and Skoog, 1962), Olive Medium (OM) (Ruggini, 1984) and Woody Plant Medium (WPM; Lloyd and McCown, 1981) were used during initiation, multiplication, rooting, and formation of callus. The media composition is shown in table1). Media were solidified using bacteriological agar (0.8%) and sterilized by autoclaving for 20 minutes at 118°C. 1mg/l of oxytetracycline was added to reduce browning tissues and bacterial contamination. During the various stages (initiation, multiplication, and rooting), cultures were kept in a controlled room with determined temperature conditions (20-22°C), light intensity (4000 lux) and photoperiod (16 h/d). Part of the material was kept in the dark for one week before its transfer to the culture chamber, under the same conditions, in order to facilitate the call ogenesis.

Initiation: Fragments, each bearing 2 axillary buds, were disinfected and planted in the 3 media with the addition of different concentrations of BAP (2, 3 and 4mg/l). For each medium, 30 cuttings were introduced. The shoot length of the newly developed explants was measured to assess the explants' ability to survive the initiation phase. The survival rates, the different infection rates (bacterial and fungal) and the browning of tissues were also evaluated.

Multiplication phase: New shoots developed 5 weeks after the bud establishment phase of buds. After their separation of the newly developed buds from the initial explant, they were divided into nodal explants and transferred on the same media added with same concentrations of cytokine in forming the first subculture. This operation was repeated five weeks after the first subculture and seedlings were transferred to new media to explore the hormones influence on the shoot proliferation and multiplication rate which is the ratio between the numbers of new shoots developed on the shoots initial number and was evaluated at the end of each subculture. New shoots were transferred and grown for 4 continuous subcultures on the same media.

In vitro **Rooting:** During elongation phase, micro cuttings were transferred for 4 weeks on media with no hormones. Rooting was induced on MS, OM, and WPM media, with the addition of auxins, IAA and NAA at a concentration of 2mg/l. Five weeks after the explants transplantation on new media, the rooting rate was recorded; then explants were transferred to a fresh medium, with no auxins, to stimulate root development.

Call ogenesis: One month after the initiation phase, calluses started to develop at the explants base. These explants were transplanted on MS medium, with the addition of NAA (2mg/l) and BAP (0.5mg/l), to promote the development of somatic embryos. A comparison recorded the percentage of explants where callus had developed over the 3 seasons (spring, summer and autumn) in order to analyze the impact of each season on callus formation.

Acclimatization: Explants, where roots developed and with minimum of 2 leaves were transplanted into plastic pots (8cm). Roots were rinsed to remove agar, then transplanted into a mix containing a combination of peat moss and black peat (3:1). The transferred plantlets were covered with a plastic film (50µ), to preserve high relative humidity (90%). This cover was removed progressively to control the plantlet transpiration.

Statistical Analysis: An analysis of variance (ANOVA) and a correlation of all the parameters (number of broken buds, explant contamination, new stems/explant, number of new leaves formed, stem length, multiplication rate and percentage of explants that developed callu) were performed using SPSS version 14.0 to study the effects of different factors (culture media, subculture, season).

RESULTS AND DISCUSSION

In order to resolve contamination issues, a pretreatment of 2 fungicides methalaxyl+mancozeb and propamocarb-HCl was used, followed by a treatment *in vitro* of fosethyl-aluminium and carbendazim (systemic fungicides), 2 bactericides chloroxylenol (2.5%) and NaOCl (15%), and one antibiotic the oxytetracycline (1mg/l). This treatment yielded the highest number of proliferated explants (56.7%). These figures are inferior to those observed by Sakunasingh *et al*. (2004) (80%) where only one fungicide (Benomyl 2%) and one detergent (25% NaOCl) were used. This difference might arise from the fact that, in order to lower production costs, the fungicide was not used on olive groves.

Initiation phase: A total of 810 cuttings were introduced *in vitro* on OM, MS and WPM media, supplemented with different concentrations of BAP (2, 3 and 4mg /l). Five weeks later, the numbers of explants lost by bacterial and fungal contamination, and browning tissues, the percentage of proliferated buds as well as the number of new explants were recorded. The season had a significant impact on the newly developed explants; 1.35 and 1.33 being the highest values recorded during the autumns of 2009 and 2010 respectively; conversely, and during summer 2009, only 1.12 new explants developed. Proliferated buds acted similarly during the studied seasons, with the best proliferation rate recorded during autumn 2009 (47.1%) then by summer 2009 (46.2%), whereas the lowest rate was observed during 2010 (31.1%). Browning tissues caused respectively losses of 23% and 29%, in the material collected in 2009 and 2010.The season highly affected the browning tissues and the rate ranged respectively between 23.7 (summer 2009) and 34.1 (spring 2009) (Figure 1). Oxidation results are consistent with the findings of Ramzan Khan *et al.,* (2002): explants from adult cuttings were subject to rapid oxidation, in spite of the preventive treatments administered by the addition of ascorbic and citric acids.

Table 1. Basal nutrient medium composition of Murashige and Skoog medium (MS), Woody Plant Medium (WPM) and Olive medium (OM)

Components	MS (mg/l)	WPM (mg/l)	OM (mg/l)
Macro-elements			
$CaCl_2.2H_2O$	440	96	0
$CaNO_3.4H_2O$	0	556	1300
KH_2PO_4	170	170	340
KNO_3	1900	0	1772
$MgSO_4.7H_2O$	370	370	731
NH_4NO_3	1650	400	412.5
K_2SO_4	0	990	0
Micro-elements			
$CaCL_2.6H_2O$	0.025	0	0.02
$CuSO_4.5H_2O$	0.025	0.25	0.25
H_3BO_4	6.2	6.2	12.4
KI	0.83	0	0.83
$MnSO_4.H_2O$	22.3	22.3	16.9
$NaMoO_4.2H_2O$	0.25	0.25	0.25
$ZnSO_4.7H_2O$	8.6	8.6	14.3
Iron			
$FeSO_4.7H_2O$	27.8	27.8	27.8
Na-EDTA.H_2O	37.3	37.3	37.5
Vitamins			
Inositol	100	100	100
Pyridoxine	1	0.5	0.5
Thiamin	1	1	0.5
Nicotinicacid	1	0.5	5
Ascorbicacid	25	25	0
Glycine	0	2	2
Biotin	0	0	0.05
Folicacid	0	0	0.5
Glutamine	0	1178	0
Others			
Saccharose	30000	30000	30000
Agar-Agar	8000	8000	8000
H_2O	QNFIL	QNFIL	QNFIL

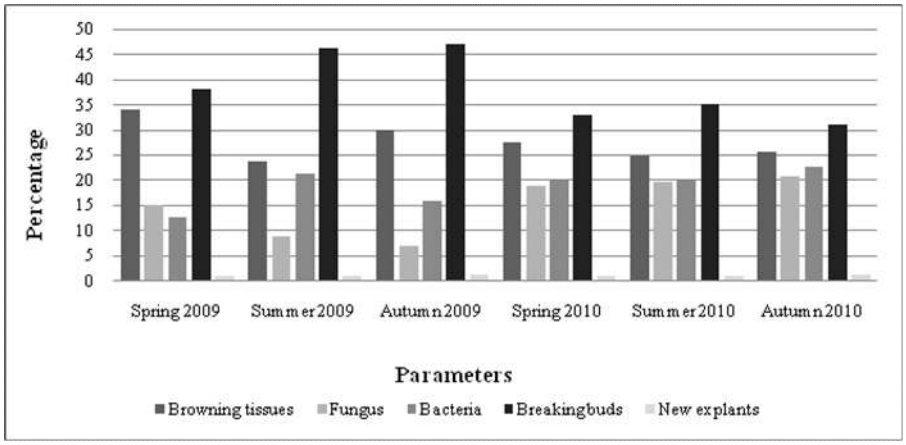

Figure 1. Effect of the 3 seasons spring, summer and autumn of 2009 and 2010 on the percentage of browning tissues, fungal and bacterial contamination, breaking buds and new explants during initiation phase

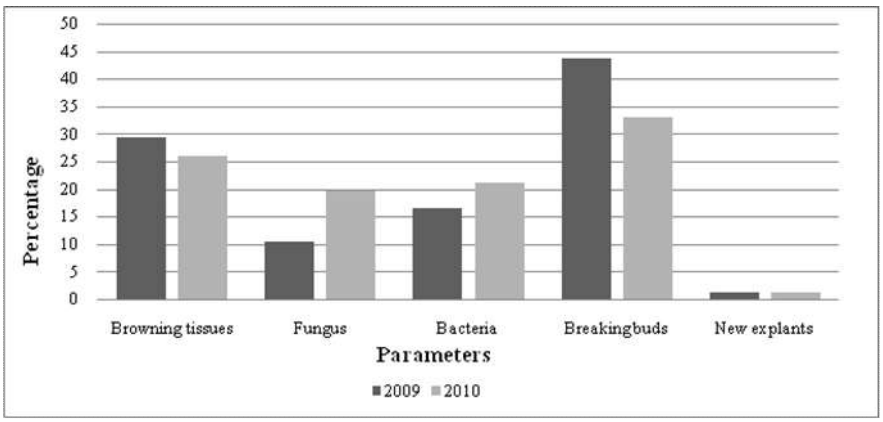

Figure 2. Effect of the years 2009 and 2010 on the percentage of browning tissues, fungal and bacterial contamination, breaking buds and new explants during initiation phase

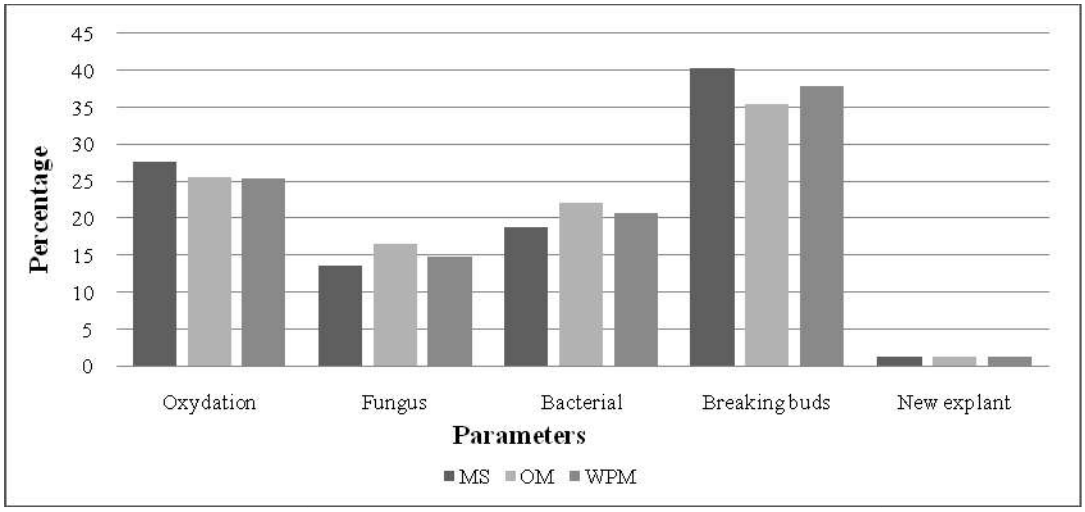

Figure 3. Effect of the 3 media MS, OM, WPM on the percentage of oxidation, fungal and bacterial contamination, breaking buds and new explants during initiation phase

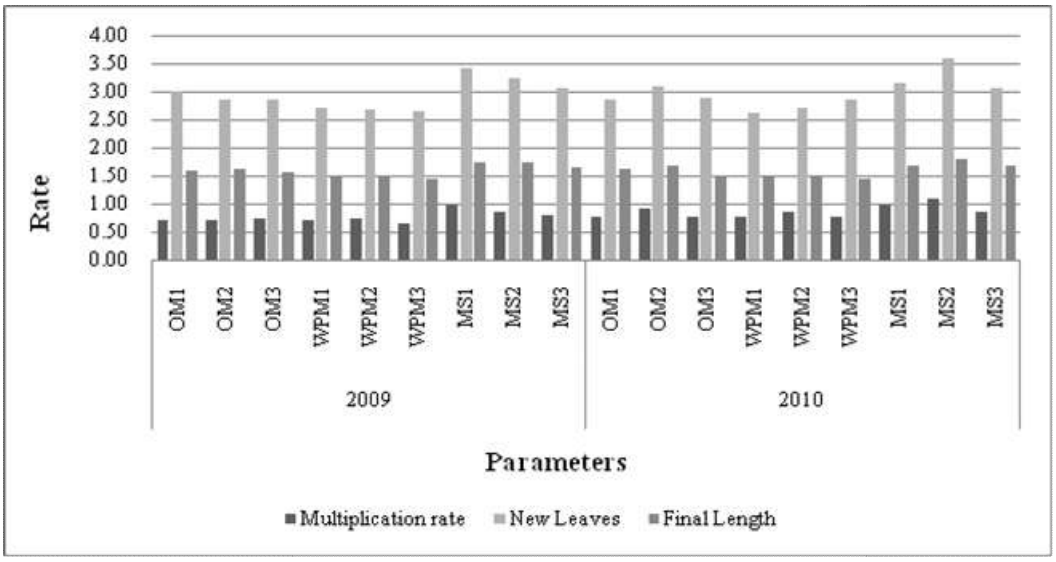

Figure 4. Influence of the 3 media on multiplication rate, new leaves and length of the explants during 2009 and 2010

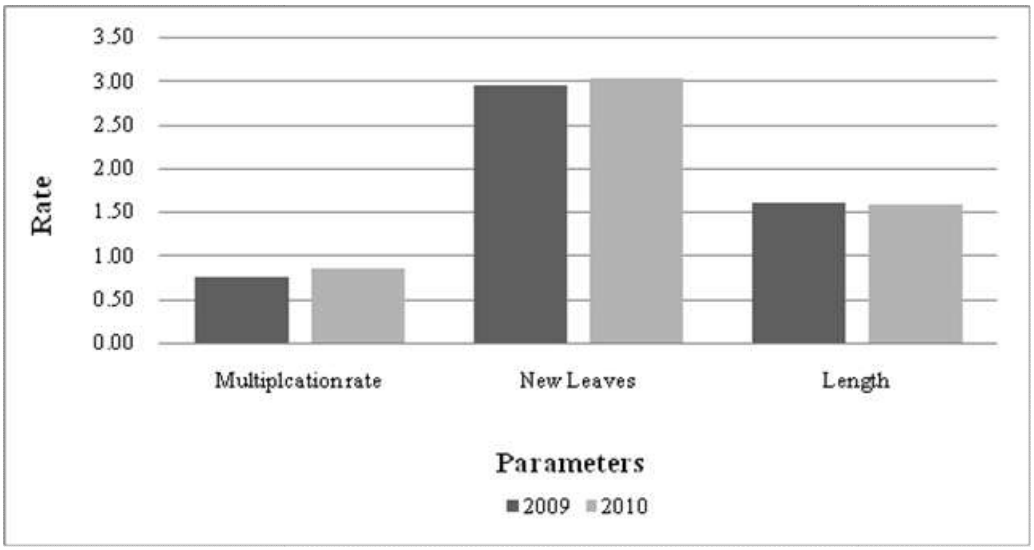

Figure 5. Influence of the years 2009 and 2010 on the multiplication rate, new leaves and length of explants

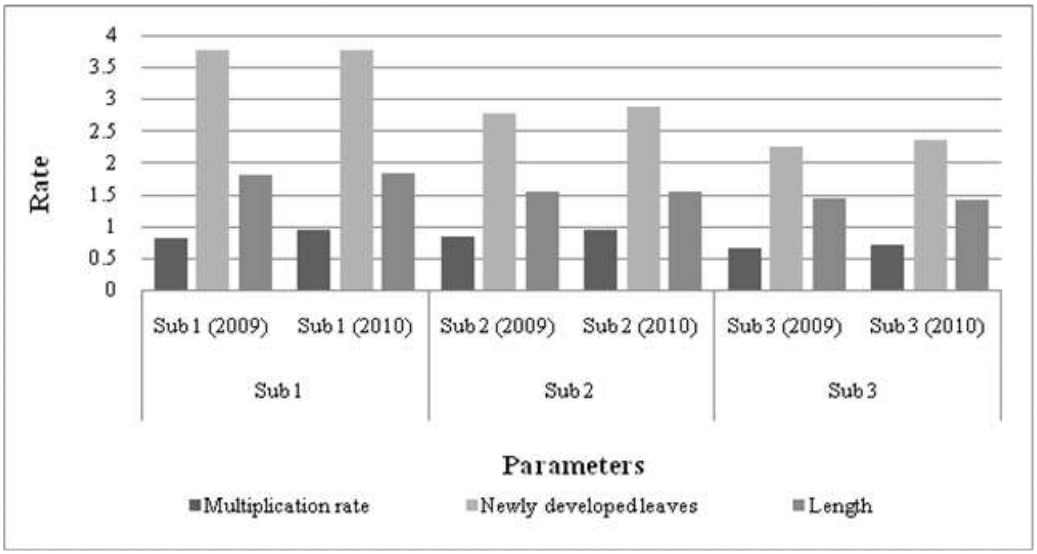

Figure 6. Influence of the subculture on the multiplication rate, new leaves and length of explants

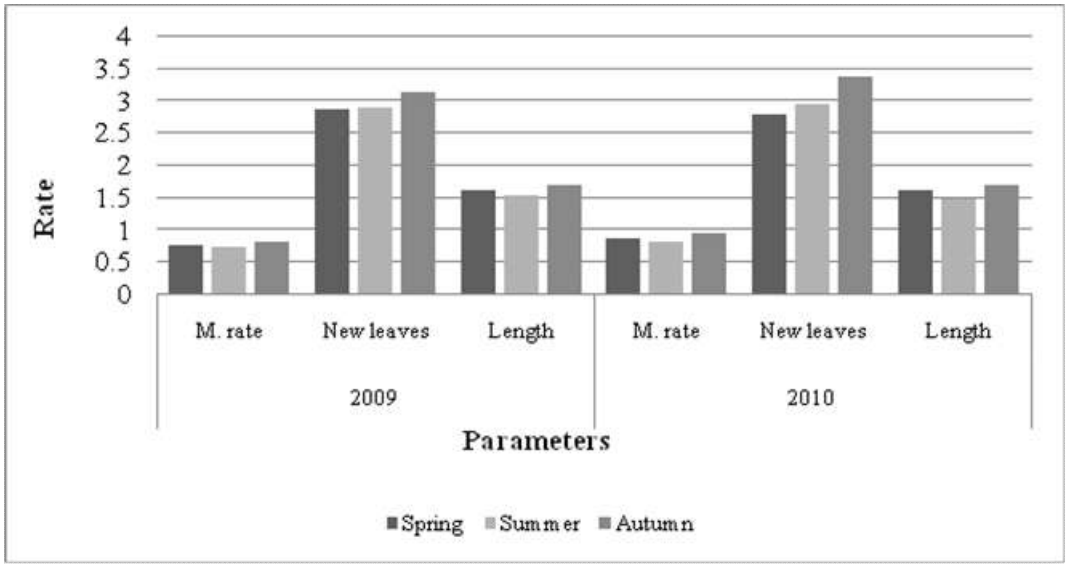

Figure 7. Influence of the 3 seasons on the multiplication rate, new leaves and length of explants

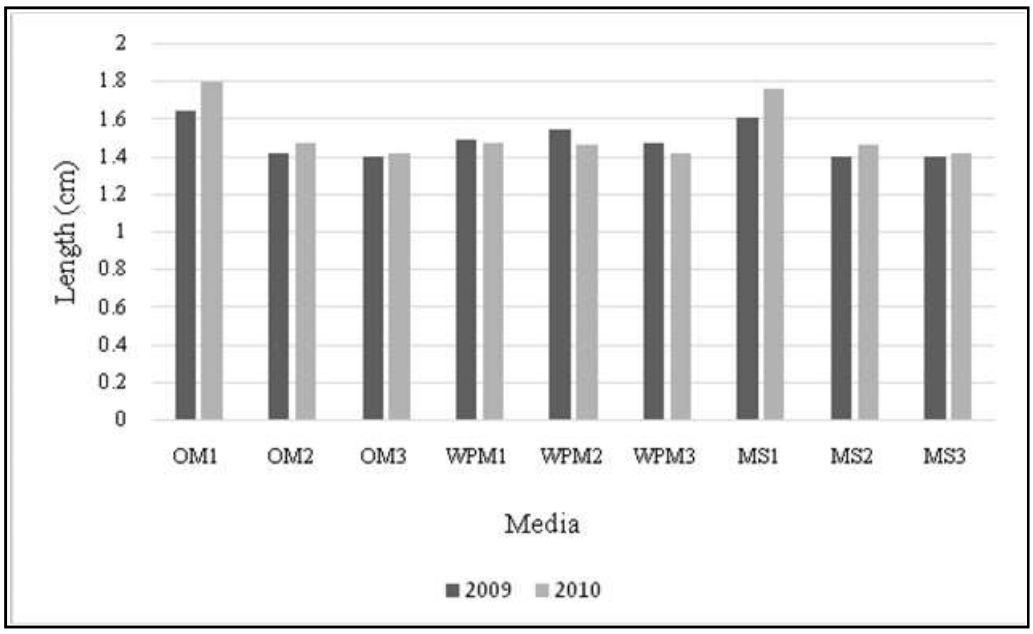

Figure 8. Influence of the 3 media on the elongation of the explants

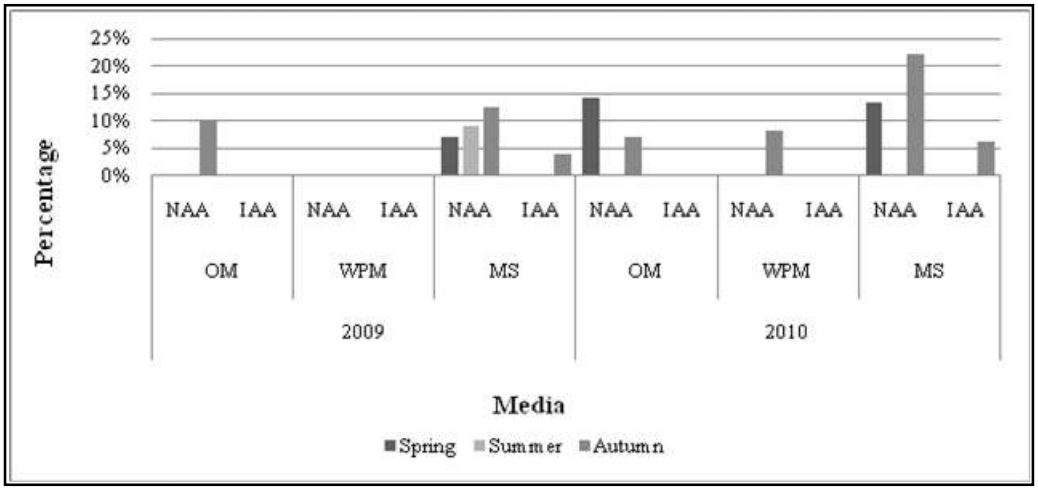

Figure 9. Influence of the added hormone on the percentage of the rooted explants

Grigoriadou *et al.* (2002) had made similar observations on the leaves and buds of *Chalkidikis* cv. where the plant material was lost. During the initiation phase, the oxidation may be due to the size of introduced axillary buds (<0.5cm) or to the explants sensitivity towards the disinfection protocol. Even when the combination of fungicides and bactericides was used, bacterial (12%) and fungal (13.3%) contaminations continued to appear. The highest rate of bacterial contamination (22.6) was recorded during autumn 2010, while the number of explants that were lost due to fungal contamination was not affected by the year (Figure 2). Multiplication results are comparable to those observed in 2other Lebanese cultivars (Baladi and Chatawi), where proliferation was best during autumn (61%), followed by spring (37.5%) and summer (24.5%) (Boustany, 2008). A full blossom and fruit set, during spring and summer, seemed to have a negative impact on the regeneration of the explants introduced *in vitro*. For this reason, the highest rate of proliferated buds was recorded during autumn. Bougdal *et al*. (2007) obtained similar results on olives with the best percentage of proliferated buds (90%) obtained during autumn, followed by spring (40%), summer (16%). Abousalim *et al.,* (2004) indicated that spring and autumn are the active seasons for olive growth.

Explants introduced during spring will produce explants with low vegetative growth, which could result from the flowering effect on the tree. The sampling year indicated no significant impact on the examined parameters. The highest percentage of broken buds (43.7%) was recorded in 2009, while the new developed explants acted similarly during 2009 and 2010, with a mean value of 1.22. As for the browning tissues, 2010 gave better results than 2009 and recorded the lowest percentage (26%; Figure 2). Bacterial (10.4%) and fungal contaminations (16.6%) were both inferior in 2009. These losses are still inferior to those observed on pendollino cv., where culture contamination varied between 50% and 60%, even with the use of $HgCl_2$ (0.1%) and NaOCl (50%) (Ramzan Khan *et al.,* 2002). The different composition of media, with the exception of the browning tissues, did not impact the other parameters. The highest percentage of browning tissues occurred on MS media (27.6%) . The media components could not have any effect neither on the fungal mycelium development noron bacterial colonies. Neither the media, nor the added hormone had any impact on the percentage of proliferated buds and new explants. The percentage of broken buds and newly developed explants varied between 35.4% and 40.3% and 1.17-1.19 respectively (Figure 3).

These results echoed the findings of a previous study of two Lebanese cultivars Baladi cv. and Chatawi cv., conducted on 23 media, where no significant difference was recorded and all explants acted similarly during the initiation phase (Boustany, 2008). Results showed statistical interaction among the year of sampling, the season and the medium: MS supplemented with 3mg/l BAP during 2009 was the best medium for the development of new explants (1.46) on MS, while the highest rate of breaking buds (60%) was recorded during autumn and summer 2009 on MS supplemented with 2 or 3mg/l . During 2010, the addition of 3mg/l BAP to MS in autumn produced the highest percentage of newly developed explants, while the addition of 2mg/l BAP to WPM yielded the highest breaking buds. These results were consistent with those obtained on the Chemlal cv. where the best proliferation resulted from explants introduced on MS media, supplemented with 2mg/l BAP or 2mg/l Kinetin in autumn, recording 75 and 90% respectively (Bougdal *et al.,* 2007). OM and MS/2 media were the most effective on Moroccan Picholine cv. where proliferated buds exceeded 90% during the initiation phase (92 and 91%) (Brhadda *et al.,* 2003). Garcia-Ferriz *et al.,* (2003) stated that MS medium fared better than OM for Dan, Sorani and Zeiti (Syrian varieties); while, *Chondroliachalikidikis* cv. displayed better proliferation on WPM than on OM and Quoirin and Lepoivre media. For the Meski cv., the best result was observed on MS mediumin comparison with OM. Our results diverge from those observed on Nebbiara cv. which proliferated better and yielded the longest shoots on OM rather than MS (Zacchini and De Agazio, 2004). The BAP (1mg/l) added to OM/2, gave the highest percentage of bud break in the Arbequina and Picual varieties (Garcia-Ferriz *et al.,* 2003). Abousalim *et al*. (2004) recorded the highest percentage of bud break (100%) and development of new shoots with 5mg/l Zeatin OM. The OMdiluted by half and added with Zeatin (4 mg/l), was the most efficient for axillary bud with rates nearing 80% for the Aglandou Tenchvariety (Binet *et al.,* 2007). Dieback symptoms started to appear 4 weeks and were followed by apical bud detachment, on different media. This loss only occurred during spring and summer; a similar phenomenon had been recorded in a previous study on two Lebanese cv., Baladi and Chatawi cv., where it was observed during spring and resulted in the loss of 23% of explants (Boustany, 208).

Multiplication phase: After the subculture of explants, a medium effect was recorded and revealed the advantage of MS, with the addition of 2 and 3 mg/l BAP. The multiplication

rate was also significantly affected and the highest number of new explants (1.08) was obtained on the MS2 followed by MS1 with 0.98 (Figure4). As for the new leaves and length of explant, the same media (MS2 and MS1) recorded the best results with respectively 3.58 and 3.41 newly developed leaves and 1.78 and 1.75 cm length. The shortest explants (1.43cm) and the lowest multiplication rate (0.64) were detected on WPM supplemented with 4mg/l BAP, while the lowest number of new leaves was seen on the same medium supplemented with 3mg/l BAP (Figure4).

The MS media recorded better results in 2009 and 2010 on the examined parameters, followed by the OM and WPM (Ruggini, 1984). These findings appeared to be in contradiction with those observed on cv. Moraiolo, where OM proved to be significantly superior than WPM and yielded the highest number of shoots per proliferated explant (0.84) (Ansar *et al.,* 2009). Regarding the concentration of the growth regulator (BAP), the largest quantity used (4mg/l) had a negative impact on the multiplication rate that also declined with subculture. This finding is consistent with Ansar *et al.,* (2004) who indicated that a high concentration of growth regulators limited significantly shoot multiplication on OM and WPM. The shoot height in Leccino cv. decreased with the increase in the cytokininconcentration levels (BAP, 2ip and Kinetin) (Ramzan Khan *et al.,* 2002). Dimassi-Theriou (1994) found that BAP alone resulted in bettershoot proliferation than 2ip, and when added at a concentration of 5 to 7.5 ml/l, BAP produced 1.2-1.8 shoots/explant and did not impact the height of Kalamon olive (Rama and Pontikis, 1990). MS medium produced the longest explants, followed by OM and WPM, respectively. These results were consistent with those recorded on Moraiolo cv. where OM yielded the highest shoot length (2.25cm) in comparison with WPM (1.24cm). In Moroccan Picholine cv., media had a significant impact on the length of developed shoots after 2subcultures. OM produced the longest shoots (1.24cm), followed by MS/2 (0.89cm) then WPM (0.3cm) (Brhadda *et al.,* 2003). The year had an important influence on the multiplication rate of Bshaaleh explants, potentially resulting from the use of foliar fertilizers during the 2009 season, which improved vegetative growth and produced well-developed twigs on the tree regardless of the seasons in 2010. The multiplication rate reached its highest in 2010, with 0.86 compared to 0.76 for the previous year (13% increase), while newly-developed leaves acted similarly and did not display any significant change (2.7%), increasing from 2.95 to 3.03; the mean value of the new explants length was unchanged during 2009 and 2010 at 1.59 (Figure 5). A subculture effect was observed and significantly affected the multiplication rate, the number of new leaves and length of explants during all three seasons of both years. The first subculture yielded the highest rates, followed by the second and the third subcultures (Figure 6). The highest multiplication rate (0.93) was observed during the first 2010 subculture, followed by the second subculture of the same year (0.925) and decreased during the third (0.71) (Figure 6). Newly developed leaves recorded their highest (3.77) during the first subculture (in 2009), while the lowest (2.26) was observed during the third subculture of the same year. The length decreased from one subculture to another, from 1.82 to 1.42cm during 2009 and from 1.45 to 1.14cm during 2010. All the examined parameters, particularly the explant length due to the vigorous shoots introduced *in vitro* after the use off oliar fertilizer, were significantly affected by the season. The highest number of new explants (0.8 and 0.94) was recorded in autumn, followed by spring and summer. The lowest number was observed during summer with 0.71 and 0.79 during 2009 and 2010 respectively (Figure 7). Lengthwise, the shortest explant developed in summer (1.48cm), while the longest was seen in autumn (1.69cm). For the newly developed leaves, the highest number (3.35) was recorded in autumn 2010, while the lowest (2.78)was observed during spring of the same year.

Elongation phase: The best elongation was recorded on explants issued from OM1, followed by MS1, for both years 2009 and 2010 with 1.79 cm and 1.76 cmrespectively during 2010; and 1.64 cm and 1.6cm during 2009. The remaining explants, in their entirety, could not exceed 1.5cm, ranging between 1.4 cm and 1.47cm,except for WPM2 (1.54cm) during 2009 (Figure 8).

Rooting phase: A significant difference was observed, indicating that MS media, with NAA added, produced the highest percentage of rooted explants (22.22%), and followed by OM , with NAA added, producing 14.3% of rooted explants (Figure 9). Ruggini (1984) indicated that NAA at 1mg/l proved to be the most efficient for the explants rooting explants issued from nonproductive shoots, yielding branches and water sprouts. Abousalim *et al*. (2005) reported that only juvenile plant material developed callus (97%) and 94.4% rooted when NAA was added to the medium at 2mg/l while the ones issued from adult olives did not develop any root system. Rooted explant on WPM developed chlorotic leaves, showing similar calcium deficiency symptoms, potentially resulting from the high concentration of chloride in the medium. For this reason, the rooted explant was transferred on MS medium to minimize the effect of chloride. The percentage of rooted plants was also affected by the season. Explants introduced during autumn displayed the highest rooting capacity, followed by spring. In total, only 4.3% (16 out of 374) of explant rooted.

Callogenesis: Callus developed only on explants introduced during autumn. Media had no impact on callus development and the rate ranged from 26.7 to 33.3%. The explants were transferred on fresh media, where they were preserved in the same conditions (dark for10days). Callus development on Bshaaleh explants was consistent with the findings of Ruggini (1984), who showed that a difficult to root cultivar "Frantoio", which was less suitable for *in vitro* culture, yielded abundant basal callus, while Moraiolo, an easy to root cultivar, grew at the fastest rate. Increased callusing at the base of the shoots was observed when highest concentrations of BAP (4 and 8mg/l) were used (Ramzan Khan *et al.,* 2002).Three weeks later, some roots started to emerge at the base of some cuttings (23.1%); the rooted explants were then transferred on MS medium added with 2mg/l of NAA. Introducing olive explants during autumn could be the fastest technique to ensure the rooting of explants with no subculture needed, as this cultivar displayed rooting difficulties.

Conclusion

Conserving the genetic resources of Millennium olive trees of Bshaaleh-Lebanon by the mean of *in vitro* micropropagation was the main objective of this study and constituted a challenge as the young branches did not show a good vegetative growth. In order to resolve contamination issues, a pretreatment with2 fungicides, a treatment with 2 fungicides, 2 bactericides and one antibiotic was done and yielded the highest number of proliferated explants (56.7%). During the

initiation phase, regardless of medium and BAP concentration, the highest percentage of broken buds was recorded on MS media (40.27%), followed by WPM (37.8%) and OM (35.4%). Autumn was the best season for this phase and produced the highest number of proliferated buds (47%) and newly developed explants (1.35). With regards to the multiplication phase and during the three seasons of 2010, the first and second subcultures produced the same proliferated rate (0.93 new explants), which is also the highest.

This rate fell to 0.71 during the third subculture. MS medium was more advantageous than the remaining two (OM and WPM) for the multiplication rate of Bshaaleh explants, producing an average of 1.08 new explants on MS added with 3mg/l BAP. The lowest rate (0.64) was recorded on WPM added with 4mg/l BAP. The season also had a noteworthy impact on the multiplication rate, where the highest number of new explants during 2009 and 2010 was produced during autumn (0.94 and 0.8 respectively). Results are comparable to the initiation phase, where the highest percentage of broken buds was seen during autumn. The highest number of newly developed leaves (3.58) and the longest explants (1.78cm) were seen during the first subculture on the MS medium, added with 3mg/l BAP. In comparison, the smallest explants (1.43cm) and the lowest number of leaves (2.6) were recorded on WPM3 and WPM1 respectively. As for rooting, NAA (2mg/l) produced the highest rate of rooted explants (22%), while IAA did not exceed 6.25% when added to MS medium. Bshaaleh explants have encountered difficulties in callus proliferation during autumn, even with proper concentrations of kinetin and NAA used on the medium. The callus, when preserved on the explant, facilitated the rooting of 23% of the plant material, with no subculture needed. This technique could be an alternative to the *in vivo* culture, requiring additional care and optimal temperature and humidity.

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Key points: The study is a challenge

The study has allowed to obtain Millennium olive trees cuttings *in vitro* conditions

Preserve the local genetic resources of Millennium olive trees in Bshaaleh - Lebanon

Glossary of Abbreviations

AFLP	Amplified fragment length polymorphism
BAP	6-benzylamino purine
IAA	Indole-3-acetic acid
MS	Murashige and Skoog
OM	Olive medium
NAA	1-Naphthaleneacetic acid
NaOCl	Sodium hypochlorite (Chlorox)
WPM	Woody Plant Medium

REFERENCES

Abousalim A., Brhadda N., Loudiyi D. 2004. Essais de prolifération et d'enracinement de matériel issu de rajeunissement par bouturage d'oliviers adultes (*Olea europea* L.) et de germination *in vitro* : effets de cytokinine et d'auxines. *Biotechnology Agronomical Society*, 9: 237-240.

Abousalim A., Walali D.M., Brhadda N. 2005. Somatic embryos development and plant regeneration in olive (*Olea europea* L.). *Fruits*, 62(2): 115-124.

Ansar A., Touqeer A., Nadeem A.A., Ishfaq H., 2009. Effect of different media and growth regulators on *in vitro* shoot proliferation of olive cultivar "Moraiolo". *Pakiston Journal of Botany*, 41(2): 783-795.

Bartolini G., Prevost G., Messeri C., Carignani G. 1998. Cultivars and World-wide collections. FAO, Roma, 462 pp.

Binet M.N., Lemoine M.C., Martin C., Chambon C., Gianinazzi S., 2007. Micropropagation of olive (*Olea europaea* L.) and application of mycorrhiza to improve plantlet establishment. *In Vitro Cellular and Developmental Biology-Plant*, 43(5): 473-478.

Brhadda N., Abousalim A., Loudiyi D., Benali D. 2003. Effet du milieu de culture sur le microbouturage de l'olivier (*Olea europea* L.) cv. *Picholine* Marocaine. *Biotechnologie, Agronomie, Société et Environnement*, 7(3-4) : 177-182.

Bougdal S., Cherifi D., Bonaly J. 2007. Production de vitro-plants *d'Oleaeuropea* var. *Chemlal*. *Cahiers Agricultures*, 16(2) : 125-127.

Boustany N. 2008. Potentiel de propagation *in vitro* de l'olivier (*Olea europea* L.). Master en Biodiversité et Biotechnologie Végétale, Faculté d'Agronomie, Université Saint-Esprit de Kaslik, 73 pp.

BouYazbeck E., Abi Rizk G., Hassoun G., El-Khoury R., Geagea L. 2018a. Ecological characterization of ancient olive trees in Lebanon Bshaaleh area and their age estimation. *IOSR Journal of Agriculture and Veterinary Science*, 11(2): 35-44

BouYazbeck E., Abi Rizk G., Battistini A., Hassoun G., Geagea L., Veronesi F. 2018b. Molecular characterization of ancient olive trees in bshaaleh Lebanon. *International journal of Plant, Animal and Environment Sciences*, 8(3): 19-29.

BouYazbeck E., Abi Rizk G., Battistini A., Hassoun G., Geagea L., El-Khoury R., Famiani F. 2019. Morphological and biochemical characterization of ancient olive trees in Bshaaleh Lebanon. Accepted at *International Journal of Current Research*. under press

Chaari-Rkhis A., Trigui A., Drira N. 1999. Micropropagation of Tunisian cultivarsolive trees: preliminary results. Third International Symposium on Olive Growing. ISHS *Acta Horticulturae* 474.

Dimassi-Theriou K. 1994. *In vitro* micropropagation of cv. Kalamon olives (*Olea europaea* L.), *Advances in Horticultural Sciences*, 8: 185-189.

Ferreira L., De Oliveira A., Fráguas C. Pasqual M. 2003. Multiplicação*in vitro* de oliveira (*In vitro* multiplication of olives)(*Oleaeuropaea* L.). *Ciencia e Agrotecnologia*, 28(1): 135-141.

García-Férriz L., Ghorbel R., Ybarra M., Marì A., Belaj A., Trujillo I. 2003. Micropropagation from adult olive trees. IV International Symposium on Olive Growing. *Scientia Horticulturae*, 113(1): 1-7.

Grigoriadou K., Vasilakakis M., Eleftheriou EP. 2002. *In vitro* propagation of the Greek olive cultivar *Chondroliachalkidikis*. *Plant Cell, Tissue and Organ Culture*, 71(1): 47–54.

Lavee S. 1990. Aims, methods and advances in breeding of new olive (*Oleaeuropaea*L.) cultivars. *Acta Horticulturae*, 286: 23–36.

Lloyd G., Mc Cown B. 1981. Commercially feasible micro propagation of Mountain Laurel, *Kalmia latifolia*, by use of shoot tip culture. Combined Proceedings – *International Plant Propagators Society,* 30: 421-427.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture: *Physiology Plant*, 15: 473-497.

Rama P., Pontikis C.A. 1990. *In vitro* propagation of olive (*Olea europaea sativa* L.) Kalamon. *Journal of Horticultural Sciences*, 65: 347-353.

Ramzan Khan M., Rachid H., Quraishi A. 2002. Development of aseptic protocols in olive (*Olea europaea*L.) cv. pendollino. *Asian Journal of Plant Sciences*, 3: 220-221.

Ruggini E. 1984.*In vitro* propagation of some olive (*Olea europaea* L.) cultivars with different rootability and medium development using analytical data from developing shoots and embryos. *Scientia Horticulturae*, 24: 123-134.

Ruggini E. 2002. Miglioramento genetico ed applicazioni biotecnologiche per la salvaguardia della biodiversità in olivo (Genetic improvement and biotechnology application for safeguard biodiversity in olive). Atti Convegno Internazionale di Olivicoltura, Spoleto, 22-23 Aprile, pp. 74-81.

Ruggini E. 2006. *In vitro* culture of the olive: an overview of the present scientific status. Third International Symposium on Olive Growing. ISHS *Acta Horticulturae* 474.

Sakunasingh P., Thepsithar C., Thongpukdee A. 2004.*In vitro* shoot culture of olive (*Olea europaea* L.) cv. *Arbequina*. Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand.

Santos C.V., Brito G., Pinto G., Fonseca H.M.A.C. 2003.*In vitro* plantlet regeneration of *Olea europaea*spp. *maderensis*. *Scientia Horticulturae*, 97: 83-87.

Van der Vossen H.A.M., Mashungwa G.N., Mmolotsi R.M. 2007. Olea *europaea* L. In: van der Vossen, H.A.M. and Mkamilo, G.S. editors. PROTA 14: Vegetable oils/Oleaginous. Netherlands.

Walali L., Abousalim A. 1993. Olive tree propagation. In: Proceedings of the first CNCPRST-CSIS seminar on oleaginous plants. October 19-21, Rabat, Morocco, pp. 63-67.

Zacchini M., De Agazio M. 2004. Micropropagation of a Local Olive Cultivar for Germplasm Preservation. *Biologia Plantarum*, 48(4): 589-592.

Walali L., 1993. La multiplication *in vitro* des espècesligneuses: état actuel et perspectives de développement. Le progress génétique passe-t-il par le repérage et l'inventaire des gènes ? AUPELF-UREF. Paris: John Libbey Eurotext, Paris, 399-409.

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