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Effects of different BA and IBA concentrations on proliferation and rooting of 'GARNEM' rootstock *in vitro* propagation

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Abstract

In this study, the regeneration shoot tip and nodal explants grown *in vitro* conditions of 'Garnem' hybrid rootstock were investigated, the effect of different phytohormone and concentrations on obtaining adventitious shoots from different explants was investigated, so an efficient protocol was developed for *in vitro* regeneration of 'Garnem' hybrid rootstock These outputs of the study can be a reference resource for future *in vitro* and biotechnological studies on the rootstock in question. The differences of PGR in MS medium culture containing node explants infinite (0.5-2.0 mg / l) BA (benzyladenine) were investigated. Upon the research, it was observed that the number of shoots andproliferationwere achieved in explants of nodal cuttings that were taken to culture in MS medium containing 2.0 mg / l BA. It has been determined that the most effective culture medium for the elongation of shoot length is MS medium containing 0.5 mg / l BA, 30 g / l sucrose and 5.5 g / l agar. Shoots growing in length were transferred to a new culture with ½ MS medium 0.5-2.0 mg / l IBA (indole-3-butyric acid) to be rooted. While rooting at a rate of 42.8% was achieved in ½ MS medium containing 2 mg / l IBA, 47.2% of rooted plantlets were acclimatized to ex- vitro conditions. Rooted plantlets obtained under *in vitro* conditions were transferred to plastic containers with 3 different environments in order to get accustomed to external conditions. At the end of the 8th week, the vitality rates of the plantlets were determined. While the viability rate of the plantlets transferred to the medium containing peat: perlite at the ratio of 1: 1 was found to be 47.2%, the viability rate of the plantlets in the environment containing only perlite was found to be 32.8%, and the viability rate of the plantlets in the environment containing only peat was found as 23.6%

Keywords: 'Garnem', Rootstock, Woody plants, Regeneration, *In vitro*, Micropropagation

Introduction

Almond cultivation has an important place in the World nut production. According to last decades data; world almond production, the United States of America (USA) ranks 1st by providing 58.8% of the total production. Spain, which provides 10.7% of the total World production, is in the second place, while Iran is in the third place with a production share of 4.4%. Turkey ranks 5th by providing 3.1% of the total World production (Eldogan, 2020). Almond, Turkey has adapted to the climate are among the important structures nuts (Akcay et al., 2005). Stone fruit species (*Prunus* sp.) are important and economically valuable fruit species in the *Prunoideae* subfamily of the *Rosaceae* family (Socias and Company, 1998; Arıcı, 2008). Although the number of almond trees grown is high, the low yield is due to non-compliance with standard production principles. The fact that old almond plantations consisted of more seeds causes variation between the types. Ensuring a standard production will only be possible with the determination of varieties suitable for ecological conditions and the use of standard rootstocks. If the new gardens to be established are established with standard varieties and these varieties are produced with grafting, a standard product and efficiency can be increased.

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When rootstocks are used in the reproduction of plants; growth of the variety grafted on rootstocks, resistance to diseases and pests, yield, fruit quality, earlyness, lateness, drought, frost, salinity, ground water resistance and plant nutrient uptake from the soil are affected. It is necessary to use a clonal rootstock for early fruiting and to obtain the maximum yield per unit area.

'Garnem' (*P. dulcis* x *P. persica*) is a hybrid rootstock obtained by crossing North American peach 'Nemared' and Spanish almond 'Garfi' in Spain. This rootstock was originally selected for almonds and showed excellent graft compatibility with peach and nectarine varieties. 'Garnem' (GN 15) rootstocks develop similar or stronger / better than GF677 rootstock. In the early stages of the growing season, they grow hairless or less hairy. The leaves are large and red, characteristically between almond and peach. It provides a high degree of shoot formation in all known varieties of almond, nectarine and peach. It is tolerant of drought and inefficient soil conditions. It has lower tolerance to iron chlorosis than GF677 rootstock. It has low tolerance to asphyxia in heavily irrigated soils. Plants inoculated with this rootstock show better graft compatibility than grafts made on GF677 rootstock. They need summer pruning to prevent excessive shoot growth and to reduce negative impact on fruit size and quality. It is resistant to root nematodes (*M. arenaria, M. hispanica, M. incognita* and *M. javanica*) which are effective in *Prunus* species.

In soils with nematodes, 'Garnem' rootstock can be used instead of GF677 rootstock. However, its resistance to overly irrigated and calcareous soils is lower than GF677 rootstock (Özden et al., 2011). The rapid production of plants under *in vitro* conditions using plant tissue culture techniques is routinely used in many plants nowadays. Since most of the fruit species have a heterozygous structure, it must be produced vegetatively. Although success in vegetative reproduction with classical methods varies according to each species and even variety, the reproduction coefficient is generally low. *İn vitro* reproduction with tissue culture techniques allows a faster production compared to conventional methods (Ak, 2018). A large number of disease-free plants can be produced in a short time, in a narrow area, out of the growing season by micropropagation.

Today, commercial production of some ornamental plants such as chrysanthemum, carnation, fuchsia, gladiol; some herbaceous (onion, peanut, asparagus, beet, brassica, vetch and chickpea species, soy, grass species, corn) and woody (such as *Malus, Prunus, Pyrus, Ribes, Atriplex, Betula)* and eucalyptus and poplar such forest trees can be produced by micropropagation. In woody plants used in fruit growing and ornamental plant cultivation, the aforementioned environments and sterilization stage may show some differences. At the same time, it is extremely important to use grafted saplings in both areas. In this study, the regeneration shoot tip and nodal explants grown *in vitro* conditions of 'Garnem' hybrid rootstock were investigated, the effect of different phytohormone and concentrations on obtaining adventitious shoots from different explants was investigated, so an efficient protocol was developed for *in vitro* regeneration of 'Garnem' hybrid rootstock These outputs of the study can be a reference resource for future *in vitro* and biotechnological studies on the rootstock in question.

Materials and Methods

Plant Material

'Garnem' (GN-15) (*P. dulcis* x *P. persica*) rootstock was used as plant material in experiment. Plant material had been given early spring time.

Explant sterilization and treatment

Plantlets brought from orchard and 20 mm green soft cuttings were obtained from the newly growing cuttings taken from 'Garnem' (*P. dulcis* x *P. persica*) rootstock, which were taken out springtime in optimum physiological cell division period. Then, after the leaves were cut and removed, with a bud on it. The cuttings kept under tap water for 30 minutes in the laboratory were kept in 70% ethyl alcohol for 1 more minute and then rinsed with sterile distilled water. After this stage, green cuttings are subjected to surface cleaning for 15 minutes in 8% commercial bleach (0.525% NaOCl) containing one or two drops of Tween 20 for surface sterilization of the plant material, and then 5 minutes each. Surface sterilization was completed by washing 3 times with sterile distilled water. After the sterilization was completed, explants were transferred to MS culture medium containing 1 mg / l BA, 30 g / l sucrose and 5.5 g / l agar and adjusted to pH 5.6 with 1 N NaOH or HCl. Then, 3 weeks after the beginning of the culture, the percentage of contaminated and healthy explants in the culture medium was determined as percent.

Culture conditions

The light intensity of the climate cabin used in this study was adjusted to 2500-3000 lux, it was programmed to apply a 16 hour (16 hours light / 8 hours dark) photoperiod, and the ambient temperature was kept at 24 ± 1 ° C.

Culturing explants in the initial environment

The buds on the green cuttings, whose superficial sterilization was completed, were separated from the wood tissue and planted in magenta plates containing 50 ml of initial culture medium. Each magenta containers were placed 5 nodes. Nodal explants were subcultured at 3-week intervals until the shape and number of leaves and shoots that can be used in regeneration experiments in the starting culture medium were obtained. The pH of the MS (Murashige and Skoog, 1962) culture medium containing 0.5, 1.0 or 2.0 mg / l benzyladenine (BA), 30 g / l sucrose, 5.5 g / l agar, minerals and vitamins used as the starting culture medium should be determined without autoclaving. just before it is set to 5.6. The prepared culture medium is then at 121°C for 20 min. sterilized. In order to maintain the shoot culture, newly obtained shoot clumps were separated as individual shoots and transferred onto fresh shoot culture medium.

In vitro rooting

Each shoot 2-3 cm long obtained from different explants was separated and transferred into media

containers. Shoot rooting medium consisted of ½ MS containing 0.5, 1.0 or 2.0 mg / l IBA (indole-3-butyric acid), 30 g / l sucrose, 5.5 g / l agar. The pH of the rooting medium was adjusted to 5.6 before sterilization.

Acclimatization to the external environment

Approximately 4-5 weeks later, the roots of the plantlets forming sufficient roots under *in vitro* conditions were washed and cleaned from agar and then they were transplanted into plastic containers containing sterile 1: 1 peat and perlite. Plantlets are covered with transparent containers in order to provide a humid outdoor environment. The pots were opened every day for increasing periods (5-10 minutes) 3 weeks after the beginning of the acclimatization process to the external conditions and the plantlets were accustomed to the external conditions. The leaves of the 'Garnem' rootstock can become green and red over time at the place where it was taken; this was seen in plants grown under *in vitro* conditions. Care has been taken to ensure that this is not particularly related to the effect of ambient pH.

Statistical evaluation

In the *in vitro* regeneration study, the statistical evaluation of the research results in terms of 'Garnem' hybrid rootstocks was made according to the randomized plot trial design, and the research was repeated 3 times independently and each independent trial was arranged to consist of 12 shoots. When the statistically significant processes were determined, the differences between the average data were subjected to LSD test at the P <0.05 level. Angle transformation was applied to the proportional (%) data obtained by counting.

Results and Discussion

One of the most important factors affecting the success in *in vitro* studies is the effective sterilization of plant materials. Therefore, the first and most important step to be done in the study is the superficial sterilization of the plant material to be used and the decision of the most appropriate sterilization method for this. The most commonly used disinfectants in the superficial sterilization of explants; ethanol, calcium, sodium hypochlorite, silver nitrate, hydrogen peroxide and mercury chloride (Babaoglu et al., 2002).

In general, surface sterilization of explants at the beginning of *in vitro* studies is done for 5 minutes after being shaken for 6- 20 minutes in 0.5-15% NaOCl, CaOCl or HgCl2 solution containing a couple drops of Tween-20 used to break the surface resistance of explants. It was completed by rinsing with sterile distilled water 3 times (Ainsley et al., 2001a; Channuntapipat et al., 2003). On the other hand, there are researchers who perform sterilization in several stages. Some researchers (Gürel and Gülsen, 1998b; Ainsley et al., 2000; Ainsley et al., 2001b; Pruski et al., 2005) used explant sources under tap water for different times (5 min-2 hours) before switching to explant superficial sterilization has done rough cleaning by keeping. Muna et al. (1999) did not consider keeping the explant sources under tap water sufficient for coarse cleaning, and after washing with a few drops of Tween-20 for 10-15 minutes, they switched to surface sterilization. Jain and Babbar (2003) wash the shoots that they will use in the same way with 10% (v / v) Teepoland 30 minutes. After keeping them under tap water, they started the actual superficial sterilization.

Espinosa et al. (2006); Jain and Babbar (2000) completed the superficial sterilization of the herbal material using commercial bleach after keeping the explants in ethanol (50-70%) for a short time (30 sec.- 5 min.). Matt and Jehle (2005), the buds they took from 1 old branches of 5 cherry varieties first in 1.5% (w / v) Benomyl for 10 minutes. After holding, they performed a more detailed sterilization by soaking in 10% (w / v) CaOCl for 20 minutes. In the study carried out by Jain and Babbar (2003), the shoots of that year taken from 30 old trees belonging to the Syzygiumcuminii species were brought to the laboratory in a container containing 50 mg / l ascorbic acid and 100 mg / l citric acid.) washed with Teepol, 30 minutes rinsed under tap water. 5 minutes in 50% (v / v) ethanol. after capture, 25 minutes with 0.2% (w / v) mercuric chloride, 20 minutes with 0.2% (w / v) bavistin (fungicide)has been treated. Finally, 10 minutes after being kept in 0.1% (w / v) NaOCl, the explant was superficially sterilized by rinsing with sterile distilled water several times. In this study, no contamination occurred in cultures in which the superficial sterilization was started with nodal explants in 8% NaOCl disinfectant solution, and they continued their development without any morphological change in *in vitro* shoots.

Therefore, it has been concluded that there is no need to apply the explant used in the superficial sterilization of this study, in which different sterilization methods used in the above studies were carried out. For the superficial sterilization of the explants used in this study, 8% NaOCl disinfectant solution was found to be the most appropriate concentration and this concentration was used in the superficial sterilization of the explants during the research process.

The medium prepared for shoot development of shoot tip explants taken from 'Garnem' rootstock contains MS medium supplemented 30 g / l sucrose and 5.5 g / l agar. The effects of node explants taken from 'Garnem' hybrid rootstock on shoot development by adding 0.5, 1.0 or 2.0 mg / l BA to this combination were investigated. 3 weeks after the initiation of culture, the explant and undeveloped explant rates that can be subcultured were determined (Table 1).

According to the findings obtained, it was determined that BA administration doses of 2.0 mg / l were more appropriate than the others. The rate of explants that can be subcultured was obtained from the best 2.0 mg BA application with 47.2%, while the lowest rate (20.2%) was found to be at 0.5 mg BA application. As with shoot tip explants, explants taken from the lateral buds of the plants were also grown in the same environment. Again, the effects of node explants taken from 'Garnem' hybrid rootstock on shoot development by adding 0.5, 1.0 or 2.0 mg / l BA in the same combination were investigated. 3 weeks after the beginning of the

culture, explants that can be subcultured, the rate of undeveloped explants (%) was determined (Table 2).Both shoot tip and nodal explants taken into culture started to persist from the 4th day in MS medium containing 2.0 mg / l BA, at the end of the 3rd week, 52.3% of the explants reached the size that can be subcultured. The nodal explants cultured in a MS medium containing 0.5 and 1.0 mg / l BA are only 7-10 and at the end of the 3rd week 41.8% of the explants taken into culture in MS medium containing 1.0 mg / l BA and 18.3% of the explants taken in culture in MS medium containing 0.5 mg / l BA reached a size that can be subcultured. The data obtained from the results of 3 separate trials conducted independently from each other were evaluated and the shoots obtained in 0.5, 1.0 and 2.0 mg / l BA culture medium were subcultured in MS medium containing the same PGR (Plant Growth Regulators) concentration and the experiment was continued (Figure 1).

At the end of the three-week development period, proliferation numbers were determined in explants taken into subculture. The effect of 0.5, 1.0 and 2.0 mg / l BA on proliferation was demonstrated by finding different proliferationnumbers of *in vitro* shoots cultured in MS medium containing 0.5, 1.0 and 2.0 mg / l BA. According to the findings obtained, it is seen that the number of proliferationis 6.2 in the 3rd subculture, 4.8 in the 2nd subculture and 2.9 in the 1st subculture in MS medium containing 2.0 mg / l BA (Table 3).

According to various sources, the rate of proliferations in subculture is low at the beginning, but it increases especially in the later periods. Studies show that when done up to the 8th subculture, the rate of proliferationincreases rapidly. However, under the current conditions, 3 subculture applications could be made. At this stage of the research, the effect of different levels of IBA in rooting *in vitro* shoots was investigated, and *in vitro* shoots were cultured in ½ MS medium containing 0.5-2.0 mg / l IBA. In the study, the effects of different levels of IBA on the rooting rates of *in vitro* shoots were found to be statistically significant (Table 4).

As seen in Table 4, the rooting rate in micro shoots cultured in ½ MS medium containing 2.0 mg / l IBA was 42.8%, and the rooting rate in micro shoots cultured in MS medium containing 1.0 mg / l IBA was determined as 31.6%. The rooting rate of the shoots rooting in the MS medium containing the lowest concentration of IBA decreased up to 23.5% (Figure 2). In this study, it was determined that the effect of 1.00 mg / l IBA added to the rooting medium on the root length of micro shoots was better and statistically different from the others.

Antonopoulou et al. (2005) stated that MS MS medium and different concentrations of auxin are generally used in rooting *in vitro* shoots of varieties belonging to the genus Prunus. In this study conducted on GF-677 almond rootstock, *in vitro* shoots obtained 100% rooting when cultured in a MS media containing 1 mg / l IBA. Fotopoulos and Sotiropoulos (2005) determined the effects of ½ MS or MS media containing different IBA concentrations on the rooting rate in *in vitro* shoots in their study on PR 204/84 peach-almond hybrid. 100% rooting was obtained in micro shoots that were cultured in MS medium containing 1.0 mg / l IBA or ½ MS media containing 0.5 mg / l IBA. Durkovic (2006), in his study, obtained 73.3% rooting by culturing *in vitro* shoots from the nodal segment explants of cherry in MS medium containing 0.3 mg / dm3 IBA. Osterc et al. (2004) in the study of cherry, 75-100% rooting rate was obtained when *in vitro* shoots were cultivated in ½ MS medium containing 1 mg / l IBA. Rooted plantlets obtained under *in vitro* conditions were transferred to plastic containers with 3 different environments in order to get accustomed to external conditions. At the end of the 8th week, the vitality rates of the plantlets were determined. While the viability rate of the plantlets transferred to the medium containing peat: perlite at the ratio of 1: 1 was found to be 47.2%, the viability rate of the plantlets in the environment containing only perlite was found to be 32.8%, and the viability rate of the plantlets in the environment containing only peat was found as 23.6% (Table 6).

When transferred to larger pots in the 8th week, the adaptation of the plantlets grown in an environment containing peat: perlitewas faster and the plantlets continued to grow and develop in a short time. Leaf colors of the plantlets transferred only to perlite medium were observed to lighten. One of the reasons for the low viability of the plantlets transferred to the peat environment is that the roots may have been damaged during planting. Therefore, in order to prevent damage to the roots during planting, the roots of the plantlets should be transferred to the lapping environment without exceeding 12-15 mm on average. Again, in parallel with this research output, Muna et al. (1999) in the study conducted on Maxma-14 cherry variety, it was emphasized that plantlets get used to external conditions more easily when they are transferred to pots shortly after rooting. The most important point in the acclimation of rooted plantlets obtained in *in vitro* studies to external conditions is to prevent water loss. It is assumed that water and nutrient insufficiency is the main reason for the low viability of the plantlets transferred to the perlite environment. While *in vitro* plantlets are accustomed to external conditions, it is also necessary to keep them in climatic rooms with high humidity and gradually reduce the humidity.

Table 1. Shoot development from shoot tip explants

BA (mg/L)	Explant that can be subcultured (%)	Undeveloped explant (%)
0.5	20.20 c	16.50 b
1.0	37.30 b	8.40 c
2.0	47.20 a	21.30 a

*The difference between the means with different letters on the same column is statistically significant($P<0.05$)

Table 2. Rooting percentage of PMPC hybrid rootstock candidate (%)

BA (mg/L)	Explant that can be subcultured (%)	Undeveloped explant (%)
0.5	18.30 c	28.30 b
1.0	41.80 b	12.10 c
2.0	52.30 a	21.80 a

*The difference between the means with different letters on the same column is statistically significant(P<0.05)

Table 3. The effect of BA applied in different concentrations on the number of proliferationobtained from nodal segment explants

CYTOKINE	PROLIFERATION NUMBERS			
BA (mg/L)	1st Subculture	2nd Subculture	3rd Subculture	Avarege
0.5	1.30	1.90	3.30	2.17 b
1.0	2.10	4.30	5.30	3.90 a
2.0	2.90	4.80	6.20	4.63 a

*The difference between the means with different letters on the same column is statistically significant(P<0.05)

Table 5. *İn vitro* rooting

IBA (mg/L)	Rooting rate (%)	Number of Roots (pieces)
0.5	23.50 c	2.80 c
1.0	31.60 b	3.10 a
2.0	42.80 a	2.20 b

*The difference between the means with different letters on the same column is statistically significant(P<0.05)

Table 6. Acclimatization of rooted plantlets ex vitro conditions

MEDIUMS		
Perlite	Peat	Peat:Perlite (1:1)
32.80 b	23.60 c	47.20 a

*The difference between the means with different letters on the same column is statistically significant(P<0.05)

Figure 1. Shoot development from explants

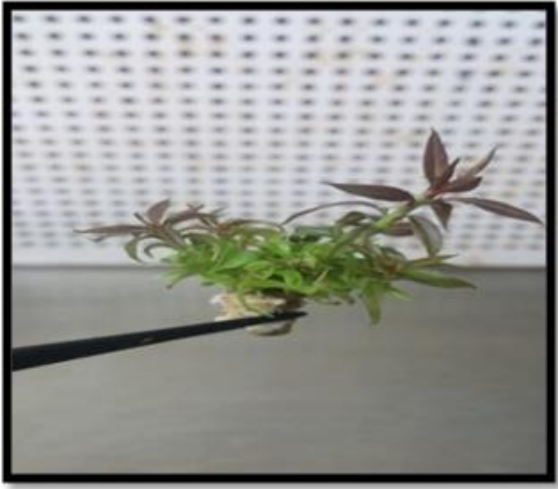

Figure 2. Views from proliferated and rooted explants

Conclusion

'Garnem' clone rootstock was chosen as rootstock because it shows good graft compatibility with almond, peach and nectarine. In the rootstock nursery, it shows a very good development at half the strength of GF 677 rootstock. The success of the budding is high with all known almond, peach and nectarine varieties. It is tolerant of poor soil conditions and arid conditions. In this study, the effects of different PGR and their concentrations on obtaining adventitious shoot regeneration of 'Garnem' rootstock, which is a hybrid of almond and peach rootstock, which has adapted to the general high lime ratio where almond growing areas in the World, were investigated. This study can be grouped under 4 main headings: 1. Surface sterilization of plant material, 2. Culturing of the node explants in the starting environment and shoot propagation, 3. Rooting of the shoots, 4. The acclimatization of rooted plantlets to external conditions. Very high values could not be obtained in terms of rooting and acclimatization. Because it can be said that especially the plant growth medium is very effective in this part of research. In the light of previous sources and information, it is thought that better results can be obtained by adding GA3 to the rooting medium and making applications in the dark in the light of some information. It may be suggested to apply them in future studies. In this study, it was determined that the ratio of explants to be subcultured was better in lateral explants when plants obtained from shoot tip and lateral (node) explants were compared. However, in general, the plants obtained from the shoot tip give better results in the reproduction of many fruit species or rootstocks. In order to be successful in plant tissue culture studies, it is essential that the laboratory and climate room conditions are complete. On the other hand, care should be taken to determine the choice of explants, PGR concentrations and combinations. The choice of media is very important in tissue culture studies. As a result of the studies, it has been determined that the reactions of each species and variety to tissue culture techniques are different. Factors such as the age of the explant, the physiological condition of the donor plant, the source of the explant affect the regeneration rate and may differ significantly. The type of culture media, its components, and the most suitable culture conditions differ for each genotype.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the aurhors to the present study is equal. All the authors verify that the text, figures, and tables are original. The authors read and approved the final manuscript.

Ethical approval

Not applicable

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Data availability

Not applicable

Consent for publication

Not applicable

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