Tr. J. of Botany
22 (1998) 363-373

Research Article

The Effects of Different Sucrose, Agar and pH Levels on *In Vitro* Shoot Production of Almond (*Amygdalus communis* L.)

Songül GÜREL
Sugar Institute, Plant Breeding Department, Etimesgut, 06790 Ankara-TURKEY
Yücel GÜLŞEN
Ankara University, Faculty of Agriculture, Department of Horticulture, Dışkapı, 06110 Ankara-TURKEY

Received: 03.03.1997
Accepted: 27.02.1998

Abstract: In this study the possibilities of *in vitro* vegatitve propagation of almond (*Amygdalus communis* L.) cv. Texas and cv. Nonpareil by shoot-tip culture were investigated. Different levels of sucrose, agar and pH were tested and shoot proliferation, shoot development and growth were observed during three successive stages, namely initiation, transplantation and multiplication. During the initiation stage, proliferation was achieved only with 5 and 6% sucrose and the best explant development occured with 0.5% agar and pH 5.5. During the second stage, again 5 and 6% sucrose and pH 5.5 gave the best results with respect to shoot production and growth. Although the medium with low agar concentrations (0.5%) was favourable for shoot formation and growth, 0.7% agar was determined to be the most appropriate level for this stage since low agar levels (0.5 and 0.6%) caused vitrification. During the multiplication stage, the highest rate of shoot production was achieved with 3 and 4% sucrose, 0.7% agar and pH5.5, and higher sucrose and agar levels increased callus formation at the base of the shoots.

Key Words: Almond, tissue culture, micropropagation, shoot-tip culture

Farklı Sakkaroz, Agar ve pH Düzeylerinin Bademde (*Amygdalus communis* L.) *İn Vitro* Sürgün Verimine Etkileri

Özet: Bu araştırmada, Texas ve Nonpareil badem (*Amygdalus communis* L.) çeşitlerinin sürgün ucu kültürü ile *in vitro* vejetatif çoğaltım olanakları araştırılmıştır. Bu amaçla farklı sakkaroz, agar ve pH düzeyleri, takip eden üç farklı kültür aşamasında (ilk dikim, şaşırtma ve çoğaltma) ayrı ayrı test edilmiş ve sürgün proliferasyonu, sürgün gelişmesi ve büyümesi incelenmiştir. İlk dikim aşamasında, sadece %5 ve 6 sakkaroz dozları proliferasyonu uyarmıştır, ve %0.5 veya 6 sakkaroz ile pH 5.5 ise en iyi eksplant gelişmesini sağlamıştır. Şaşırtma aşamasında, yine %5 veya 6 sakkaroz ile pH 5.5 içeren ortamlarda sürgün gelişmesinin ve büyümesinin en iyi olduğu görülmüştür. Her ne kadar, düşük agar dozlarında sürgün verimi ve gelişmesi iyi olmuşsa da, bu dozlarda sürgünlerde camlaşma meydana geldiğinden, bu aşama için 0.7% agar en uygun doz olarak belirlenmiştir. Çoğaltma aşamasında ise, en yüksek sürgün verimi %3 ve 4 sakkaroz, %0.7 agar ve pH 5.5 düzeylerinde sağlanmıştır. Yüksek sakkaroz ve agar dozlarının, sürgünlerin dip kısımlarında kallus oluşumunu artırdığı gözlenmiştir.

Anahtar Sözcükler: Badem, doku kültürü, mikroçoğaltım, sürgün-ucu kültürü

Introduction

Almond *(Amygdalus communis* L. [syn. *Prunus amygdalus* Batsch]) has long been grown in Turkey, mostly along the coast of the Aegean and Mediterranean regions, as Anatolia has been one of the main germplasm bases of this fruit species (1). Turkey ranks in seventh place among the largest producers of almond in the world with a 2.5% contribution (2). Since it is a cross-pollinated species, a continuous genetic variation has occurred in almond through the years, which has led to the formation of very different types in terms of crop development, fruit yield and quality, and tolerance to environmental stresses. To minimize the problem of enormous genetic variation and to obtain genetically identical populations, vegetative propagation via layerage or cutting is inefficient due to the great problems of this fruit species in rooting *in vivo*. This, therefore, makes the plant tissue culture techniques more valuable for the clonal propagation of almond trees.

* To whom correspondence should be addressed.

Tissue culture techniques have been intensively used for the clonal propagation of many tree species, of which the fruit and timber trees are of great economic importance (3-5). However, many tree species are not suitable for vegetative multiplication and in those which are suitable, to a certain extent, the process is rather slow. There have been few reports on the propagation of almonds through tissue culture. Mehra & Mehra (6) were the first to report organogenesis and plant regeneration from callus obtained from various parts of the almond seedlings. More reports have since appeared on shoot regeneration from callus (7-9) and embryo cultures in almonds (10-12) but they achieved very limited success in rooting these shoots. *In vitro* plant regeneration of almonds through shoot-tip culture also gained much attention in the late 19701s, with several researchers reporting on clonal propagation by culturing isolated shoot-tips on medium containing 2-3% sucrose, 0.7-0.9% agar and 0.7-1.0 mg/l BAP (13-15).

The present study was carried out to determine the optimum levels of sucrose, agar and pH essentially used in the culture medium with the aim of developing a reliable protocol for *in vitro* propagation of two almond cultivars (cv. Texas and cv. Nonpareil), which are commonly grown in the USA, the largest producer of almonds in the world, and were found to be very adaptable to Turkey's conditions (1). The effects of different auxin and cytokinin concentrations and combinations on *in vitro* shoot production in the same cultivars were also investigated and the results will be presented in a future report (16).

Materials and Methods

In spring, 5-10 cm long shoots, which were still actively developing, were taken from almond (*Prunus amygdalus* Batsch) trees cv. Texas and cv. Nonpareil, grown in Ankara and washed thoroughly, first with tap water and then with sterile water. 1.0-1.5 cm long shoot-tips excised from these shoots were then surface-sterilized in 2% sodium hypochloride for 10 min followed by 4 rinses (5 min each) with sterile-distilled water. Then, 2-4 mm long shoot-tip meristems with 4-5 leaf primordia were excised under a dissecting microscope in a laminar flow hood and then incubated in either glass culture tubes (160x15 mm) or in 250 ml Erlenmeyer flasks, containing MS (17) basal medium supplemented with 0.1 mg/l GA_3, 0.1 mg/l IBA and 1.0 mg/l BAP, which had been sterilized by autoclaving for 15 min at 103.5 kPA. For testing the effects of sucrose, the medium solidified with 0.7% agar at pH 5.5 was supplemented with 2, 3, 4, 5 and 6% sucrose. The effects of agar were epamined by keeping the sucrose concentration at 3% at pH5.5 while changing the agar concentration from 0.5 to 0.9% at 0.1 intervals. Finally, different levels of pH (4.5, 5.0, 5.5, 6.0 and 6.5) at 3% sucrose and 0.7% agar were used for testing the effects of pH on shoot proliferation. Explants were incubated in a controlled environment (25±1°C, 16 h photoperiod, 2200 lux light intensity, 75±5% humidity) and kept in culture for 18 weeks, subculturing every 3 weeks. The experiments involed three culture stages; i) the initiation stage (the first three weeks of culture), ii the transplantation stage (the next three weeks of culture following the initiation stage) and iii) the multiplication stage (the remaining 12 weeks of culture with four subcultures at three week intervals). At the end of each subculture during the last (multiplication) stage, multiple shoots were singled out and then the individual shoots were subcultured. A 1-4 scoring system was developed for the determination of the growth rate of shoots that developed (Table 1). Mean shoot number per explant and the mean growth rate of the shoots were recorded at the end of each stage, except for taking the mean of four subcultures for the multiplication stage since this stage lasted 12 weeks. The experiments were laid out according to randomised plot design with 12 replicates per treatment and the results were evaluated using the method of analysis of variance, and statistically significant differences were indicated in letters according to the Duncan Test at p=0.01 (18).

Growth Rate	Shoot Length (cm)		
	Inititiation Stage (0-3 weeks)	Transplantation Stage (4-6 weeks)	Multiplication Stage (7-18 weeks)
1.0 (Poor)	up to 0.5	up to 1.0	up to 1.0
2.0 (Medium)	0.6-1.0	1.1-1.5	1.1-2.0
3.0 (Good)	1.1-1.5	1.6-2.0	2.1-5.0
4.0 (Very Good)	1.6 or longer	2.1 or longer	5.1 or longer

Table 1. The scoring system developed for measuring the growth rate of the shoots developed. During the inititiation stage, the growth rate of both initial and newly proliferating shoots was recorded while during the transplantation and multiplication stages only the growth rate of the newly developing shoots was

Results

Effects of Different Sucrose Concentrations on Shoot Development

Initiation Stage

Shoot proliferation was observed only with 5 and 6% sucrose concentrations in both the cultivars tested, with a mean of 3.6 and 3.3 shoots per explant with 5% sucrose, and 4.0 and 4.1 shoots per explant with 6% sucrose in cv. Texas and cv. Nonpareil, respectively. The differences between varieties and sucrose treatments (i.e., between 5 and 6% sucrose) were not found to be significant at p=0.01 (Figure 1). The first shoot proliferation occurred on the 6th day of incubation with 5 and 6% sucrose concentrations and by day 10, nearly 100% of the shoot-tip explants had already proliferated in both cultivars. No shoots were produced with other sucrose concentrations (i.e., 2, 3 and 4%). Shoot growth capacity, expressed as the growth rate of the developing shoots (Table 1), showed a steady increase with increasing concentrations, with 5 and 6% sucrose concentrations producing more vigorous shoots than the lower concentrations (Figure 1). In addition, the growth of those explants, which did not produce any shoots at all, continued to increase with increasing sucrose concentrations. It was also observed that tissue of the new explants and of the newly forming shoots remained green throughout the culture but small amounts of brown callus fromde at the base of the shoots with higher sucrose levels.

Transplantation Stage

As seen in Figure 2, it is apparent that during this stage that there were no clear-cut differences between the effects of different concentrtaions of sucrose on shoot development in either cultivar, with the exception of 2% sucrose which significantly lower numbers of shoots per explant than the other concentrations. However, the highest shoot production (7.3 shoots per explant) was obtained in cv. Nonpareil when 3% sucrose was used in the culture medium while concentrations of 3-6% sucrose induced similar numbers of shoots in cv. Texas, ranging from a mean of 5.3 to 6.1 shoots per explant. In terms of the growth rate of the shoots, as in the initiation stage, there was a steady increase as the sucrose level increased, with 5 and 6% sucrose concentrations resulting in a signigicantly higher growth rate, from 2.0 (Medium) to 3.5 (Good-Very Good), in both of the cultivars. The shoots that developed on

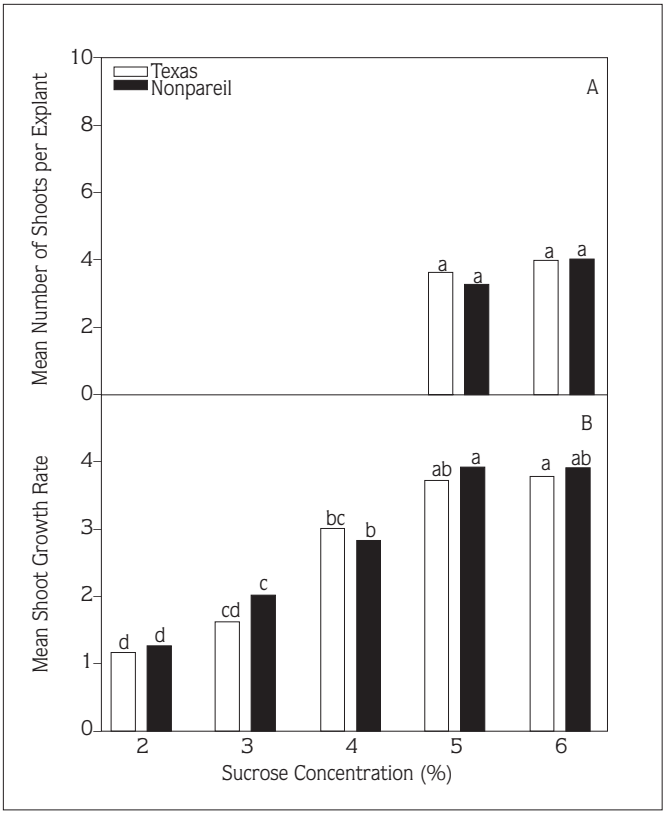

Figure 1. The effects of different sucrose concentrations on shoot development from shoot-tip explants (A) and their subsequent growth (B) during the initiaiton stage in cv. Texas and cv. Nonpareil.

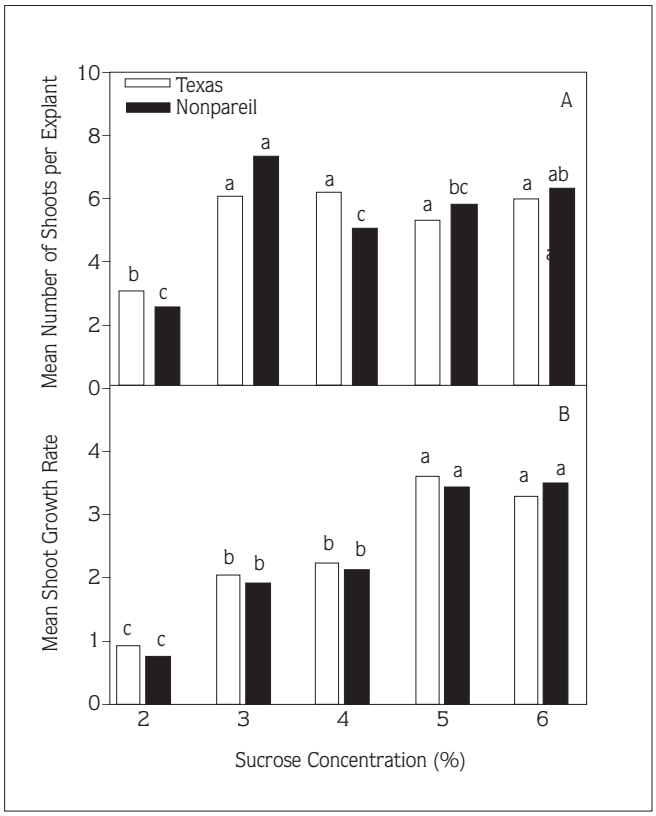

Figure 2. The effects of different sucrose concentrations on shoot development from shoot-tip explants (A) and their subsequent growth (B) during the transplantation stage in cv. Texas and cv. Nonpareil.

medium containing 2% sucrose had a greenish-yellow appearance whereas the others, cultured with 3-6% sucrose, remained green and healthy throughout the subculture. Small amounts of brown callus also formed during this stage with only 5 and 6% sucrose concentrations.

Multiplication Stage

During this stage, 3 and 4% sucrose levels were found to be significantly better than the other concentrations used in both cultivars with respect to both shoot production, with a mean ranging from 6.3 to 7.8 shoots per explant, and growth rate of the developing shoots, with a mean rate of 2.4-3.8 compared with 1.3-1.9 obtained with 2, 5 and 6% sucrose levels (Figure 3). 2% sucrose produced the least numbers of shoots and led to a much lower shoot growth rate in both cultivars. The shoots that developed on media containing 2, 5 or 6% sucrose concentrations were mostly greenish-yellow whereas those that developed which 3 and 4% sucrose were green. Large amounts of callus developed with 5 and 6% sucrose during only this stage.

Effects of Different Agar Concentrations on Shoot Development

Initiation Stage

No new shoot proliferation was observed during this stage and the explants were, therefore, evaluated for their growth rates only (Figure 4). It was clear that the increasing concentrations of agar caused a steady decrease in the growth of shoot tip explants in both cultivars, with 0.5% agar resulting in a significantly higher shoot growth rate than the other agar concentrations, except for 0.6% agar which was as good as 0.5% agar in cv. Nonpareil. No visible differences in the explant colour were observed between different agar concentrations as all the explants were green. However, 0.8 and 0.9% agar concentrations produced small amounts of callus at the proximal cut ends of the shoots, and no vitrification occurred in the explants during this stage.

Transplantation Stage

During this stage, 0.5-0.8% agar concentrations seemed to have similar effects on shoot production in

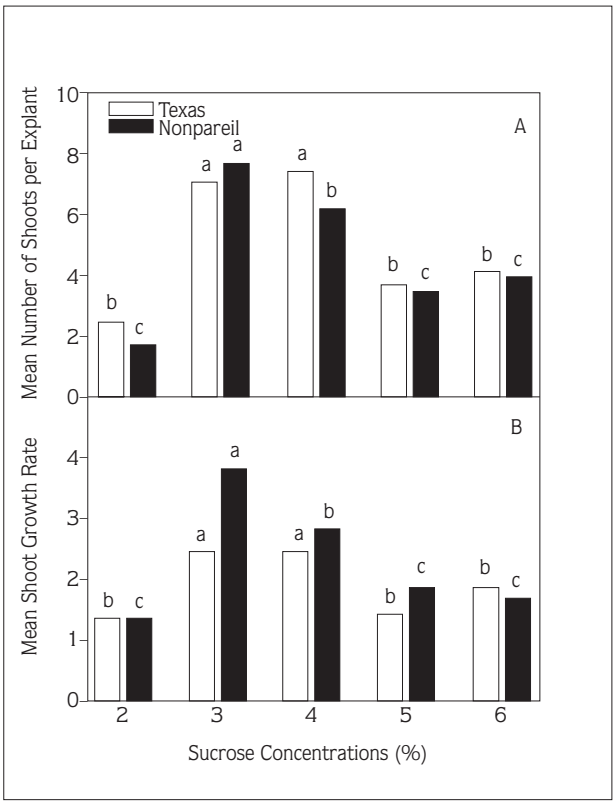

Figure 3. The effects of different sucrose concentrations on shoot development from shoot-tip explants (A) and their subsequent growth (B) during the transplantation stage in cv. Texas and cv. Nonpareil.

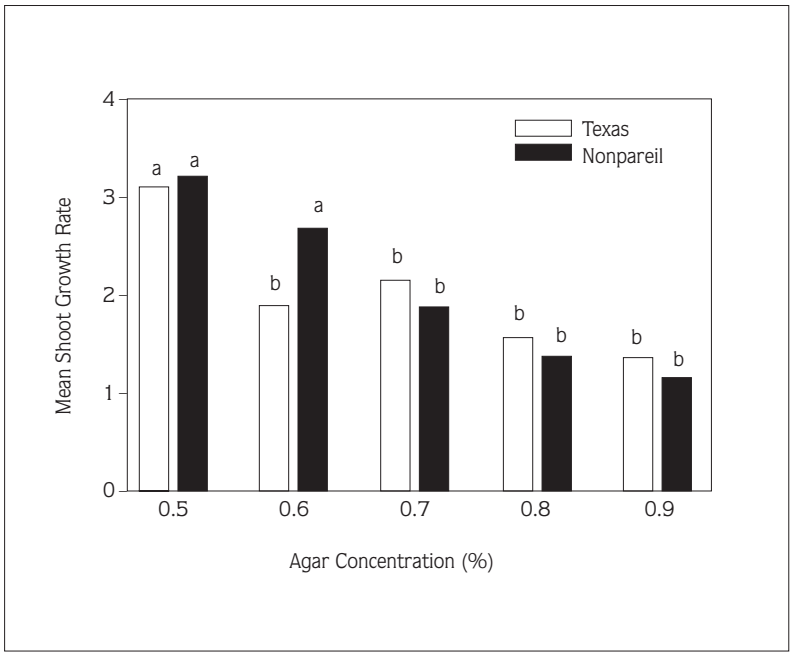

Figure 4. The effects of different sucrose concentrations on shoot development from shoot-tip explants (A) and their subsequent growth (B) during the transplantation stage in cv. Texas and cv. Nonpareil.

both cultivars, with a mean of 5.0-7.0 shoots per explant and the highest agar concentration (0.9%) reduced the number of shoots in a significant manner (Figure 5). No clear pattern was observed in the growth rate although concentrations of 0.5, 0.6 and 0.7% seemed noticeably better than 0.8 and 0.9% concentration, which is broadly consistent with the effects on shoot production during this stage (Figure 5). All explants cultured were green but the lower agar concentrations (i.e., 0.5 and 0.6%) caused a low frequency of vitrification which was not observed during the initiation stage. On the other hand, 0.8 and 0.9% agar (i.e higher concentrations), induced a small amount of brown callus at the proximal ends of the shoots.

Multiplication Stage

A considerable difference between the cultivars was observed in terms of their response patterns to different agar concentrations. Significantly more shoots were obtained with 0.5% agar in cv. Texas than at the other concentrations which led to a gradual decrease, up to a mean of 2.3 shoots per explant, as the agar level increased (Figure 6). In cv. Nonpareil, concentrations of 0.5-0.8% agar produced similar numbers of shoots with a mean ranging from 5.9 to 8.5 shoots per explant. As in cv. Texas, 0.9% agar was also the least effective for shoot production in cv. Nonpareil. For the growth rate of the shoots, 0.5, 0.6 and 0.7% agar concentrations were significantly better than 0.8 and 0.9% agar with each group inducing a similar growth rate. A high level of shoot vitrification occurred with lower agar concentrations (i.e., 0.5 and 0.6%) in both cultivars while high agar levels (0.8 and 0.9%) caused an increased amount of brown callus development at the proximal end (base, of the shoots whose leaves were greenish yellow in appearance.

Effects of Different pH Levels on Shoot Development

Initiation Stage

As in the agar treatments, no new shoot proliferation occurred during this initial stage and the shoot explants were, therefore, evaluated for their growth rates only (Figure 7). The results clearly indicated that pH 5.5 was the optimum level for shoot growth in both cv. Texas and cv. Nonpareil. As the pH level decreased from 5.5 to 4.5 or increased from 5.5 to 6.5, there were significant decreases in the growth rate of the shoots in both

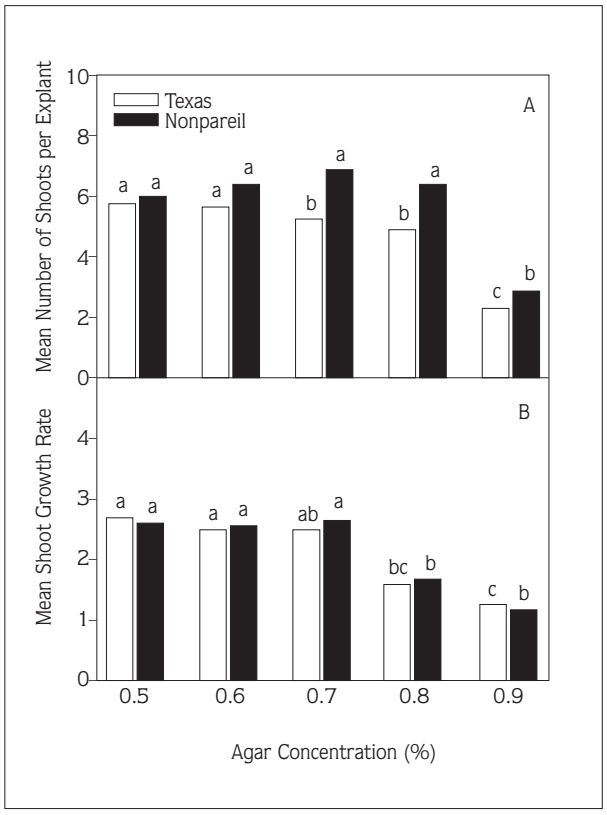

Figure 5. The effects of different agar concentrations on shoot development from shoot-tip explants (A) and their subsequent growth (B) during the transplantation stage in cv. Texas and cv. Nonpareil.

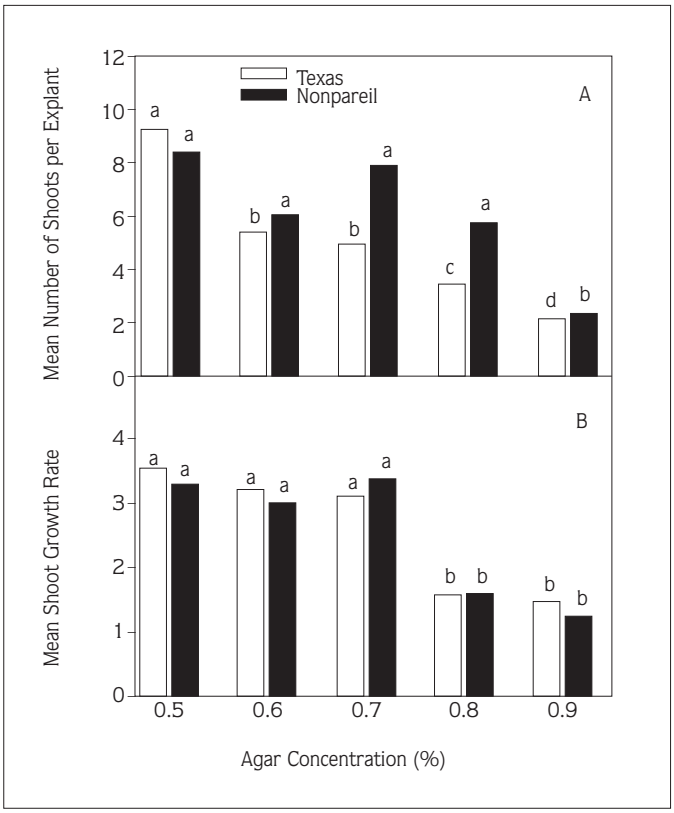

Figure 6. The effects of different agar concentrations on shoot development from shoot-tip explants (A) and their subsequent growth (B) during the transplantation stage in cv. Texas and cv. Nonpareil.

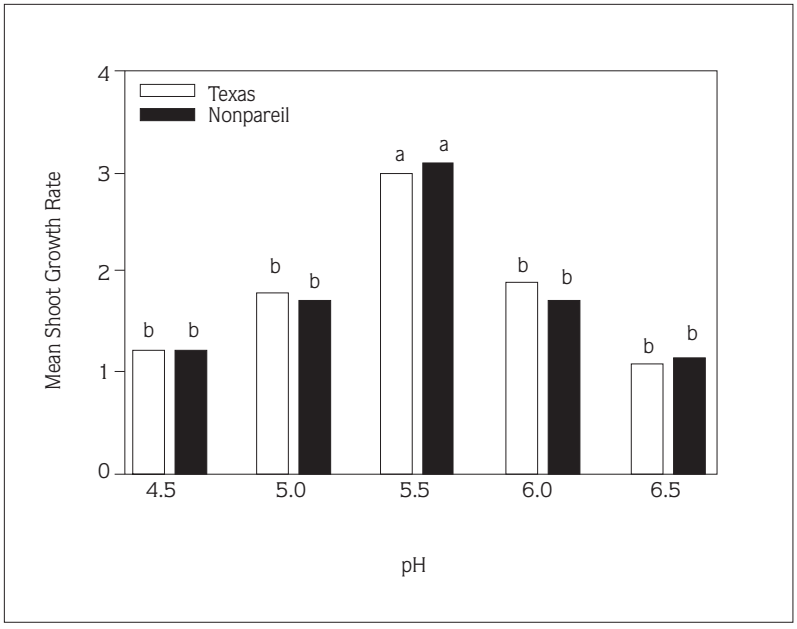

Figure 7. The effects of different pH levels on shoot development from shoot-tip explants (A) and their subsequent growth (B) during the transplantation stage in cv. Texas and cv. Nonpareil.

cultivars. No detectable differences were seen between the different pH treatments with regard to the colour of the shoots, all having a green and healthy appearance. It is important to point out here that no callus formation or vitrification occurred during this stage.

Transplantation Stage

In both cultivars, pH 5.5 and 6.0 produced significantly more shoots than the other pH levels, with mean shoots production ranging from 4.5 to 6.8 shoots per explant (Figure 8). With respect to shoot growth rate, pH 5.5 was again significantly better than the other pH levels tested. At all pH levels, explants were green with no vitrification but at pH 6.5, small amounts of greenish-white callus fromed at the base of the shoots.

Multiplication Stage

Again pH 5.5 was found to be significantly more effective than the other pH levels for both shoot production and shoot growth rate in both cultivars (Figure 9). Unlike during the inititation and transplantation stages, low pH levels, especially pH 4.5, caused shoots to turn yellow in both cultivars. In addition, the lowest (4.5) and the highest (6.5) pH levels resulted in the development of shorter shoots with narrow, curled and sharp-pointed leaves (i.e., leaf malformation). Large amounts of greenish-white callus developed at the base of the shoots again at pH 6.5 only but no vitrification occurred.

Discussion

Since most fruit trees and ornamental plants are highly heterozygous, their seed progeny is not true-to-type. Asexual (vegetative) reproduction, on the other hand, gives rise to plants which are genetically identical to the parent plant and thus permits the perpetuation of the unique characters of the cultivars (19). The almond is a cross-pollinated species and a reliable method for the clonal propagation would be of great value for its

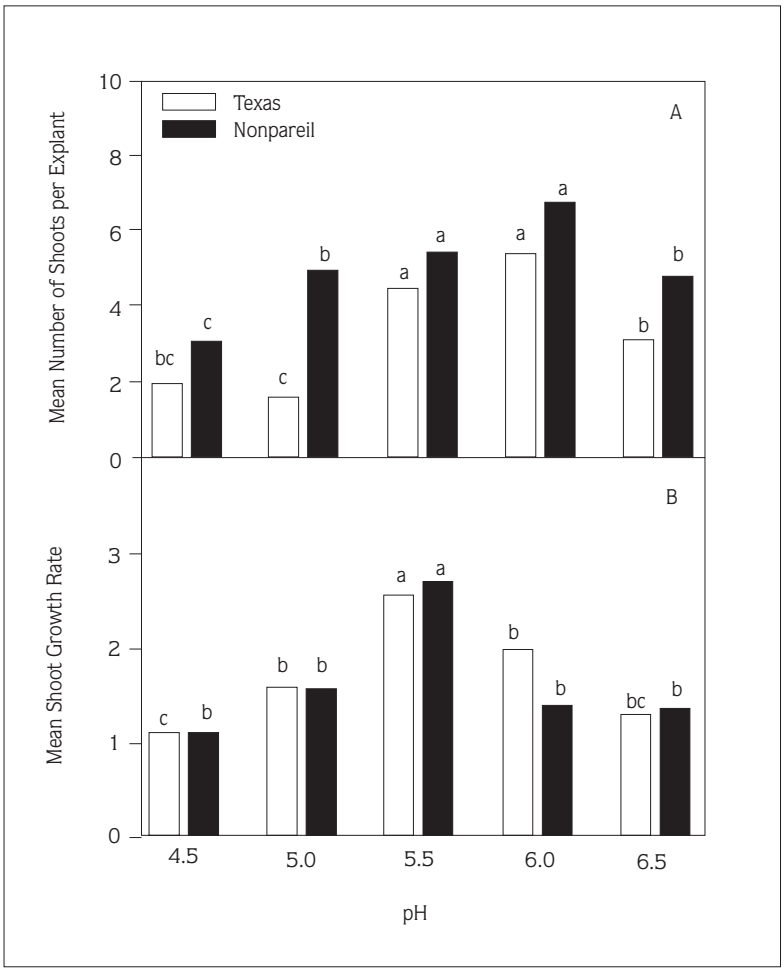

Figure 8. The effects of different pH levels on shoot development from shoot-tip explants (A) and their subsequent growth (B) during the transplantation stage in cv. Texas and cv. Nonpareil.

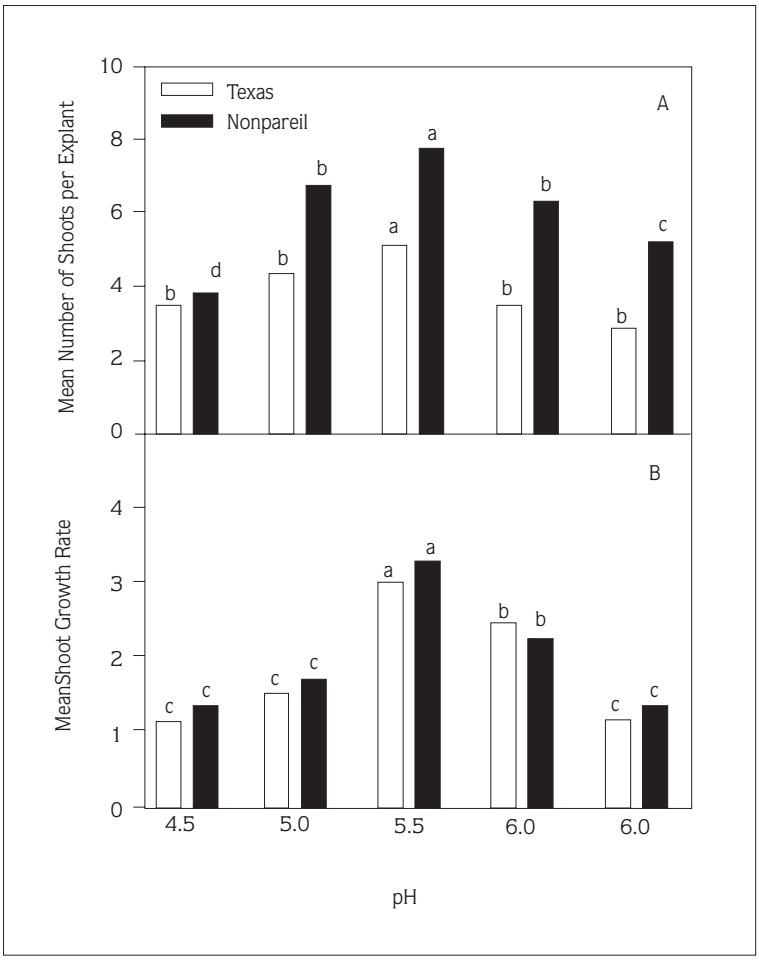

Figure 9. The effects of different pH levels on shoot development from shoot-tip explants (A) and their subsequent growth (B) during the transplantation stage in cv. Texas and cv. Nonpareil.

breeding. We therefore attempted to develop an effective protocol for the clonal propagation of two almond cultivars (cv. Texas and cv. Nonpareil) through shoot-tip culture, examining the effects of sucrose, agar and pH during three successive culture stages.

All the factors studied were found to be highly effective on shoot development and growth in both cultivars. During the proliferation and trasplantation stages, a steady increase in the mean shoot production and mean shoot growth rate per explant was observed with increasing sucrose (5 and 6%) which was used as a carbon (energy) source. This may be mainly due to the fact that high sugar levels avaliable in the culture medium may speed up cell division thus leading to an increase in the volume and weight of tissues cultured, as suggested by other researchers (20). In contrast with the proliferation and transplantation stages, lower sucrose concentrations (3 and 4%) were significantly better during the multiplication stage in terms of shoot production rate and their subsequent growth, which was consistent with the findings of Hisajima (11, 12), Rugini & Verma (15) and Rugini (8) who suggested that 3% sucrose should be used in all culture stages for the *in vitro* multiplication of almond. However, our results differed in that younger cultures (i.e., during the proliferation and transplantation stages) required a higher sucrose level, 5 or 6% and as cultures proceeded further (i.e., during the multiplication stage), the need for sucrose decreased considerably to 3 or 4%, suggesting a direct relationship between the rate of cell division and energy (sucrose) consumption in the cultured tissues since cell division in ageing tissues decreases with time (21).

We tested a range of agar concentrations from 0.5 to 0.9% and our observations demonstrated that agar levels of 0.5-0.8 were broadly effective in promoting early shoot proliferation, shoot development and growth during all stages. Nevertheless, lower levels (0.5 and 0.6%) caused severe vitrification during the transplantation and multiplication stages. Some researchers have reported that the leaves or leaf explants

cultured at low agar levels were able to uptake more water from the medium and, therefore, exhibited much higher vitrification (15, 22, 23). On the other hand, higher agar concentrations may result in a decrease in shoot production and growth as agar would increase the solidness of the medium from which nutrient uptake would be limited. Our results demonstrated that shoot proliferation was considerably delayed, and shoot production and growth were greatly inhibited when 0.9% agar was used. In addition, 0.9% agar increased callus formation which then probably became inhibitory for shoot development. Based on our experimental results and observations, we can suggest that 0.7% agar is the most suitable concentration for shoot-tip culture of the almond cultivars that we used since lower or higher levels had adverse effects. However, the use of agar at 0.5 may also be suggested during the initiation stage as vitrification may not necessarily occur at a limiting level at this early stage of the culture.

The other factor we examined was pH. It has been reported that the pH of the medium has a regulatory effect on nutrient uptake by the explant tissue (23, 24). Our results indicated that pH 5.5 was the optimum during all stages in both cultivars and either low or high pH levels caused serious abnormalities, producing shorter shoots with narrow, curled and sharp-pointed leaves. There are conflicting reports on the optimum pH level for almond tissue culture, with some determining pH 5.5 as optimum (11, 12), which is consistent with our findings, while others have reported that a higher level (pH 5.9) produced the highest number of regenerants (13). This might be attributed to gnetypic variation as it can be a serious problem in tissue culture work with many plant species. As a matter of fact, in our experiments we observed differences between the cultivars in terms of shoot production, with cv. Nonpareil usually producing 10-30% more shoots than cv. Texas cultured on the same medium although no statistical analysis was carried out to confirm this observation.

In conclusion, it can be summarised that a high sucrose level (5-6%) should be used during the first and second stages of the culture but reduced to a low level (3-4%) during the final stage. As for agar, unlike sucrose, the first stage needs a low agar level (0.5%) while the second and third stages require an increase up to 0.7%. The pH seems to be the most consistent variable as all stages produced their highest yield at pH 5.5.

Acknowledgement

The authors wish to thank The Research Fund Directorate of Ankara University for their financial support.

References

1. Dokuzoğuz M., Gülcan R., Badem Yetiştiriciliği ve Sorunları. TÜBİTAK XV. Kuruluş Yılı Bilimsel Yayınları, Ankara, Turkey (1977).
2. Anonymous. F.A.O. Yearbook Production. Volume 41, Rome, Italy (1987).
3. Henry P.H., Blazich F.A., Hinesley L.E., Vegetative propagation of eastern redcedar by stem cuttings. HortSci. 27(12), 1272-1274 (1992).
4. Gjuleva V., Atanassov A., Micropropagation of Platanus acerifolia in vitro. Silvae Genetica 43(4), 215-218 (1994).
5. Gomez M.P., Segura J., Axillary shoot proliferation in cultures of explants from mature Juniperus oxycedrus trees. Tree Physiol. 15(9), 625-628 (1995).
6. Mehra A., Mehra P.N., Organogenesis and plantet formation in vitro in almond. Bot. Gazet. 135(1), 61-73 (1974).
7. Kester D.E., Tabachnik L., Negueroles J., Use of micropagation and tissue culture to investigate genetic disorders in almond cultivars. Acta Horticul. 78, 95-101 (1977).
8. Rugini E., Progress in studies on in vitro culture of almonds. In: Plant Tissue Culture and Its Agricultural Applications, 41st Conference in the Easter School Series in Agriciltural Science, England (1984).
9. Rugini E., Almond. In: Evans D.A., Sharp W.R., Ammirato V. (eds.), Handbook of Plant Cell Culture, Techniques and Applications, Volume 4, MacMillan Publishing Company, New York, USA (1986).
10. Hansman D., Novoa C.O., Micropropagation of temperate nut trees. Horticultural Abstracts 56(6), 403-416 (1986).
11. Hisajima S., Multiple shoot formation from almond embryos. Biol. Plant 24(3), 235-238 (1982).
12. Hisajima S., Multiple shoot formation from almond seeds and an excised single shoot. Agricul. Biol. Chem 46(4), 1091-1093 (1982).
13. Tabachnik L., Kester D.E., Shoot culture for almond and almond-peach hybrid clones in vitro. HortSci. 12(6), 545-547 (1977).

14. Rugini E., Verma D.C., Micropropagation and cell suspensions of a difficult to propagate almond (Prunus amygdalus Batsch) cultivar. In: Fujiwara A. (ed.), Plant Tissue Culture 1982, The Proceedings of the 5th International Congress on the Plant Tissue and Cell Culture, Tokyo, Japan (1982).

15. Rugini E., Verma D.C., Micropropagation of a difficult-to-propagate almond (Prunus amygdalus Batsch) cultivar. Plant Sci. Let. 28, 273-281 (1983).

16. Gürel S., Gülşen Y., The effects of IBA and BAP on in vitro shoot production of almond (Prunus amygdalus Batsch). Tr. J. Bot., (this issue).

17. Murashige T., Skoog F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473-497 (1962).

18. Daniel W.W., Biostatistics: A Foundation for Analysis in the Health Sciences, 6th Edition, John Wiley & Sons, Inc., Singpore (1995).

19. Bhojwani S.S., Razdan M.K., Plant Tissue Culture: Theory and Practice, Elsevier Science Publishers B.V., Amsterdam, The Netherlands (1990).

20. Chong C., Taper C.D., Mallus tissue culture. I. Sorbitol (D-glucitol) as a carbon source for callus initiation and growth. Can. J. Bot. 50, 1399-1404 (1972).

21. Gürel E., Wren M.J., In Vitro Development from leaf explants of sugar beet (Beta vulgaris L.): Rhizogenesis and the effect of sequential exposure to auxin and cytokinin. Ann. Bot. 75, 31-38 (1995).

22. Gaspar T., Kevers C., Debergh P., Maeng L., Paques M., Boxus P., Vitrification: Morphological, physiological and ecological aspects. In: Bonga J.M., Durzan J. (eds.), Cell and Tissue Culture in Forestry, General Principles and Biotechnology, Volume 1, Martinus Nifhoff Publications, Dordreacht, The Netherlands (1987).

23. Hussey G., Vegetative propagatation of plants by tissue culture. In: Yeoman M.M. (ed.), Plant Cell Culture Technology, Blackwell Scientific Publications, Oxford (1986).

24. Quak F., Meristem culture and virus-free plants. In: Reinert J., Bajaj Y.P.S. (eds.), Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, pp. 598-615, Springer-Verlag, Berlin, Germany (1977).

25. Pennell D., Micropropagation in Horticulture, Grower Books, London, UK (1987).