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Full Length Research Paper

In vitro storage of synthetic seeds: Effect of different storage conditions and intervals on their conversion ability

Muhammad Ikhlaq, Ishfaq Ahmad Hafiz[1]*, Maurizio Micheli[2], Touqeer Ahmad[1], Nadeem Akhtar Abbasi and Alvaro Standardi[2]

[1]Department of Horticulture, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan.
[2]Department of Agricultural and Environmental Sciences, Faculty of Agriculture, University of Perugia, Italy.

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In vitro **derived shoots of olive cv. Moraiolo were employed in synthetic seeds preparation by alginate encapsulation, and then stored in artificial endosperm solution at cold (4°C) and room storage (21 ± 2°C) conditions in interaction with different storage intervals of 0, 15, 30, 45 and 60 days to evaluate the comparative regrowth and conversion capacity of synthetic seeds. Cold stored synthetic seeds were superior in terms of their regrowth capacity than that of room stored ones for all the growth parameters studied. A promising degree of interaction was observed between 4°C and 45 days of storage interval for regrowth percentage as well as for shoot and root development. Moreover, an ascending trend was recorded in conversion potential with an increase in storage intervals up to 45 days (S_3) whereas there was a declining trend after that up to 60 days (S_4). Moreover plantlets regenerated from synthetic seeds, with 4 - 6 fully expanded leaves and well developed root system were successfully acclimatized under *ex vitro* conditions. The protocol can be used for germplasm exchange of woody trees and preparation of synthetic seed.**

Key word: Synthetic, seed, olive, encapsulation, storage, conversion

INTRODUCTION

Olive (*Olea europaea* L.) belonging to the family Oleaceae is a schlerophyllous evergreen tree with a high degree of drought tolerance (Bacelar et al., 2006). Owning to its nutritive and therapeutic values and ability of olive trees to grow on poor soils even in arid conditions, many countries are interested in bringing their areas under cultivation of olive orchards. There is a great demand of its germplasm exchange and good quality plants. Micro-propagation techniques can be used to get true to type, disease free and certified plant material of a few olive cultivars efficiently (Rugini, 1984).

Synthetic seed technology is a good substitute to traditional seeds and micropropagation systems as the establishment of germplasm repositories of traditionally micropropagated plants for further study is difficult; due to limited space, huge amount of money is required for their maintenance. Moreover, exchange of stock cultures between laboratories is also problematic in consideration with temperature fluctuations and danger of infestation with microorganisms. Synthetic seeds provide an alternative dependable way for mass scale production, efficient delivery of cloned plantlets and also to meet the interna-tional quarantine requirements. Exchange of axenic plant material between laboratories and successful plant regeneration from synthetic seeds has been reported in several plant species (Fabre and Dereuddre, 1990; Standardi and Piccioni, 1995; Maruyama et al., 1997). However, storage is a critical factor for exchange and preservation purposes, which determines the success of synthetic seeds after their transportation abroad. Therefore, appropriate storage conditions and definite storage periods required to maintain viability during exchange of

*Corresponding author. E-mail: decenthafiz@gmail.com.

Abbreviations: OMM, Olive medium modified; **RIM,** root induction medium; **IBA,** indole-3-butyric acid; **MCTS,** microcutting; **ABA,** abscisic acid.

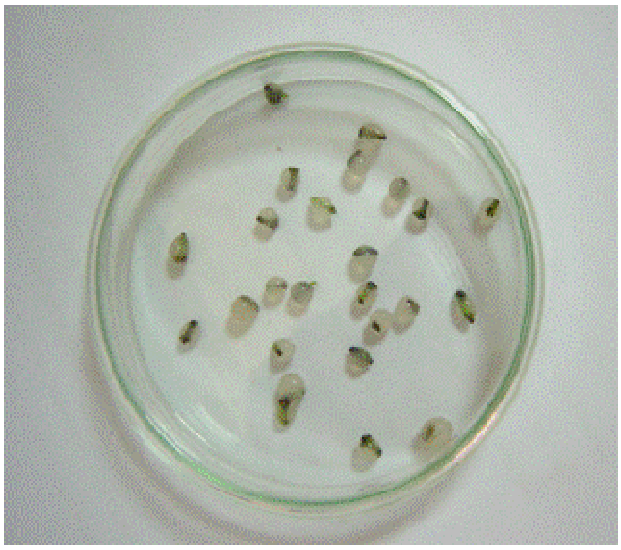

Figure 1. Prepared synthetic seeds.

germplasm are prerequisites for synthetic seed technology and its commercial appli-cation. In addition, development of appropriate storage techniques is also obligatory for successful conversion of the synthetic seeds of clonal germplasm of elite and endangered cultivars in the near future (Maruyama et al., 1997). The use of synthetic seeds for obtaining plants has been reported for several crops of economic interest (Redenbaugh et al., 1987; Gray and Purohit, 1991) but for only very few woody species (Rao and Bapat, 1993; Lulsdorf et al., 1993). Keeping in view all these aspects, the present study was therefore undertaken to (1) Study the effect of cold and room storage at different storage intervals on survival, proliferation and conversion abilities of synthetic seeds; (2) formulate a protocol and composition of nutritive medium for room storage in subsequent experiments as energy is a big crises for storage of synthetic seeds into freezer/refrigerator in developing countries.

MATERIALS AND METHODS

Stock cultures

Synthetic seeds of five Olive (*Olea europaea* L.) cultivars; Dolce Agogia, VP1, PS1, Chietina and Moraiolo were prepared according to the method described by Micheli et al. (2007) and stored at 4°C for one week in Biotech Lab., University of Perugia Italy. The glass bottles of synthetic seed were packed in cartons and sent through DHL Courier Service, under ambient temperature conditions within four days to Biotech Laboratory, Arid Agriculture University Rawalpindi Pakistan. These synthetic seeds after one week storage at 4°C were cultured successfully on olive medium modified (OMM) (Mencuccini et al., 1997), supplemented with 4 mg l^{-1} zeatin, 30 g l^{-1} sucrose and 6.5 g l^{-1} agar. For maintenance of cultures of the five varieties, subculturing of single uninodal segments of about 10 - 15 mm length, with two opposite leaves having axillary buds was done after every 45 days. For further studies on storage of synthetic seeds of cv Moraiolo, after establishment of sufficient stock, first four nodes of each proliferated shoot were excised (1 or 2 mm either side of node) to prepare uninodal microcutting (MCTS) of 3 - 4 mm in length, without leaves and having two axillary buds. MCTS were inserted into culture jars containing root induction medium (RIM), comprising of half strength OMM supplemented with 0.5 mg l^{-1} indole-3-butyric acid (IBA), 20 g l^{-1} sucrose + 10 g l^{-1} glucose, 100 mg l^{-1} brilliant black dye and solidified with 6.5 g l^{-1} agar. After 11 days, MCTS with root primordia were taken out and used for encapsulation with sodium alginate (Medium viscosity, Sigma code A-2033) solution 2.7 % (w/v), enriched with artificial endosperm composed of half strength OMM supplemented with 1 mg l^{-1} zeatin and 20 g l^{-1} sucrose + 10 g l^{-1} glucose. The MCTS were immersed for a few seconds in the autoclaved alginate solution followed by complexation with a mixture of $CaCl_2$ 1.1 % (w/v) and endosperm solution for 35 min. After complexation, the hardened alginate capsules were washed twice for 15 min each time with autoclaved rinse solution, consisting of the artificial endosperm without further additions. For study of temperature effects, re-growth potential of synthetic seed was evaluated after storage of cold (4°C) and room (23 ± 1°C) temperatures at various intervals of 0, 15, 30, 45 and 60 days. Capsules were stored in glass bottles along with artificial endosperm solution to maintain the relative humidity inside the bottle during the storage period.

After defined period of storage, the synthetic seeds (Figure 1) were cultured on half strength OMM medium supplemented with 20 g l^{-1} sucrose, 10 g l^{-1} glucose, 6.5 g l^{-1} agar and 100 mg l^{-1} brilliant black dye. The pH of all media was adjusted to 5.8 before autoclaving at 121°C for 15 min and cultures were incubated at 25 ± 1°C under 16 h light (2,000 lux) with white fluorescent tubes (Philips TL 40W/54). All the manipulations of the plant material were carried out in sterile conditions under laminar flow cabinet. Data were recorded on germination percentage, number of shoots per explant, shoot length (cm), number of nodes, rooting percentage, number of roots per explant and root length (cm). The treatments were arranged according to completely randomized design (CRD) consisting of four replications per treatment and twenty synthetic seeds per replication. Statistical analysis of the data was carried out by using analysis of variance (ANOVA) technique and differences among treatment means were compared by using least significance difference (LSD) test at 5% probability level (Steel et al., 1997).

RESULTS AND DISCUSSION

Synthetic seeds imported from Italy were evaluated none statistically for their regrowth parameters. Rough estimation of data yielded regrowth percentage (93.90%), shoot length (3.72 cm), number of shoot per synthetic seed (1.83) and number of node per plantlet (5.55). These results are also comparable to Micheli et al. (1998, 2007). It was also observed that yielded plantlets from synthetic seeds were healthy, green and free from any morpho-logical disorder. These results also ensure that synthetic seed technology is an authentic method for exchange of plantlets grown *in vitro* from one laboratory to another aseptically as well as without danger of any loss. Stock cultures of imported synthetic seeds were maintained for clonal propagation and were also acclimatized successfully in glass house.

Germination percentage

Results in Table 1 shows the effect of storage temperatures

Table 1. Effect of different temperature and storage intervals on germination percentage (%) of the synthetic seeds of Olive cv. Moraiolo.

Treatment (storage interval)	Mean germination percentage (%)		Mean
	Cold storage (4°C)	Room storage (21 ± 2°C)	
S_0 (0 days)	65.00 cd	65.00 cd	65.00 C
S_1 (15 days)	77.50 b	77.50 b	77.50 B
S_2 (30 days)	95.00 a	73.75 bc	84.75 A
S_3 (45 days)	97.50 a	70.00 bc	83.75 AB
S_4 (60 days)	60.00 d	47.50 e	53.75 D
Mean	79 A	66.75 B	
$LSD_{0.05}$	Storage 4.04	Interaction (S×I) 9.03	Treatment 6.38

Any two means not sharing a letter differ significantly at $p<0.05$; S = storage conditions; I = interval (time).

(4°C and 21 ± 2°C) on germination (shoot or root growth > 3mm) percentage of synthetic seeds. Cold storage (4°C) gave the highest rate of germination percentage (79%) which was significantly different from that yielded by room storage (21 ± 2°C) that is 66.75%. Similar results of improvement in regrowth of synthetic seeds after cold storage were also experienced by Micheli et al. (1998) in olive and Sicurani et al. (2001) in apple rootstock. It might be possible that cold storage slow down the metabolic activities of the synthetic seeds hence, they remained in a visual quiescent state that is helpful for preservation of nutritive reservoir in the synthetic seeds during cold storage. Contrarily, it was observed that most room stored synthetic seeds elongated, protruded out from encapsulating gel and ultimately showed necrosis during storage. Hydrated synthetic seeds were difficult to store at room storage because they lack quiescence and deplete nutritive reservoir which resulted to low germination percentage (Nieves et al., 2001). However, the potential of cold and room stored synthetic seeds to convert into complete plants indicated that trimming and encapsulation had no negative effect on their regrowth and they still maintained meristematic characteristics.

Statistical analysis showed the non significant interaction between the storage conditions (cold and room storage) and storage intervals (0, 15, 30, 45 and 60 days) at $p<0.05$ regarding the germination percentage of the synthetic seeds (Table 1). It is also interesting to put in evidence that germination percentage of 15 days stored synthetic seeds at both storage conditions increased in comparison to synthetic seeds sown immediately after encapsulation (control). This is because, synthetic seeds were stored along with endosperm solution which might have diffused into the encapsulation matrix and consequently gave better results. Moreover, interaction indicated that cold storage at 45 days of interval (S_3) resulted in maximum regrowth percentage (97.5%) of the synthetic seeds. This might be due to the maximum diffusion of endosperm solution which remained preserved along with the accumulation of endogenous abscisic acid (ABA) at cold storage. Nieves et al. (1998) reported that ABA kept MCTS dormant and enhanced accumulation of other organic compounds which helped in the mobilization of storage reserves. Other biochemical changes proceeded as synthetic seeds started their regrowth. Contrarily, there was a progressive decrease in the germination percentage of room stored synthetic seeds. It was more abrupt as storage interval increased from 45 to 60 days at both storage conditions. Progressive decline in germination percentage of room stored synthetic seeds might be due to necrosis of most elongated MCTS which was first observed in the centre of MCTS and later on, across the MCTS as days of storage elapsed. However, the reason for sudden decrease in germination percentage after 60 days of cold storage is still unknown.

As far as the influence of different storage intervals on germination percentage of synthetic seeds is concerned, a non significant variation was also recorded among them. However, rate of germination percentage increased with increasing storage intervals up to certain extent and after that there was a drop. The treatments of 30 days (S_2) and 45 days interval (S_3) showed maximum germination of 84.37 and 83.75%, respectively. As treatments showed combined effect of cold and room storage, maximum spermidine accumulation at cold storage for 30 and 45 days intervals might be a reason for good germination. Jouve et al. (1995) advocated that increased in endogenous spermidine content plays an important role in cell protection and acclimatization in wild cherry shoots stored at low temperature for 15 days. Moreover, these compounds are mandatory in the regulation of organogenesis (Nieves et al., 1998). These compounds are also bound with specific proteins in the presence of transglutaminase enzyme forming protein-Glu-PA which play an important role in the post-translational modifications of proteins and stabilize the configuration and function of proteins by preventing them from denaturing during storage (Serafini-Fracassini, 1995). However, the

Table 2. Effect of different temperature and storage intervals on number of shoots per synthetic seed of olive cv. Moraiolo.

Treatment (storage interval)	Mean shoot number per synthetic seeds		Mean
	Cold storage (4°C)	Room storage (21± 2°C)	
S_0 (0 days)	1.47 de	1.47 de	1.47 B
S_1 (15 days)	1.67 ab	1.51 cd	1.59 A
S_2 (30 days)	1.54 cd	1.69 ab	1.61 A
S_3 (45 days)	1.71 a	1.60 bc	1.66 A
S_4 (60 days)	1.37 e	1.56 cd	1.46 B
Mean	1.55 A	1.56 B	
$LSD_{0.05}$	Storage 0.04	Interaction (S×I) 0.10	Treatment 0.07

Any two means not sharing a letter differ significantly at $p < 0.05$.

minimum germination, 53.75%, was recorded at 60 days interval (S_4). This might be due to limited availability of oxygen in meager environment of the synthetic seeds. Encapsulated MCTS are living and respiring tissues, withdrawing oxygen from adjacent intercellular spaces and evolving CO_2 into it. Therefore, any decline in germination percentage of the synthetic seeds is due to inhibited respiration of plant tissues by alginate cover (Redenbaugh et al., 1987).

Number of shoots

Numbers of shoots per synthetic seed were almost similar in response to cold (1.55) and room (1.56) storage although statistically different (Table 2). This might be due to the morphology of encapsulated MCTS that bear two opposite axillary buds each of which produced a shoot regardless of the storage temperature. Micheli et al. (2007) reported that the nodal MCTS contain two opposite buds and they usually produce two shoots in each nodal cutting. In this respect, the phenomenon of apical dominance might be suppressed by cutting the shoots into individual nodal segments for the purpose of microcuttings preparation by which shoots from each bud were regenerated successfully. On the other hand, under normal sub-culturing conditions, a single olive shoot always shows apical dominance/suppressing effects on the tiny/rudimentary shoot from the second axillary bud on the same node of *in vitro* maintained shoots.

The interaction between the storage conditions (cold and room) and storage intervals was also not significantly different for number of shoots (Table 2). After 45 days interval, cold and room storage produced 1.71 and 1.60 shoots per synthetic seed, respectively. With an increasing storage interval, number of shoots per synthetic seed remained almost unaffected (a very minute change). Moreover, it was also observed that all entrapped (in encapsulation gel), viable and healthy synthetic seeds mostly produce two shoots. This might be due to the protective action of alginate coating. An alginate-gelled matrix surrounding MCTS slows the process of desiccation and provides the mechanical support to protect the tissue (buds) within encapsulation medium during storage (Sujatha and Kumari, 2007). Effectiveness of the protective coating and possibility to store the propagules was also confirmed by Ballester et al. (1997) who reported that survival percentage of encapsulated MCTS was better than those of non encapsulated ones.

In consistency with the above results pertaining to shoot number, storage intervals have also shown non significant effect for this parameter (Table 2). After storage interval of 0, 15, 30, 45 and 60 days, number of shoots produced were 1.47, 1.59, 1.61, 1.66, and 1.46, respectively. These results clearly showed no effect of storage intervals on shoot number of the synthetic seeds. However, it is suggested that the presence of zeatin and its concentration (1 mg l^{-1}) is optimum which assume to improve shoot emergence from encapsulated nodal explants. Rout et al. (2001) reported that alginate beads prepared in the MS medium without growth regulator has a low percentage of bud break and slower growth than the synthetic seeds having MS medium with growth regulators.

Shoot length

Shoot length followed the same pattern of results as that of germination percentage (Table 3). Higher score for shoot length (1.67 cm) was observed at cold storage relative to 1.17 cm shoot length of room stored synthetic seeds. The most probable reason for better shoot length of cold stored synthetic seeds might be due to the accumulation of growth substances during cold storage in primordia responsible for shoot development. Fuiji et al. (1993) stated that cold storage contributes to a better maturation and accumulation of reserve compounds like storage proteins and carbohydrates in the explants. These carbon sources help the synthetic seed in its shoot elongation as shoot development *in vitro* is a highly energy consuming process with rapid metabolic rates

Table 3. Effect of different temperature and storage intervals on shoot length (cm) of the synthetic seeds of olive cv. Moraiolo**.**

Treatment (storage interval)	Mean shoot length (cm)		Mean
	Cold storage (4°C)	Room storage (21 ± 2°C)	
S_0 (0 days)	1.45 d	1.45 d	1.45 B
S_1 (15 days)	1.67 c	1.48 d	1.57 A
S_2 (30 days)	1.96 b	1.22 e	1.59 A
S_3 (45 days)	2.15 a	1.13 e	1.64 A
S_4 (60 days)	1.15 e	0.58 f	0.86 C
Mean	1.67 A	1.17 B	
$LSD_{0.05}$	Storage 0.05	Interaction (S×I) 0.12	Treatments 0.08

Any two means not sharing a letter differ significantly at $p < 0.05$.

involving increased breakdown of starch and free sugars accompanied with increased respiration rates in shoot regeneration (Luttage and Ratajczak, 1997).

As far as the interaction between storage conditions (cold and room storage) and storage intervals (0, 15, 30, 45 and 60 days) is concerned, better response was established by cold storage after 45 days interval (S_3) with a maximum outcome of 2.15 cm shoot length while at room storage, 1.13 cm shoot length was produced at same storage interval (Table 3). An eminent shoot length development after 45 days of storage interval at 4°C (cold storage) might be due to the synthesis of specific proteins which reached a maximum extent at 45 days of storage intervals. Saliveit (2000) reported that low storage temperature involves changes in total protein contents and composition of soluble proteins. In this respect, Thomashow (2001) also documented that synthesis of specific proteins are common to cold storage. Moreover, proteins present in plasma membrane are permeases which formed sucrose permease complex on bonding with carbohydrates. Thus, permease shape change which results in rotation of complex in such a way that sucrose move to the direction where energy is required (shoot and root primordia). Moreover, these proteins are involved in growth and developmental processes as a result of their metabolic and storage functions (Laurie and Halford, 2004).

Treatments (storage intervals) differed non significantly with regards to their effect on shoot length. Promising result was given by S_3 (45 days interval) with maximum shoot length of 1.64 cm followed by 1.59 cm long shoots in S_2 (30 days interval). Reduced shoot length (0.86 cm) was observed at an increased storage duration of 60 days that is S_4. It may be inferred from the above results that storage intervals have shown the positive interaction with storage conditions in general up to 45 days interval (S_3). These results are combined effect of cold and room storage. However, reduced shoot length after 60 days interval (S_4) might be due to browning of the synthetic seeds observed during storage. Schall (1987) linked browning with ethylene production. Moreover, *In vitro* grown plant tissues gradually accumulate ethylene in the culture vessels (Minocha and Jain, 2000) which can cause stunted growth (Ma et al., 1998). Furthermore, Gasper et al. (1996) documented that ethylene act as growth inhibitor of regeneration and delay DNA synthesis and cell division in the meristems (Apelbaum et al., 1981). However, the shoot length was found smaller in the present work than that of previous studies (Micheli et al., 2007), the most plausible reason is that MCTS were given sprouting induction and sprouting initiation treatments prior to encapsulation which resulted to more shoot length. In our study, we applied only root induction treatment that led to the development of root primordia. It seems like an exhaustive and competitive process to get growth of both organs viz shoot and root from tiny MCTS of 3 - 4 mm in size encased in alginate matrix.

Number of nodes

Maximum number of nodes (2.79) was recorded in shoots developed from cold treated synthetic seeds (Table 4), whereas, shoots of room stored synthetic seeds bear only 2.33 nodes per shoot. These results confirm that cold stored synthetic seeds were more vigorous than that of room stored synthetic seeds. Micheli et al. (2007) reported that number of nodes increase proportionately with an increase in shoot length in olive.

Different storage intervals in interaction with cold storage, exhibited an increasing trend for number of nodes up to 45 days of interval which went to a decline afterwards up to 60 days. Maximum number of nodes recorded after 45 days interval with cold storage was 3.23. In contrast, room storage resulted in a quite fair outcome of 2.86 at 15 days interval with a progressive decrease in number of nodes by increasing storage interval. It might be possible that at room storage, carbohydrates and other storage products continue to be broken down by respiring synthetic seeds. It was also confirmed by Ding et al. (1998) that during storage, carbohydrate reserves decline rapidly and the rate of breakdown greatly increases with

Table 4. Effect of different temperature and storage intervals on number of nodes per shoot of the synthetic seeds of olive cv. Moraiolo.

Treatment (storage interval)	Mean Number of nodes per shoot		Mean
	Cold storage (4°C)	Room storage (21± 2°C)	
S_0 (0 days)	2.46 c	2.46 c	2.46 C
S_1 (15 days)	3.03 ab	2.86 b	2.94 A
S_2 (30 days)	3.05 ab	2.30 cd	2.67 B
S_3 (45 days)	3.23 a	2.08 de	2.65 B
S_4 (60 days)	2.18 d	1.94 e	2.06 D
Mean	2.79 A	2.33 B	
$LSD_{0.05}$	Storage 0.17	Interaction (S×I) 0.23	Treatments 0.16

Any two means not sharing a letter differ significantly at $p < 0.05$.

Table 5. Effect of different temperature and storage intervals on rooting percentage (%) of the synthetic seeds olive cv. Moraiolo.

Treatment (storage interval)	Mean rooting percentage (%)		Mean
	Cold storage (4°C)	Room storage (21± 2°C)	
S_0 (0 days)	15.00 cd	15.00 cd	15.00 B
S_1 (15 days)	30.00 ab	18.75 c	24.37 A
S_2 (30 days)	32.50 ab	17.50 c	25.00 A
S_3 (45 days)	35.00 a	10.00 de	22.50 A
S_4 (60 days)	27.50 b	7.5 e	17.50 B
Mean	28.00 A	13.75 B	
$LSD_{0.05}$	Storage 2.9	Interaction (S×I) 6.6	Treatments 4.6

Any two means not sharing a letter differ significantly at $p < 0.05$

Increasing storage temperature as well as storage intervals.

Statistically, there was also no significant difference among treatments with reference to number of nodes. Storage interval of 15 days gave the maximum number of nodes (2.94) followed by 30 and 45 days interval with 2.67 and 2.65 nodes, respectively.

Rooting percentage

Comparison of the two different storage conditions proved cold storage to be superior with an acquisition of 28.00% rooting while at room storage; merely 13.75% rooting was observed (Table 5). The most probable reason for better rooting response by cold stored synthetic seeds might be due to the synthesis of endogenous rooting co-factors parallel to the shoot growth. These rooting co-factors, morphogen and rhizocaline, were reported to stimulate the root initials along with root induction treatment (Haq et al., 2009). Results also show that cold stored synthetic seeds produce more shoot length, hence rooting co-factors synthesis in good amount which consequently gave better rooting response.

Storage conditions (cold and room) and storage intervals (0, 15, 30, 45 and 60 days) interacted non significantly at $p<0.05$ for rooting percentage of the synthetic seeds (Table 5). An elevated rate of rooting (35.0%) was observed at cold storage after 45 days interval while at room storage, the maximum rooting (18.75%) was recorded after 15 days interval. According to perusal of data, a synergism was found between rooting percentage, shoot length and number of nodes per shoot of the synthetic seed. Therefore, it is suggested that increase in shoot length and number of nodes might be a factor for better rooting response. Nordstrom and Eliasson (1991) reported that auxin is produced in shoot and moves basipetally to trigger the rhizogegesis. Moreover, rooting co-factors are believed to be produce in the nodes and are essential for rooting because these cofactors combine with auxin to form a complex that directs RNA to activate enzymes that cause root initiation (Hartmann et al., 2007).

A non significant difference was noted between treatments at $p<0.05$ for the rooting percentage (Table 5). A fair rooting response (25.00%) was observed after 30 days interval after which as storage interval increased, a gradual decrease in rooting percentage was recorded. However, over all, low rooting percentage was observed in spite of root induction treatment. It is because of that in

Table 6. Effect of different temperature and storage intervals on root length (cm) of the synthetic seeds of olive cv. Moraiolo.

Treatment (storage interval)	Mean root length (cm)		Mean
	Cold storage (4℃)	**Room storage (21± 2℃)**	
S_0 (0 days)	0.850 f	0.850 f	0.850 C
S_1 (15 days)	1.85 bc	1.42 de	1.63 A
S_2 (30 days)	2.05 ab	1.25 e	1.65 A
S_3 (45 days)	2.21 a	0.64 fg	1.42 B
S_4 (60 days)	1.63 cd	0.420 g	1.02 C
Mean	1.71 A	0.91 B	
$LSD_{0.05}$	Storage 0.12	Interaction (S×I) 0.28	Treatments 0.19

Any two means not sharing a letter differ significantly at $p < 0.05$.

Table 7. Effect of different temperatures and storage intervals on number of roots per synthetic seed of olive cv. Moraiolo.

Treatment (storage interval)	Mean number of roots		Mean
	Cold storage (4 ^{0}C)	**Room storage (21± 2 ^{0}C)**	
S_0 (0 days)	1.0 b	1.0 b	1.0 B
S_1 (15 days)	1.25 ab	1.25 ab	1.25 AB
S_2 (30 days)	1.5 a	1.25 ab	1.37 AB
S_3 (45 days)	1.5 a	1.5 a	1.5 A
S_4 (60 days)	1.5 a	1.25 ab	1.37 AB
Mean	1.35 A	1.25 A	
$LSD_{0.05}$	Storage 0.31	Interaction (S×I) 0.69	Treatments 0.43

Any two means not sharing a letter differ significantly at $p < 0.05$.

difficult-to-root cultivars, the exogenous factors either failed to promote rooting or promote it slightly (Wiesman and Lavee, 1994). Micheli et al. (2006) have also reported low rooting ability of Moraiolo cultivar. Poor rooting ability of many other olive cultivars has also been reported by Aviadan and Lavee (1978), Wiesman and Epstein (1987) and Hartmann et al. (2007).

Number of roots

Results reveal that storage conditions as well as storage intervals and treatment has no significant effect on the number of roots per synthetic seed (Table 7). However, maximum average number of roots (1.50) was recorded after 45 days of interval and minimum (1.0) number of roots was observed in the control treatment. These results depicted that number of roots increased as storage interval elapsed. Thus, it leads to a conclusion that synthetic seeds of woody crops take time for root formation. Chand and Singh (2004) reported that woody perennial trees generally have a long time for root formation and they are mostly difficult to root. A well rooted plantlet is shown in Figure 2 prior to acclimatization.

Root length

The better root length (1.71 cm) was recorded at cold storage (Table 6). Whereas, only 0.91 cm long root were produced by the synthetic seeds stored at room temperature. Similarly, Machii and Yamonouchi (1993) observed better root development in the synthetic seeds of mulberry only when these were stored at 4℃ before sowing. Higher root length after cold storage might be due to accumulation of carbohydrates in root primordia as Ahmad et al. (2007) stated that root development is an energy requiring process and continuous supply of carbohydrates is necessary for normal root growth. Moreover, the internal carbohydrate pool is reported to have an important role in morphogenesis of several woody species (Kromer and Gamian, 2000; Li and Leung, 2000). Aeschabacher et al. (1994) reported that root formation is a process of cell division which need more energy to switch predermined cells from their morphogenetic path to act as mother cells for root development. However, comparatively, lesser root length after room storage might be attributed to the competition between shoot and root growth, in which these carbohydrates might be consumed in shoot elongation resulting in the limitation of

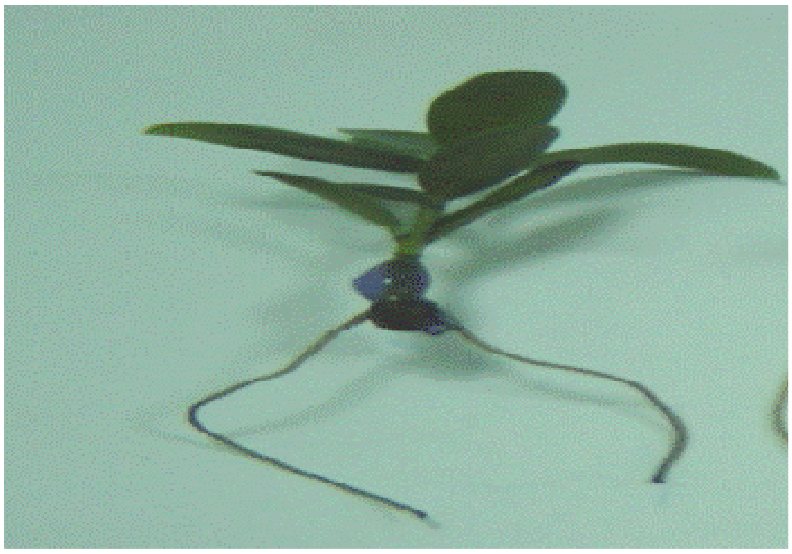

Figure 2. Plant with well developed roots.

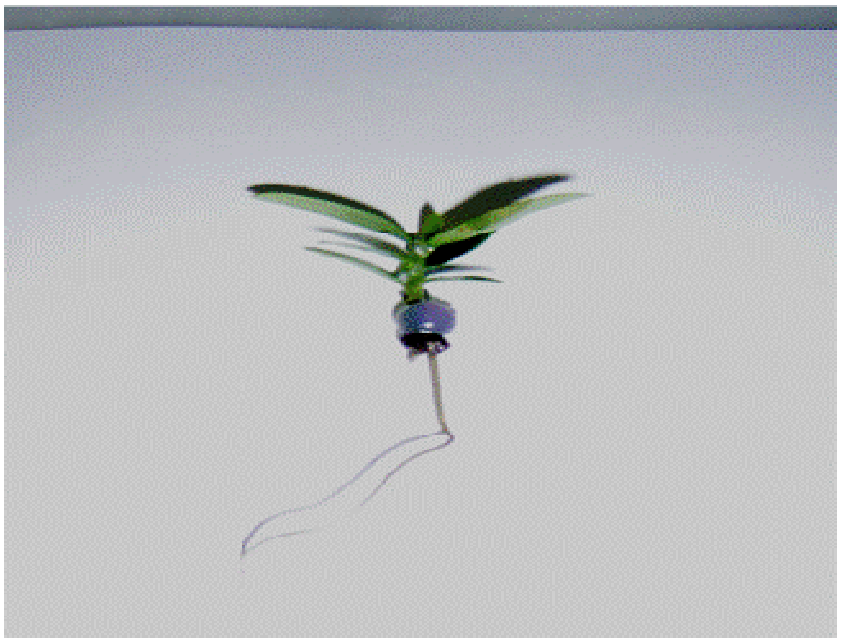

Figure 3. The plant with maximum root length (4 cm) obtained from a synthetic seed for 45 at 4°C.

root development.

Same trend was put forth for interaction between storage conditions (cold and room) and intervals (0, 15, 30, 45 and 60 days) as seen for preceding character of rooting percentage (Table 6). Best interaction was observed after 45 days interval at cold storage with maximum root length 2.21 cm (Figure 3). The difference between the interaction of storage conditions (cold and room) and storage intervals for root length might be due to various levels of accumulation of ABA which is maximum at 45 days of cold storage. With this respect, Senaratna et al. (1995) reported that exposure of explants to low temperature (4°C) enhance ABA accumulation. Moreover, ABA is considered to be a signal molecule in the stimulation of various physiological processes through expression of selected ABA-responsive genes (Giraudat et al., 1994). Davies and Zhang (1991) documented that ABA acts as a root signal and affects the translocation of sugars and amino acids and the synthesis of reserve materials. A beneficial effect of endogenous ABA and applied auxin

Table 8. Acclimatization of plantlets regenerated from synthetic seeds of olive cv. Moraiolo.

Treatment (storage Interval)	Mean survival percentage (%)		Mean
	Cold storage (4℃)	Room storage (21± 2 ℃)	
S_0 (0 days)	10	10	10
S_1 (15 days)	25	18	21.5
S_2 (30 days)	35	20	27.5
S_3 (45 days)	44	22	33
S_4 (60 days)	28	17	22.5
Mean	28.4	17.4	

Figure 4. Successfully acclimatized plant.

together on root growth of GF 677 was also found by Tsipouridis et al. (2006).

A synergism was found between the results of rooting percentage and root length of the synthetic seeds (Table 6). The results showed that 30 days interval (S_1) produced maximum root length (1.65 cm) for the synthetic seeds which was statistically similar to 30 days interval (S_2). However, a gradual decline in root length was observed as storage interval increased, as according to the data, only 1.02 cm long root was observed after 60 days interval which was relatively poor than other treatments.

Acclimatization

Ex vitro acclimatization was achieved by a simple procedure already proposed by Roussos and Pontikis (2002) that simplified the acclimatization process. Plants were directly transferred to pots containing soil and sand (1:1), and placed in glass house having light intensity of 4000 – 10,000 lux and relative humidity of 90 - 95% with a temperature ranging from 26 - 28℃. Data regarding *in vivo* survival percentage of plantlets derived from synthetic seeds are presented in Table 8. Synthetic seeds stored under cold storage conditions were better acclimatized (28.4%) as compare to those stored at room storage (17.4%). The maximum survival (44%) was recorded in the plants obtained from the synthetic seed stored at 4℃ for 45 days. Furthermore, plants regenerated from cold stored synthetic seeds resulted in a higher shoot length during acclimatization than those regenerated from room stored ones (Figure 4). This depicts that cold storage positively increased the success rate during hardening or acclimatization of olive cv. Moraiolo. The paper demonstrates a possibility of transfer of axenic plant material (Olive cultivars) from one country to another in the form of synthetic seed.

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