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Optimization of Aseptic Conditions for Micropropagation of Olive (*Oleaeuropaea*L.*)* Cultivar Uslu

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Abstract: Efforts were made to develop proper protocol for establishment of aseptic conditions as well as efficient initiation and multiplication of in vitro cultures of olive cultivar "Uslu". Our work inquires that the main factors such as media composition; growth regulators, fungicide (mancozeb) and bacteriocide (Clorox) have prominent effects on the micropropagation of the selected olive cultivar. The effectiveness of different treatments, in terms of concentration and time, was investigated for optimization of aseptic conditions.For controlling fungal contamination; the best results were achieved when 5gm mancozeb was used for 75 minutes, while 50% Clorox treatment for 5 minutes was found to be the best for controlling bacterial contamination. After sterilization, medium for direct shoot regeneration and proliferation were optimized. The explants were cultured on olive media containing different concentrations and combinations of auxins and cytokinins. Among the treatments used, the highest sprouting rates were observed in the media containing 6-Benzyleaminopurine3.5 mg/L and 1-Naphthaleneacetic acid 1.5 mg/L. Our findings accentuate on the fact that the applicability of the optimized methods should be evaluated for olive cultivars of commercial value.

Key words: Browning, Callus, Contamination, Olive media, Phytohormone, Proliferation, Sprouting

Introduction

Olive (*Oleaeuropaea* L.) is one of the oldest cultivated crop trees of mankind and a major fruit of the Mediterranean region consumed all over the world. Olive cultivation from Mediterranean basin is presently expanding into areas of Australia, Central and South Asia, South and North America and South Africa (Rugini and Fedeli, 1990). The consumption of olive oil is increasing day by day due to its large nutritional values but the olive tree and its fruit products can be largely damaged by many diseases and pests (Haber and Mifsud, 2007). The conventional method of olive

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tree propagation is based on vegetative multiplication using cuttings, grafting or suckers (Fabbri et al., 2004). Although occasionally yielding satisfactory results, these methods have been frequently criticized as being very slow and inefficient for some highly valued cultivars. Nowadays, several studies have been conducted with the aim of developing alternative micro-propagation methods that can help to overcome the limitations associated with the traditional techniques currently used for the propagation of olive trees. One of the driving forces behind this growing interest is the challenging attempt to increase the number of plants produced while decreasing the interval of plant production time. As chances of success are fewer with conventional cuttings, micro-propagation of olive is done to get a large number of desired plants in minimum possible time. Moreover, the plants obtained in this way are free of any diseases or pests (Rugini et al., 1999).

Large number of plants can be produced through in vitro propagation. Success of propagation depends on the genotype, selection of explants, media/culture conditions and levels of growth regulators. The micro-propagation of olives was first achieved a quarter of a century ago, however, progress in improving the technique has been relatively slow, due to the inherently slow growth of olive explants. The rate of proliferation of olive explants is limited by the low frequency of bud sprouting and poor growth rates of secondary shoots (Leva et al., 2012). The first report of somatic embryogenesis in olive used a portion of cotyledons from immature embryos (Leva et al., 1995). Improved protocols have enabled the induction of somatic embryogenesis from mature tissues (petioles) obtained from shoots grown in vitro portions of the radicle and cotyledons (Peyvandi et al., 2010), ovaries, stamens, leaves and petioles (Mazriet al., 2011) and cell suspension cultures derived from mature olive tissue (Trabelsi et al., 2011). For the successful preservation of endangered cultivar Nebbiara of olive, sterilization and growth conditions was optimized (Zacchini and De Agazio, 2004).

The genetic improvement of olive requires more research work so as to increase resistance to drought, salinity, cold and biotic stress. Although conventional breeding does make a great contribution to the genetic improvement of woody plants including olive plants (Hussain et al., 2014), classical improvement programs in trees such as olive are slow and tedious due to their long generation cycle and the high level of heterozygosity (Rugini and Gutierrez, 2006). Therefore, in vitro biotechnological methods such as micro-propagation and somatic embryogenesis are being used with commercial tree species such as olive tree (Aderkas and Bonga, 2000). The above literature dictated the significance of micro-propagation but only a few studies have been carried out with an objective to speed up the propagation time of olive plants and the requirements that govern olive in vitro development. Accordingly, the present study was undertaken to acquire new information that might contribute to the development of an in vitro protocol for the rapid and efficient micro-propagation of olive cultivar “Uslu” which is of high commercial value.

Materials and Methods

Preparation of Explants

The healthy olive suckers (explants, ~20-30cm long) of the cultivar “Uslu”

cultivated in the orchards of National Agricultural Research Council (NARC), Islamabad, Pakistan, were collected and cut into uninodal segments (from 2.0 to 4.0 cm in length) and taken into the lab, which were then thoroughly washed under running tap water for at least 30 minutes until the dirt was removed from the explant. These uninodal segments were then agitated with detergent for 20 minutes to remove undesirable materials from explants. After that a final wash with distilled water (DW) was given before sterilization.

Explant Sterilization

The explants were then surface sterilized under laminar flow hood by being dipped and constantly agitated in different concentration and treatments of fungicide (Mancozeb 64% w/w + Metalaxyl 08% w/w) such as 0 (T0), 2 (T1), 3 (T3), 4 (T4), 5 (T5) and 6gm (T6) for 25, 45 and 75 minutes respectively. After treating with fungicide, the uninodal explants were washed with sterile DW and then treated with different dilutions of commercially available bacterioside Clorox (containing 50% sodium hypochlorite). The strength of different treatments made from stock Clorox were 25%, 50%, 75% and 100% accordingly and utilized for different time periods of 5, 10 and 15 minutes and finally washed five times with sterile DW before inoculating on media.

Media Preparation and Explant Culturing

Initiation shooting and proliferation medium i.e. olive media (OM) was prepared as proposed by Rugini (1984) as shown in Table 1. The medium was supplemented with auxins and cytokinins in the combination as 6-Benzyleaminopurine (BAP) 1mg/L + 1-Naphthaleneacetic acid (NAA) 0.5 mg/L, BAP 1.5 + NAA 0.5 mg/L, BAP 2 + NAA 1 mg/L, BAP 3 + NAA 1mg/L, BAP 3.5 + NAA 1.5 mg/L and BAP 4 + NAA 2mg/L. The pH of the medium was adjusted to 5.8. The medium was poured in test tubes after adding agar (Gelzan, 1.7 g/ L), plugged, covered with paper, labeled and subjected to autoclaved for 20 minutes at 121^{o}C and 15 psi. After the sterilization of explants is over and moisture dried up then explants were carefully inoculated into the culture tube containing media under laminar flow hood autoclaved forceps and petri plates were used for handling explants once they are sterilized. The explants were kept under illumination for 16 hours (Grolux lights, 80mmol m-2 sec-1) at 25±2^{o}C.

Shooting Initiation and Proliferation

For initiation of shooting and proliferation, different range of concentrations of auxins and cytokines were tried separately as well as in combinations. Such as for shoot initiationBAP3.5 mg/L, NAA1.5 mg/L,2-isopentenyladenine(2iP) 100µl/L, Indole-3-butyric acid(IBA) 50µl/L. For proliferation, combination of 200µl BAP and 200µl Thidiazuron (TDZ) were used.

Optimized Growth Conditions

Inoculated cultures were maintained in a growth chamber at 25±2^{o}C and under white fluorescent lights (140µmol $m^{-2}s^{-1}$) with a 16 hour photoperiod. The explants were sub cultured after 3 weeks. The explants developed into shoots within 1-3 weeks of sub culturing and after one month of culturing, the sprouted nodes were transferred to multiplication medium i.e. modified OM with some more supplements containing 0.1mg/L 2iP, 0.05mg/L IBA, 1mg/L BAP and 1.5 mg/L NAA. To induce leaves multiplication OM was further supplemented with 0.2mg/l concentrations of BAP combined with 0.2 mg/L of TDZ.

Table 1. Composition of Basic Culture Media (Olive Media) for Initiation.

Macronutrients	Quantity (mg/L)	Micronutrients	Quantity (mg/L)	Vitamins	Quantity (mg/L)
NH_4NO_3	1031	H_3BO_3	12.4	Glycine	2
KNO_3	1500	$MnSO_4.4H_2O$	22.3	Myo-inositol	100
$CaCl_2$. $2H_2O$	440	$ZnSO_4.7H_2O$	14.3	Thiamine	0.5
$MgSO_4$. $7H_2O$	935	KI	0.83	Pyridoxine	0.5
KH_2PO_4	255	$Na_2MoO_4.2H_2O$	0.25	Nicotinic Acid	100
KCl	250	$CuSO_4.5H_2O$	0.25	Biotin	0.05
$Ca(NO_3)_2$. $4H_2O$	300	$CoCl_2.5H_2O$	0.25	Glutamine	200
		$FeSO_4.7H_2O$	27.80	Sugar/ Mannitol	30000
		Na_2-EDTA	37.2		

Results

Explant Sterilization

Fungicide (Mancozeb 64% w/w + Metalaxyl 08% w/w) and Clorox were used for sterilization so that the treatment of explants is essential for effective and persistent control against fungal and bacterial contamination. Mancozeb was used against fungal contaminants but afterwards Clorox must be followed to limit bacteria arising in culture tubes. It is critically examined that the survival of the explants was totally dependent upon the concentration and time of the treatment of both fungicide and Clorox. Moreover, treating explants with higher concentrations of Clorox or due to the treatment of both for longer period of time affect survival of explants. The effects of different treatments of Mancozeb and Clorox for different time periods on the suckers are shown in the Table 2 and 3 respectively. The explants burnt and became brownish black due to the very high doses of Clorox, whereas, higher doses of fungicide does not significantly affect vigor of explants.

Sprouting

Sucker explants showed positive result which sprouted rapidly and with maximum care, sucker explants have shown results near to 70% in terms of sprouting (Table 4). BAP with NAA was seen as most responsive supplement. The hormonal combination of BAP 3 + NAA 1.5 mg/L exhibited good result for the culturing of sucker on OM. At the 3rd week of sub culturing sprouting of shoots up to 5cm was formed from axillary bud of explants (Fig. 1). Proliferation of explants on multiplication media was obvious and successive results were obtained as the development of new leaves and shoots started as shown in Fig. 2.

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Table 2. Effect of different concentrations of fungicide on Suckers (explants) to control fungal contamination.

Fungicide(g/250ml)/ Treatment	Exposure time (min)	Contamination (%)	Browning (%)	Survival rate (%)
0 (Control)	25	100	0	**0**
	45	100	0	**0**
	75	100	0	**0**
2 (T_1)	25	98	0	**2**
	45	95	0	**5**
	75	93	0	**7**
3 (T_2)	25	93	0	**7**
	45	92	0	**8**
	75	80	0	**20**
4 (T_3)	25	81	0	**19**
	45	74	0	**26**
	75	56	0	**44**
5 (T_4)	25	49	0	**51**
	45	36	0	**64**
	75	12	0	**88**
6 (T_5)	25	49	1	**50**
	45	36	2	**62**
	75	**22**	**4**	**74**

Table 3. Effect of different concentrations of Clorox on Suckers (explants) to control bacterial contamination after fungicide treatments.

Clorox (%)	Exposure time (min)	Contamination (%)	Browning (%)	Survival rate (%)
25	5	92	0	**8**
	10	91	0	**9**
	15	84	0	**16**
50	5	40	20	**40**
	10	30	44	**26**
	15	20	69	**11**
75	5	56	14	**30**
	10	34	46	**20**
	15	21	70	**9**
100	5	12	67	**21**
	10	9	79	**12**
	15	**3**	**94**	**3**

Table 4. Sprouting rate of suckers under varying combinations of BAP and NAA on Olive Media.

BAP (mg/L)	NAA (mg/L)	Weeks to sprout	Sprouting Rate (%)
1.0	0.5	No Sprouting	0
1.5	0.5	No Sprouting	0
2.0	1.0	2.0	01.0
3.0	1.0	2.0	10.0
3.5	1.5	1.0	70.0
4.0	2.0	1.0	65.0

Fig. 1. Sprouting of Olive cv. Uslu (sucker) explant till 3 weeks on olive media

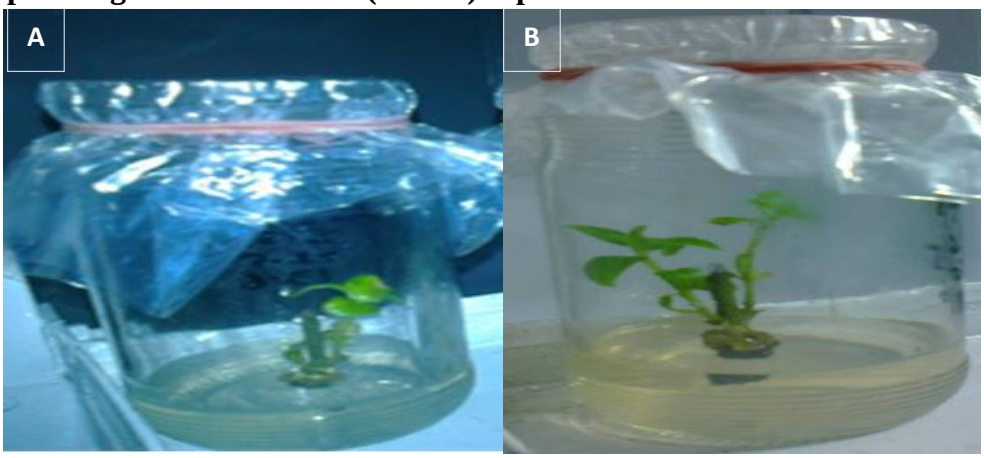

Fig. 2. Proliferation of sucker explants after sub culturing on multiplication media (A) Sub cultured explants after three weeks, (B) Successive proliferation showing leaf formation.

Discussion

The composition of the medium is a crucial factor for the achievement of high multiplication rates (Şasertkaya and Çınar, 1999). Numerous studies have been reported to assess the effect of alternative medium compositions (Revilla et al., 1996; Lambardi et al., 2006) on the propagation of explants taken from adult plants or vegetative explants cultured in vitro. Olive medium accounted to be effective for the micro-propagation of a wide array of olive cultivars (Rama and Pontikis, 1990; Zuccherelli, 2002).

It was observed that the sterilization is very important against bacterial and fungal contamination before inoculation of explants in test tubes containing OM. The best result against contamination was found when explants were treated with 5gm of fungicide (mancozeb) and for 60 min and these treatments showed 88% percent of survival of explants and our results coincide with the findings of Farah et al. (2011) who indicated a significant role of mancozeb on surface sterilization and percentages of contamination in the in vitro culturing. Similarly other fungicides (like Kanker-X) when used for the surface sterilization of nodes, such as 0.1% Kanker-X for 25 min and 2% Benlate for 10 min, showed 80.87% of survival in sterilized conditions (Sakunasingh et al., 2002). From the number of treatments given, it was suggested that neither high nor low amounts of mancozeb used, showed effective result. Fungicide treatment for 60 minutes showed more positive result. Higher amount of fungicide resulting in browning of explant and less amount was not able to overcome the fungal contamination. Time was strongly related with the amount of fungicide used.

Clorox was used to overcome bacterial growth. More explants survived with the 50% treatment of Clorox and the time given to that treatment was 3 min depended upon the cultivars because some explants' ends become black due over exposure to Clorox. There is greater chance of burning of explant when treatment time is more than 5 min. High concentration of Clorox resulted in oxidation of explant and less concentration was not enough to overcome bacterial contamination so time factor is very important.

Different concentrations of BAP (cytokines) and NAA (auxins) were used for the sprouting of explants and both proved to be very effective growth hormones for olive sprouting. Different concentrations of BAP and NAA when tested in combination, the best result obtained when BAP used was 3.5mg/l and NAA 1.5mg/l. These results were related to the earlier findings of Sakunasingh et al. (2002) i.e. they used BAP with combination of NAA to induce sprouting from axillary buds. The results of present study are in contrast with the reports of earlier workers in term of combination of hormones i.e. the highest regeneration was achieved directly from the proximal part of the petioles after 2 to 3 weeks in media containing 5 to 40 µM TDZ, or with both 10 µM 2-isopentenyladenine + 2.2 µM 6-benzyladenine with or without low auxin concentration (Mencuccini and Rugini, 1993).

When cultured explants after sprouting were transferred to the multiplication media and they showed positive result by increase in number of leaves after 3rd week on the multiplication media containing 0.2mg/l BAP combined with 0.2mg/l TDZ. Similar results were observed by Chaari-Rkhis et al. (2003), they used OM medium supplemented with zeatin (1 and 2 mg /L).

Conclusion

Protocols were optimized for initiation of micro-propagation process of *Oleaeuropea* cv Uslu. The data revealed

treatments compatible for the control of contamination and in vitro sprouting for the mass propagation of the olive cultivar. For control of fungi, treatment was best optimized at 5 gm fungicide for 75 minutes. Similarly elimination of bacteria was carried out by using 50% Clorox treatment for 5 minutes. Olive explants with best sprouting was examined on olive medium supplemented with 3.5 mg/L BAP and 1.5mg/L NAA. The same treatment also induced callus in those nodal segments which did not sprout in light. For shoot proliferation olive medium with 0.2mg/L BAP combined with 0.2mg/L TDZ yielded satisfactory results. It can be suggested that the best sterilization treatments be utilized for other olive cultivars also for efficient olive micro propagation without any contamination.

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