Turk J Agric For
24 (2000) 465–473

Somatic Embryogenesis From Mature Seed Cultures of *Pistacia atlantica*

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Received: 15.07.1998

Abstract: Embryogenic mass was produced from kernels of mature fruits of *Pistacia atlantica* cultured in liquid Murashige and Skoog media, supplemented with 100 mg/l casein hydrolysate, 100 mg/l l-ascorbic acid, and benzylaminopurine (BAP). Embryogenic masses were differentiated directly from the kernel explants after culture for 3 weeks in liquid medium with 0.5-4 mg/l benzylaminopurine. After transfer of the embryogenic masses into the same medium, but after a few subcultures with benzylaminopurine, somatic embryos appeared. Clusters of somatic embryos were transferred to the agar solidified MS medium for maturation. Matured somatic embryos, germinated on the maturation medium without growth regulators, developed into plantlets.

Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BAP - benzylaminopurine (N^6-benzyladenine); EMS -embryogenic mass; IAA - Indole-3-acetic asid; MS - Murashige and Skoog medium; NAA - α- naphthalene acetic acid; PGR - plant growth regulator; SE(s) - somatic embryo (s);

Pistacia atlantica'nın Olgun Tohumlarında Somatik Embriyogenesis

Özet: 100 mg/l hidrolize-kazein, 100 mg/l l-askorbik asit ve benzilaminopurin ile desteklenen sıvı Murashige ve Skoog (MS) besi ortamında kültürelınan *Pistacia atlantica*'nın tohumlarından embriyogenik doku rejenere edilmiştir. Embriyogenik dokular sıvı besi ortamında benzilaminopurin (0.5-4 mg/l) ile kültüre alındıktan 3 hafta sonra tohum eksplantlarından direkt olarak farklılaşmışlardır. Rejenere olan dokular benzilaminopurin ile aynı besi ortamında birkaç kez altkültürü yapıldıktan sonra somatik embriyolar üretilmiştir. Olgunlaşmamış somatik embriyo grupları olgunlaşma için agarla katılaştırılmış MS besi ortamına transfer edilmiştir. Olgunlaşmış somatik embriyolar bitki büyüme düzenleyicisi olmayan besi ortamında çimlendirilerek fideler elde edilmşitir.

Introduction

Pistacia is a genus of the family *Anacardiaceae*, which comprises such widely known trees and shrubs as *Anacardium occidentale*, *Mangifera* spp., *Rhus toxicodendron*, *R. radicans*, and *R. coriaria* (1-7). Turkey is one of the gene centres of origin of pistachio species. The most recent monographic study is that by Zohary (3) who recognized eleven species of the genus *Pistacia*, including *P. atlantica, P. cabulica, P. chinensis, P. falcata, P. integerrima, P. vera, P. kurdica, P. mutica, P. Palestine, P. terebinthus* and *P. khinjuk*. *P. vera* is the only edible nut producing species in the genus *Pistacia*. Beside its commercial use, some of the other *Pistacia* species are used for ornamental planting or as a source of ornament and shade and a windbreak for erosion control, because some species such as *P. lentiscus* are evergreen, varying from a low, shrubby bush of 3 to 6 feet in height and 6 to 10 feet in width to a small dense tree 8 to 12 feet in height and 10 to 20 feet in width (8).

Due to cross-pollination, commercial pistachio nut trees are virtually as variable as wild populations. The breeding strategy is to exploit this genetic variation using seed orchards and controlled crossing. Currently, in Turkey like elsewhere in the world pistachio trees are propagated by grafting or budding mature scions onto seedling rootstocks due to difficulties in rooting cuttings. Rootstocks for pistachio are still obtained from seeds, because a successful vegetative propagation method for *Pistacia* species has not yet been found. Rootstocks for pistachio are of two kinds: wild pistachio species which are grafted at the place where they happen to grow, and pistachio seedlings which are used for establishing commercial orchards (9). Several *Pistacia* species may be used as rootstocks. The rootstock diameter should be large enough to accommodate pistachio buds which are broad and large as compared with most fruit tree buds (10, 11). In the US, "Kerman" (pistillate) and "Peters" (staminate) are normally budded onto *P. atlantica* or *P. integerrima* rootstocks (9, 12) which have a greater

resistance to nematodes and *Verticillum* with than *P. vera*. In Turkey, Iran and other Middle Eastern countries, in general, *P. vera* is used as a rootstock for new orchards. Seedlings of this species make more lateral roots and thicker stems than the others and they can reach budding size in a shorter time (9). However, there are growth differences among seedlings, so between rootstocks and cultivars stock-scion incompatibility may be seen requiring intergrafting. Seedlings of *P. atlantica* and *P. khinjuk* show a rapid increase in length, but make thinner seedlings than the others so they reach budding size later. However, there is no stock-scion incompatibility between cultivated varieties and these rootstocks (9). Seedlings of *P. atlantica* and *P. terebinthus* are widely used for commercial production (13, 11, 14). Top worked cultivars on these two rootstocks quickly outgrow and outyield those grafted onto *P. vera* in spite of the characteristically slow growth in the nursery of the two former rootstocks (10). *P. atlantica* and *P. terebinthus* have been reported to be highly susceptible to *Verticillium* (14). Because of its very slow growth habit, *P. terebinthus* when grafted at the place where it grows, makes dwarf trees and begins to produce early, large and quality fruit. Concerning root knot nematode susceptibility, seedling progeny of *P. vera* x *P. atlantica*, *P. vera* x *P. interregima* have been found to be highly resistant compared with *P. vera* x *P. terebinthus* seedling, which proved to be the least resistant (15). However, *P. terebinthus* was reported to be tolerant to *Phytophthora* spp (16). In Turkish pistachio-growing regions 'budding' is the most common propagation technique. This technique can only be used during a very short period of the year (17).

The outlook seems to be that traditional methods of vegetative propagation in pistachios still suffer from many problems. A potential solution to these problems would be the development of methods for vegetatively propagating the rootstocks. It would also be advantageous, for example, to propagate only preferred rootstocks which impart a low production of non-bearing trees while maintaining the other superior characteristics associated with the rootstocks (18). Unfortunately, attempts to propagate rootstocks vegetatively by conventional methods of soft and hard wood cuttings have given inconsistent results (19-21). Thus, plant protection is expected to be a widespread and abundant problem. Therefore, there is an urgent need for large numbers of improved, fast growing trees to establish the new pistachio orchards. The traditional methods used for pistachio propagation and improvement are apparently not adequate to meet these demands. Therefore, new techniques to supplement the traditional methods must be developed. Plant tissue, organ and cell culture techniques provide a promising and alternative approach to the traditional methods. The aim of the present study was to develop an effective method for micropropagating *P. atlantica* used as pistachio nut rootstock.

Materials and Methods

Source of plant material: Mature fruits were used as a source of primary explants. These fruits were harvested in September 1996, in Gaziantep province, South-east Turkey.

Surface sterilisation: Mature kernels, from which the outer pericarp and shells had been removed, were pre-sterilised by immersion in absolute ethanol for 2 min followed by a rinse with sterile distilled water. These pre-sterilised kernels were then exposed to 10% (w/v) hydrogen peroxide solution for 5 min followed by a 20% (v/v) sodium hypochlorite solution (10-14% available chlorine) for 20 min. The testas were then removed and the kernels washed five times with sterile distilled water before being placed in contact with the liquid culture medium.

Initiation of EMS: The auxins NAA and 2,4-D and cytokinins BAP and TDZ were tested in liquid MS medium at concentrations of 0.5 to 10 mg/l for all tested plant growth regulators. Combinations of NAA and IAA at 1 to 4 mg/l with BAP at 1 and 2 mg/l were also tested in liquid MS media. Usually, the media were supplemented with 2% (v/w) sucrose and adjusted to pH 5.7 before autoclaving. An EMS was initiated by culture of mature fruits for 21 days in liquid MS medium with 100 mg/l casein hydrolysate, 100 mg/l l-ascorbic acid, 0.5-2 mg/l BAP, and 88 mM sucrose. This EMS was then subcultured regularly for 2 weeks on an embryogenesis expression medium having the same composition, but supplemented with or without 1 mg/l BAP. Subcultured explants were maintained in continuous light at 25°C, in 250 ml culture tubes sealed with aluminium foil.

Maintenance and proliferation of EMS: In order to study the influence of various carbohydrate sources on embryogenic mass proliferation, embryogenic mass subcultured at least 3 times was used in experiments with different carbohydrate concentrations on a MS medium modified by the addition of 2, 4, 6, 8 and 10% of sucrose, glucose, lactose and fructose. The mother EMSs thus obtained were transferred to a tissue proliferation medium that consisted of liquid MS medium supplemented with 100 mg/l casein hydrolysate and 100

mg/l l-ascorbic acid, either with 1.0 mg/l BAP or without plant growth regulator. Embryogenic suspension cultures were started from EMSs (0.5 g wet weight) using Erlenmeyer flasks of 250 ml capacity containing 50 ml of liquid MS medium without agar. The flasks were then sealed with a double layer of aluminium foil and placed on an orbital shaker at 98 rpm at a light intensity of 25 µmol. m^{-2} sec^{-1} photon flux density and a temperature of 25°C. The effects of the carbon sources sucrose, glucose, fructose and lactose on maintenance and proliferation were tested at concentrations of 2, 4, 6, 8 and 10%. They were subcultured every two weeks. Growth was determined by taking wet and dry weight measurements rather than by cell count data, because the cells are in very tight clusters, and the clusters are not completely digested with enzyme peroxidase and speed shaking. For fresh weight (FW) measurements the embryogenic tissue was weighed in a plastic Petri dish, and was left at room temperature for at least two hours in order to remove the liquid medium. Then total dry weight of the embryogenic mass was obtained by putting the embryogenic mass on a pre-weighed paper case previously stored in a desiccator in an oven at 80°C for at least 48 h. They were then weighed, after cooling in a desiccator, placed in the oven for another 24 h, cooled and weighed. If there was no decrease in weight for any of the treatments, it was assumed that they were oven dry. All flasks were sampled after 10 days of culture, and the fresh weight and dry matter were determined as explained above. Each growth measurement represents the mean of 5 flasks per treatment. Each experiment was performed at least twice. All the embryogenic mass suspension experiments described in the subsequent tables were performed with the same embryogenic line (originating from a single fruit).

Maturation on agar-solidified MS medium: Ten days after subculture on the liquid proliferation medium, pieces of actively-growing EMS were transferred onto agar-solidified MS medium with combinations of BAP at 0.0, 1.0, 2.0 and 4.0 mg/l together with sucrose at 2, 4, 6, 8 and 10%, and incubated at 25°C. The embryogenic potential of the EMS is defined as the number of somatic embryos produced in 4 weeks per 250 mg fresh weight of EMS.

Germination of somatic embryos: Germination studies were carried out in full-strength MS medium without PGRs, supplemented with sucrose, 100 mg/l casein hydrolysate, 100 mg/l l-ascorbic acid and 0.7% agar.

Transfer of plantlets to soil: In vitro germinated embryos were washed overnight in running water before being potted up in a sterile 1:1 mixture of peat and perlite. Plantlets were covered with a pyrex beaker to maintain relative humidity for 4-5 weeks before transfer into glasshouse conditions (25°C day; 20°C night; 18 h daylength).

Statistical analysis: The cultures in all experiments were set out in a completely randomised block design. Descriptive analysis was performed to provide information about the central tendency and variability of data. To detect significant differences among treatment levels, data from experiments were subjected to ANOVA (analysis of variance) using the Minitab package. Student's *t*-test was adjusted at P = 0.05 probability level to separate mean differences when significant treatment effects were detected. In the maturation experiments, each treatment used two blocks, 5 replicates (Petri dishes) per block and 5 explants (ca. 50 mg) per replicate.

Results and Discussion

EMS induction in liquid medium:

Among several PGRs and combinations tested, only the BAP treatments initiated friable embryogenic tissue, depending on the concentration of BAP in the induction medium. After 3 weeks of culture in the liquid MS medium supplemented with BAP, kernel explants gave greyish-yellow nodular structures (Fig. 1A). Compact nodular structures initiated in liquid media were subcultured regularly in liquid media by biweekly subculture onto MS medium supplemented with or without BAP (Fig. 1B). The first stages of development of EMS visible on the external part of the explants were never observed before 2 subcultures in culture (Fig. 1C). The explants cultured on the highest concentration of BAP did not induce EMS and formed degenerate tissues. The effectiveness of BAP in stimulating somatic embryogenesis from mature seeds (22) and immature fruits of *P. vera* L. have also been reported by Onay et al. (23) who found that EMS was induced when seeds of *P. vera* L. were first grown on liquid MS medium with the cytokinin BAP and subsequently maintained on a medium with or without the lower BAP concentrations. In the present study EMS was induced from *P. atlantica* with a frequency of 90% with mature seed explants cultured on BAP alone, but only in a liquid medium. In addition, the competence for somatic embryogenesis acquired in the presence of BAP was maintained after sub-culture on the same medium. Although the formation of EMS was greatest in MS medium with BAP, these EMS persisted on media containing sucrose with or without BAP.

Maintenance of EMS:

Four tested sugars (sucrose, glucose, fructose and lactose) in various concentrations in the liquid MS medium had no pronounced effect on the dry matter and the fresh weight (Tables 1-4).

Effect of sucrose on dry matter content and fresh weight in EMS cultures of pistachio:

The effects of sucrose concentrations on dry matter as percentage of the fresh weight are shown in Table 1. Cultures grown on a growth regulator-free medium attained approximately 15% higher dry matter than cultures grown on a BAP treated medium.

The statistical analysis of the data showed that there were very highly significant differences in the dry matter content of EMSs between the tested treatments ($P<0.001$). There was a steady increase in the dry matter content of the embryogenic mass on both media with BAP and growth regulator-free, and with an increase in the concentrations of sucrose up to concentration 6% of sucrose and a slight decrease thereafter (Table 1).

The effects of sucrose concentrations on fresh weight are also shown in Table 1. With increasing sucrose the fresh weight production declined. In all the other treatments tested, the ratio of final to initial fresh weight of the embryogenic tissue ranged from 9 to 3 on MS medium supplemented with 4% and 10% sucrose, respectively. New embryogenic tissue proliferated during the mid-level sucrose treatments was slightly friable deep green. In general, the embryogenic tissues of all treatments proliferated with a fine texture and just visible immature somatic embryos after 14 days of growth (Fig. 1C). The results presented in Table 1 indicate that the EMSs showed the best growth response between 6% and

Table 1. Effects of sucrose concentrations (%) on dry matter (%) and fresh weight (g) of EMS on media supplemented with 1 mg/l BAP and growth regulator-free media.

Sucrose	Dry matter (%)		Fresh weight (g)	
(%)	with BAP	without BAP	with BAP	without BAP
2%	15.6 ± 9.56a	20.1 ± 1.20a	3.20 ± 0.35a	4.00 ± 0.25a
4%	19.7 ± 0.61b	24.8 ± 0.80bc	4.50 ± 0.34d	4.40 ± 0.30a
6%	23.2 ± 1.18cd	27.7 ± 1.14c	3.20 ± 0.24a	4.00 ± 0.25a
8%	25.8 ± 1.25d	26.7 ± 2.08cb	2.30 ± 0.21c	2.50 ± 0.26b
10%	20.7 ± 0.77bc	22.5 ± 1.18ab	1.50 ± 0.22b	2.10 ± 0.23b

Different lowercase letters above any two treatments in a column indicate that these two means are statistically different at p=0.05 according to Student's t-test. The data for the mean of two experiments with 5 replicates.

Table 2. Effects of glucose concentrations (%) on dry matter (%) and fresh weight (g) of EMS on media supplemented with 1 mg/l BAP and growth regulator-free media.

Glucose	Dry matter (%)		Fresh weight (g)	
(%)	with BAP	without BAP	with BAP	without BAP
2%	14.7 ± 0.70a	16.6 ± 0.58a	4.40 ± 0.40a	5.10 ± 0.37a
4%	13.9 ± 0.87a	13.5 ± 0.50b	5.40 ± 0.49a	4.30 ± 0.30a
6%	22.4 ± 0.76b	18.7 ± 0.70c	4.60 ± 0.45a	4.70 ± 0.47a
8%	15.3 ± 0.85a	18.6 ± 1.11c	4.80 ± 0.41a	3.10 ± 0.31b
10%	21.2 ± 0.78b	27.4 ± 1.01d	2.40 ± 0.26b	1.80 ± 0.20c

Different lowercase letters above any two treatments in a column indicate that these two means are statistically different at p=0.05 according to student's t-test. The data for the mean of two experiments with 5 replicates.

8% sucrose in terms of dry matter content, and between 2% and 6% sucrose in terms of fresh weight.

Effect of glucose on dry matter content and fresh weight in EMS cultures of pistachio:

The effects of glucose concentrations on dry matter as percentage of the fresh weight are shown in Table 2. Statistical analysis of the data showed that there were very highly significant differences in the dry matter content of EMSs between the tested treatments ($P<0.001$). In general, an increase in glucose concentrations increased the dry weight. There was a steady increase in the dry matter content of the embryogenic mass on media with or without BAP, and with an increase in the concentrations of glucose up to 10%. The EMSs treated with 10% of glucose without BAP had a mean dry weight about 3 times higher than the initial one.

An analysis of the results showed that the fresh weight was also significantly influenced by the concentrations of glucose (Table 2). The EMS treated with 4% glucose with BAP increased in fresh weight at a maximum of 10.8 after 12 days. The ratio of final to initial fresh weight of the embryogenic tissue ranged from 3.6 to 10.8 on MS medium supplemented with 10% and 4% glucose, respectively.

Effects of fructose on dry matter content and fresh weight in EMS cultures of pistachio:

The effects of fructose concentrations on dry matter as % of the fresh weight are shown in Table 3. Statistical analysis of the data showed that there were highly significant differences in the frequencies of dry matter content of EMSs between the tested treatments ($P<0.001$). There was a steady increase in the frequencies of dry matter content of the embryogenic mass on media with or without BAP, and with an increase in the concentrations of fructose up to 10% (Table 3).

Cultures grown up to 10% fructose on a growth regulator-free media attained approximately 65% higher dry matter than cultures grown on BAP treated media. With increasing fructose the fresh matter production declined but dry matter production increased. However, non-embryogenic and black tissue clusters were also observed in all treatments after 14 days of culture. Apparently there was decreasing cell division because there was visible black mucilaginous material secreted by the embryogenic tissues that was clearly evident in all treatments. The ratio of final to initial fresh weight of the fructose embryogenic tissue ranged from 5.8 to 1.68 on MS medium supplemented with 2% and 10% fructose, respectively (Table 3).

Effects of lactose on dry matter content and fresh weight in EMS cultures of pistachio:

The effects of lactose concentrations on dry matter as percentage of the fresh weight are shown in Table 4. Analysis of the data indicated that there was a significant difference in the dry matter content as well as in the fresh weight of EMSs between the treatments. The dry matter content increased up to 6% of lactose and thereafter decreased sharply (Table 4). However, the fresh weight of EMSs decreased with increasing lactose concentrations. The ratio of final to initial fresh weight of the lactose treated embryogenic tissues ranged from 0.94 to 3.44 fold on MS medium supplemented with 10% and 4% lactose, respectively. As in the fructose case, all lactose treatments tested produced visible black material which clearly inhibited the embryogenic masses in most of the treatments.

Since EMS cultures consist only of densely cytoplasmic embryogenic cells and highly vacuolated suspensors, the dry matter in these cultures may also be directly correlated to the proportion of embryogenic cells (24) and is an indicator of proliferative capacity (25). Accordingly attention was focused on the fresh matter and dry matter content of EMSs in MS medium supplemented with different carbohydrate sources over a 14-day period. It was found that there was a relation between the fresh weight or dry matter content of EMS and the concentrations of carbohydrate tested. Overall, from the above results it was concluded that the frequency of embryogenic mass formation varied with the type and concentration of BAP used.

The analysis of the data shows that the embryogenic mass induction was highest with MS medium supplemented with sucrose and in the absence of growth regulators. Furthermore, the viability of embryogenic masses could only be maintained when transferred from sucrose to sucrose and sucrose to glucose supplemented media. The generation of phenols, however, was also observed in glucose treatments. Similar observations were noted in the proliferation of *P. vera* EMSs (22). On the basis of these results it could be postulated that sucrose had a conditioning effect at the initial step of the culture. Thus, sucrose could be used as a carbohydrate source for the proliferation of pistachio embryogenic mass cultures.

Table 3. Effects of fructose concentrations (%) on dry matter (%) and fresh weight (g) of EMS on media supplemented with 1 mg/l BAP and growth regulator-free media.

Fructose	Dry matter (%)		Fresh weight (g)	
(%)	with BAP	without BAP	with BAP	without BAP
2%	6.80 ± 0.41a	10.4 ± 0.65b	2.90 ± 0.34a	2.70 ± 0.36a
4%	7.70 ± 0.51a	12.6 ± 0.68b	1.80 ± 0.24b	1.40 ± 0.16b
6%	9.50 ± 0.45b	14.7 ± 0.47cd	1.40 ± 0.16b	1.40 ± 0.22b
8%	11.1 ± 1.32b	16.9 ± 0.84d	1.40 ± 0.16b	0.92 ± 0.03c
10%	14.8 ± 0.43c	15.3 ± 1.20c	0.84 ± 0.09c	0.66 ± 0.07d

Different lowercase letters above any two treatments in a column indicate that these two means are statistically different at p=0.05 according student's to t-test. The data for the mean of two experiments with 5 replicates.

Table 4. Effects of lactose concentrations (%) on dry matter (%) and fresh weight (g) of EMS on media supplemented with 1 mg/l BAP and growth regulator-free media.

Lactose	Dry matter (%)		Fresh weight (g)	
(%)	with BAP	without BAP	with BAP	without BAP
2%	8.30 ± 0.47a	11.0 ± 1.09a	1.07 ± 0.11a	1.52 ± 0.11a
4%	11.1 ± 0.58b	13.7 ± 0.51bc	1.31 ± 0.09ab	1.28 ± 0.13a
6%	12.2 ± 0.51b	13.9 ± 0.62c	1.72 ± 0.12c	0.70 ± 0.06b
8%	15.2 ± 0.77c	17.7 ± 0.95d	1.50 ± 0.07cb	0.66 ± 0.05b
10%	11.0 ± 0.47b	12.4 ± 0.54abc	0.69 ± 0.08d	0.47 ± 0.03c

Different lowercase letters above any two treatments in a column indicate that these two means are statistically different at p=0.05 according student's to t-test. The data for the mean of two experiments with 5 replicates.

Maturation of somatic embryos on agar solidified medium:

Pieces of EMS transferred to an agar solidified maturation media exhibited changes in proliferation ability and progressive development of SEs in the ABA treatments. The proliferative ability was affected both by sucrose concentration and ABA concentration (Table 5). By day 7, pieces of actively growing EMS transferred to the maturation medium had increased in size enormously. The colour of the clusters of EMSs was generally greyish or slightly yellowish especially in the control treatment, and deep green in the ABA supplemented treatments. The EMSs showed sustained proliferation in all treatments tested but partial inhibition of growth on the medium with 2 and 4 mg/l ABA. After 7 days of culture, the effects of ABA and sucrose were clear and the SEs underwent stages of late embryogeny in the control and 1.0 mg/l ABA treatments tested. After 14 days of culture, the developing embryos started developing elongated cotyledons. At this time the effects of ABA and sucrose became evident in the number of SEs developed (Fig. 1D). Some of the treatments tested produced no precociously germinated SEs during 2 or 3 weeks of culture in the agarified medium. Fig. 1E shows fully matured and isolated SEs after 4 weeks of culture on the agarified maturation medium.

Analysis of variance showed significant effects of sucrose and ABA concentrations on the number of mature SEs. An interaction was also evident between ABA concentration and sucrose concentration.

Table 5 shows the mean number of mature SEs on the agarified MS media supplemented with different ABA and sucrose concentrations. There was a significant difference in the mean number of mature SEs between the treatments tested. The number of SEs on the control

ABA	Sucrose(%)				
(%)	2%	4%	6%	8%	10%
0.0	36.4 ± 1.25g	39.8 ± 0.87h	50.4 ± 1.61k	32.0 ± 1.55f	23.8 ± 1.63e
1.0	47.1 ± 1.40i	45.9 ± 1.19i	44.6 ± 1.50i	39.1 ± 1.76gh	30.7 ± 0.96f
2.0	19.1 ± 1.48d	19.4 ± 1.12 d	20.3 ± 1.22de	14.6 ± 3.16c	12.3 ± 0.7bc
4.0	10.6 ± 0.83ab	13.1 ± 1.28bc	10.4 ± 0.8ab	10.8 ± 0.84ab	8.90 ± 1.01a

Mature SEs with cotyledons were counted by eye. Different lowercase letters above any two treatments indicate that these means are statistically different at p=0.05 according to student's t-test.

Table 5. Numbers of mature SEs per 250 fresh weight of *P. atlantica* embryogenic mass obtained on media with different ABA and sucrose concentrations after 4 weeks on agar solidified medium.

treatment with sucrose concentrations 6% was significantly higher ($P < 0.05$) than on the remaining treatments (Table 5). Media devoid of ABA and 6% sucrose induced the most embryos (50.4 per 250 mg fresh weight). Increasing the ABA concentration from 1.0 to 4.0 mg/l resulted in less mature SEs. ABA concentration above 2.0 mg/l were not beneficial. Sucrose concentrations 4% and 6% were the most beneficial treatments. Further increases in the sucrose concentration above 6% had an inhibiting effect, so that the highest concentrations of ABA and sucrose produced the lowest yields of SEs.

From results presented in Table 5, it may be concluded that the maturation of SEs was best achieved on low concentration of ABA i.e. 1.0 mg/l ABA or PGR-free-agarified MS medium. Embryos of various shapes and sizes visible in the same clusters indicated that the process of embryogenesis was synchronous. However, there was partial inhibition of cultures tested with 2 and 4 mg/l ABA treatments. The growth was minimal and some of the tissues turned brown. Thus, medium devoid of growth regulators was selected as the maturation treatment.

Germination and plantlet development from matured somatic embryos:

Many of the somatic embryos developed normally with a reduced shoot but with well-grown roots (Fig. 1F). Most of the somatic embryos also produced new somatic embryos, and somatic embryos germinated within 20 days from 90% of the mature somatic embryos transferred to MS medium without growth regulators. Although most of the somatic embryos germinated and gave rise to roots in full strength MS medium with or without growth regulators, the differentiation of the plumule end was restricted. Incorporation of various plant growth regulators in the embryo germination medium was ineffective in improving the frequency of somatic embryo germination (data not shown). Although 100% of the embryos formed well differentiated roots, less than 10% of the somatic embryos differentiated to plantlets. When somatic embryo-derived plantlets were successfully established in soil they showed normal growth after transfer to the greenhouse. However, some fungal contamination of the soil was observed, and the number of successful plantlet conversions from adventive embryos was low.

Conclusions

This appears to be the first report of the development of embryos to germination and beyond for *P. atlantica*. Sucrose was the most favourable carbon source for maintenance of the embryogenic line. Efforts are now being made in order to optimize the maturation and the healthy germination of somatic embryos, and the acclimatization of plantlets. The progress in somatic embryogenesis of *P. atlantica* described here shows promise as a model system for mass clonal rootstock propagation of *P. atlantica*. This also provides a sound basis for the adaptation of more sophisticated culture methods for *elite* mature trees of Pistachio. The exploitation of somatic embryogenesis for cloning requires developed protocols to regenerate or rejuvenate mature elite trees under in vitro conditions. It will not be possible to realize this potential without further extensive research on pistachio. Therefore, efforts are now under way to develop a workable micrografting system for pistachio.

Acknowledgements

The research was funded by a grant from Dicle University, Faculty of Science and Art, Diyarbakır, Turkey, and performed under a Scottish Office Agricultural and Fisheries Department licence.

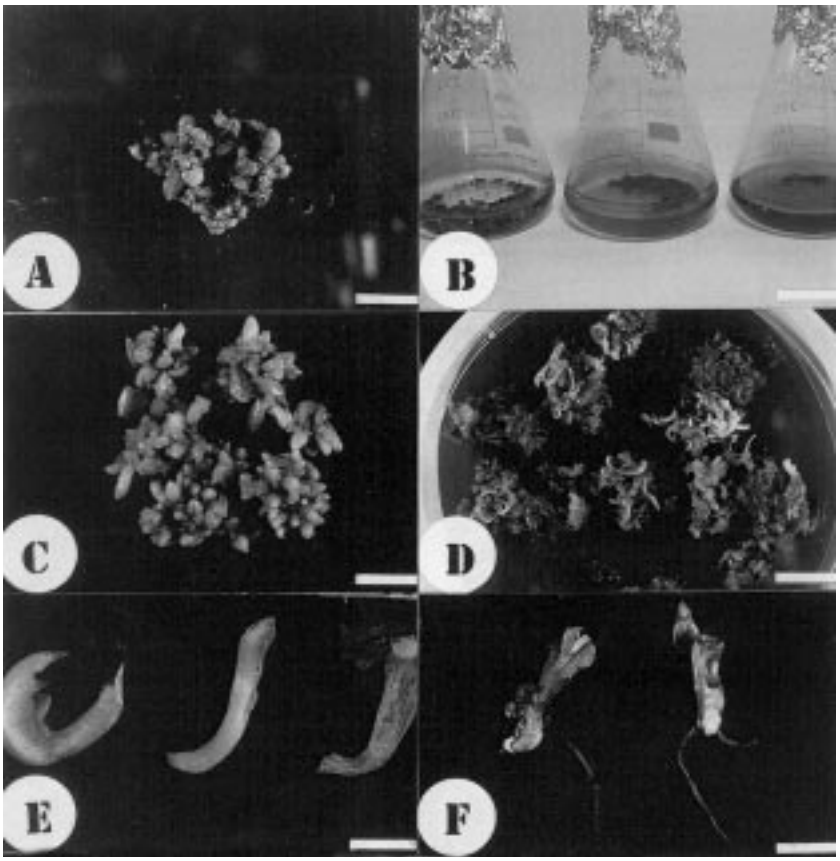

Figure 1. Somatic embryogenesis from mature seeds of *Pistacia atlantica:*

(A) Greyish compact nodular structures after 3 weeks in liquid MS medium supplemented with 1 mg/l BAP, bar = 8 mm); (B) Maintenance of nodular structures in liquid media by biweekly subculture, bar = 42.5 mm; (C) Embryogenic tissue and just visible immature SEs after 14 days of culture in liquid medium, bar = 8.7 mm; (D) Maturation of somatic embryos (after 2 weeks proliferation on liquid MS medium in the absence of PGR) on the agarified MS medium, bar = 11.9 mm; (E) Isolated germinating SEs after 4 weeks maturation on agar solidified MS medium in the absence of growth regulator, bar = 3.0 mm; (F) Developed plantlets 4 weeks after germination on MS medium, bar = 11.8 mm.

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