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Development of Aseptic Protocols in Olive (*Olea europa* L.) cv. Pantaloon

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Abstract: An efficient protocol was developed for the establishment of aseptic conditions, as a pre-requisite for meristem tip culture of olive *(Olea europa* L.*)* CV. Pantaloon. Shoot tips 0.5-0.8mm in length were used as explants source from the field-grown olive plants. Before cultured on Olive Initial Medium (OIM) for shoot proliferation and callus induction, these shoots were disinfected with four different disinfection procedures. Sterile and growing cultures were obtained, only from explants sterilized with 2.5mm $HgCl_2$ for 3 min followed by 50% Chlorox for 15min. Sterile explants responded to the growing media either by developing into leafy shoots or by producing callus. This paper outlines the aseptic protocols for obtaining the sterile explants as the starting material to initiate *in vitro* cultures in olive CV. Pantaloon.

Key words: *Olea europa* L., explant disinfection, meristem tip culture, 2-isopentenyladenine

Introduction

The olive tree (*Olea europa* L.) belongs to the family Oleaceae and has approximately 30 genera with 600 species (Cronquist, 1981). Olive trees are distributed on every continent and comprise a large number of garden plants and trees cultivated for economical purposes. According to Taylor (1945), the basic chromosome number of the genus *Olea* is x=23(2n=46). About 96% of the olive production are concentrated in the Mediterranean countries, mostly for oil extraction; only about 7-8% is table olive. The use of olive oil has greatly increased in recent years due to its appetizing taste and nutritional quality. It has a high oleic acid content, which has been found to be an important dietary value and is the only fluid oil, which can be used, in the crude form (Rugini *et al.*, 1995).

The Agriculture and Forest Departments have run a lot of experiments and projects on the feasibility of olive cultivation in Pakistan. The Forest Department has not been successful in attaining successful grafting of the present wild plants with the cultivated species imported form Europe. A project was run by PARC at Fateh Jang, which has shown good success and with berry yield up to 40kg $plant^{-1}$. The Agriculture Department (Extension Wing) has brought a tremendous success in top working of existing wild plants with Italian varieties like Killing gaut, Palmilo from Afghanistan and plantation of other varieties like Frantio, Lesio and Caratina, from Italy have been very successful (Quresh, 2000).

Research is needed therefore, to improve productivity of existing varieties, to develop new varieties and to raise olive nurseries, better suited for local environment. However, its long period of juvenility, and the prevailing self-sterility, has discouraged the genetic improvement of this species (Martino *et al.*, 1999).

Conventional programmes like grafting, cutting and vegetative propagation being time consuming and labor-intensive need to be supplemented with other non-conventional and biotechnological approaches such as tissue culture, micropropagation and genetic engineering to overcome some of the difficulties encountered.

Unfortunately, field grown olive plants are heavily contaminated. Consequently, it is particularly difficult to obtain sterile explants to start *in vitro* culture (Rugini, 1990).

Nearly 50 years ago the elimination of viruses from *Dahlia* and potato (*Solanum tuberosum*) by the use of sterile culture of apical meristem was demonstrated for the first time by Morel and Martin (1955). Apical meristem culture is one of the most successful methods of obtaining disease free plant material (Murashige and Jones, 1974). Since then, pathogen free apical meristem cultures have been obtained in several economically important species such as apple (Lane, 1978), pear (Faggioli *et al.*, 1997), grapevine (Galzy, 1972; Barlass *et al.*, 1982; Barba *et al.*, 1992), prunus (Boxus and Quoirin, 1974) and chestnuts (Chavin and Salesses, 1988). The percentage of apical meristem established *in vitro* is dependent on the disinfection procedure and the size of explants (Martino *et al.*, 1999).

Various attempts to culture olive meristems from field or green house grown plants proved unsuccessful, due to rapid oxidation of the explants (Rugini, 1986; Mencuccini, 1995). This investigation was under taken to find out the response of the sterile explants to shoot proliferation and callus induction on OIM media.

Materials and Methods

In vitro cultures were established from the apical shoots. Actively growing shoots of the olive CV. Pantaloon were collected from 15 years old field-grown olive plants. Expanded leaves were removed and 2-3cm long pieces were washed thoroughly in running tap water for 2-3 hr. and immersed for 30 min in a solution of ascorbic acid (100 mg l^{-1}) plus citric acid (150 mg l^{-1}). The following different disinfection procedures were adopted: Firstly, shoots were immersed for 15min in 100% Chlorox and rinsed three times in sterile distilled water (sdw). Secondly, shoots were immersed in 70% ethanol (C_2H_5OH), rinsed in sdw, placed for 10min in 100% Cholorx and rinsed again three times in sdw. Thirdly, shoots were dipped in a solution of 2.5mm $HgCl_2$ (677mg l^{-1}) plus 30 drops l^{-1} Tween-80 for 3min, rinsed in sdw, placed for 15min in 50% Chlorox and rinsed three times in sdw. Fourthly, as in three, but Tween-80 was omitted.

Shoot tips, approximately (0.5-0.8mm) in length were cut from the sterilized stem and the explants were then placed on OIM media supplemented with 0.5mg l^{-1} of 2 isopentenyladenine (2iP) to induce shoot proliferation or OIM supplemented with 0.5mg l^{-1} of 2iP and 150mg l^{-1} of filter sterilized cefotaxime (Rugini, 1984). The pH of the each medium was adjusted to 5.8 before, gelled with gelrite of Sigma Chemical Co. @ 2g l^{-1} and autoclaved at 121°C for 20min.

Culture conditions were 23-25 °C. 10hr. photoperiod at 48μ mol. S^{-1} M^{-2}. 40 single node explants per sterilization treatment and per media were used and subcultured every 20-25 days. After 3 subcultures in the same media the success was measured in terms of percentage of explants survived the sterilization treatment.

Results and Discussion

Different experiments were conducted with a view to find out the optimum culture conditions for shoot proliferation and callus induction from apical meristem. The data demonstrates that the percentage of apical meristem established *in vitro* was dependent on the disinfection procedure. When the disinfection was according to procedure 1, no explant died after 3 days, but all resulted contamination at the end of 70 days of culture (Table 1). It can be inferred that Chlorox, used as the sole disinfectant, was insufficient to achieve sterilization. It does not penetrate into the apical meristem tips to cause the death of explants as reported by Martino *et al.* (1999). Fungi were the main contaminant, which made their appearance with in 7 days. Using ethanol (procedure 2), almost all the explants rapidly appeared brown and a few surviving ones resulted in contamination at the end of 70 days of culture. Only a few explants died after disinfection with procedure 3 and 4 (Table 1). In addition, 60% (procedure 3) and 70% (procedure 4) of surviving explants remained free of contamination and grew well in *in vitro* conditions. The best results were obtained with the combined use of $HgCl_2$ 2mM and Chlorox 50%. This may be attributed to the fact that $HgCl_2$ and Chlorox are active only on the mature and differentiated tissues. Tween-80 did not prove to be a good disinfectant as 20% explants died within 3 days. From this investigation it appears that Tween-80 like ethanol (C_2H_5OH), also caused the death of explants.

The use of antioxidants at the end of sterilization has no effect on the growth of explants in any of the media tested (Otero and Docampo, 1998). Rugini (1984) also demonstrated that antioxidants can only delay the browning of explants. Our findings in this investigation also reveals that the ascorbic acid and citric acid at the end of the sterilization dose not improve the explant survival as reported by Martino *et al.* (1999).

The response of shoot tips to shoot proliferation and callus induction media refers to those explants surviving after 70 days of culture. On shoot proliferation media, enlargement of explants was observed after 20 days. After two successive subcultures elongated and expanded leaves were observed (Fig. 1).

Table 1: Comparison of four different disinfection protocols applied to olive meristem tips taken from apical shoots after 70 days of culture on OIM

Disinfection procedure	No. of explants		Contaminated by			No. of explants survived	Survival frequency
	Used	Died after 3 days	Fungi	Bacteria	Fungi + Bacteria		
1	40	-	40	-	-	-	-
2	40	35	-	5	-	-	-
3	40	8	8	-	-	24	60
4	40	2	6	-	4	28	70

Fig. 1: Developing shoots of CV. Pantaloon after 70 days of culture on Olive Initial Medium (OIM) containing 0.5mg l^{-1} of 2iP

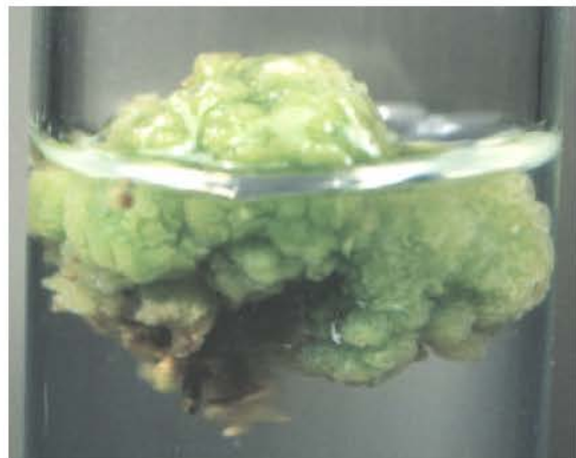

Fig. 2: Proliferating callus from meristem tips of CV. Pantaloon after 70 days of culture on Olive Initial Medium (OIM) supplemented with 0.5mg l^{-1} of 2iP and 150mg l^{-1} of cefotaxime.

Morphogenic and compact calli were observed on callus induction media after 70 days of culture (Fig. 2). Callus initiation was observed on surviving explants about after 15-16 days.

In this study the size of explants is taken as 0.5-0.8mm, which is neither very small nor large. The presence of mature cells which are known to be rich in phenolic compounds resulted in the rapid browning and death of the larger explants (Martino *et al.*, 1999). However, too small a size may fail to regenerate or to develop into complete plants unlike larger explants (Quak, 1977; Shabde-Moses and Murashige, 1979). This aspect was considered in choosing the size of the explant.

From the results of research work it is concluded that suitable sterilization procedure results in the initiation of *in vitro* cultures from apical meristems of field-grown olive plants. Disinfection with 2.5mM $HgCl_2$ and 50% Chlorox (procedure 4) proved to be the best sterilization procedure for shoot tips of field-grown olive plant CV. Pantaloon.

These findings could open up new prospects for the production of olive pathogen-free propagation material as well as for the creation of germplasm bank of olive meristems, for instance, through cryopreservation (Siebert, 1976; Withers, 1978).

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