

In Vitro Micro propagation of Begonia (*Begonia Lucerna Hort.*)

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Abstract - Various researches are conducted on the micro-propagation of a plant by using micro-samples of *B. lucerna Hort.*'s leaves. The micro-samples of the leaves were disinfected for one minute with 70% ethyl alcohol solution and 15 minutes with 10% Clorox solution. In the first experiment, the micro-samples were cultivated in Murashige and Skoog medium with 0, 1, 2, 3 and 4 mg/L of Benzyl adenine and 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L naphthalene acetic acid in order to increase the proliferation of the shoots. In this experiment, a combination of treatments was carried out with 0 mg/L Benzyl adenine and the naphthalene acetic acid concentration increase to 0.4 mg/L. Accordingly, it was observed that the percentage of live micro-samples was increased and the samples produced roots and callus. In another combination of treatments, one mg/L Benzyl adenine and up to 0.2 mg/L the naphthalene acetic acid was used. In this treatment, the percentage of live micro-samples were increased, but no roots and callus were produced. In the second experiment, the micro-samples were cultivated in the same medium with 0, 0.5 and 1 mg/L Benzyl adenine and 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L naphthalene acetic acid. The combination of 0.5 mg/L Benzyl adenine and 0.2 mg/L naphthalene acetic acid showed a significant difference compared to other treatments in shoot productivity at 5% level. According to the results of two previous experiments, this test was conducted for determining the best hormonal treatment for shoots. The micro-samples were cultivated in Murashige and Skoog medium containing 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mg/L Benzyl adenine and 0, 0.2, 0.4 and 0.6 mg/L naphthalene acetic acid. The combination of 0.6 mg/L Benzyl adenine and 0.2 mg/L naphthalene acetic acid had a significant difference with other treatments in shoot productivity at 5% level. In another study, the micro-samples were cultivated four times with the best hormone treatment in the 4-week intervals on a fresh medium. The second cultivation that was conducted at the level of 5% had a significant difference with other treatments in the proliferation of the shoots. The Murashige and Skoog medium containing 3% sucrose and 8 mg/L agar was used for rooting while a hormonal composition of 0, 0.1, 0.2 and 0.3 mg/L Benzyl adenine and 0, 0.2, 0.4 and 0.6 mg/L the naphthalene acetic acid was used for determining the best hormonal treatment. After 8 weeks, it was indicated that the best hormonal treatment for rooting was 0.1 mg/L Benzyl adenine and 0.6 mg/L the naphthalene acetic acid, which had a significant difference with other treatments at 5% level. Rooted plants were transferred to disinfected environments of sand, peat, loamy soil and their composition in a one-third volume ratio. 80% of the plants survived in a mixture of soil and peat.

Keywords: Micro-propagation, micro-samples, proliferation, shoots, under cultivation, rooting and adaptation.

Introduction

The primary origin of *Begonia* is moist and warm tropical areas. *Begonia* is from *Begoniaceae* family that has three groups: fibrous-rooted, rhizomatous and tuberous-root *Begonias*. The genus contains more than 1200 different plant species (1,4).

The general method for growing *Begonias* includes using seed, leaf cuttings, stem cuttings, and tissue cultivation (2, 11, 16).

Using seed for growing and increasing the plant is less favored compare to other methods, due to the production of hybrid varieties. Increasing the plant with the use of stem and leaf cutting cannot meet the market demand for this plant (2).

In-vitro growth methods can enable mass production of this plant by a rapid increase. Today, rapid mass production of this plant can be carried out from all parts of different species including leaf, petiole, peduncle, protozoan, shoots and meristem micro-samples. In most researches, the Benzyl adenine and naphthalene acetic acid has been used to grow the shoots and roots, respectively (20,26).

By studying various researches, it was realized that the different species of *Begonia* have a different reaction to the hormonal composition of shoots and roots regeneration (12).

In *B. cheimantha* Everett species, the highest number of regeneration was obtained at the temperature of 21-18 °C. Fewer roots and shoots were obtained at higher temperatures, and lower temperatures prevented the plant growth. The highest number of shoots was obtained at 21-18 °C; the most extended shoots were obtained at 24-21°C. Furthermore, the highest number of roots and the longest roots were respectively obtained at 18 °C and 24°C (14). The plants that were regenerated from the callous of Begonia's leaves were different in flower morphology, flower size, plant morphology and flower number in the plant. It has been indicated that the presence of Benzyl adenine and indole acetic acid was necessary to form the maximum number of inappropriate shoots, especially on the peduncle and petiole. Moreover, the flower buds formed on the leaves and the peduncle but did not form on the petiole, and the flowers were all male. In tissue cultivation method, the responses of the growing plants in the production of inappropriate buds were different (26).

In growth mediums with 1.5% to 4% concentration, Sucrose is used as a source for carbohydrates nutrition. However, in most cultivation, 3% concentration was used (23).

In a study, the micro-samples of fibrous-rooted begonia get inflated after 4-3 weeks with benzyl adenine and naphthalene acetic acid hormones in Murashige and Skoog medium (22). After 14 weeks, the shoots were transferred to a medium containing indole butyric acid for rooting. This method was used by Deberg and Muen to produce roots in hard rooting varieties (13, 23). The purpose of this study was to investigate the micropropagation of Begonia and determine the appropriate cultivation medium and hormonal requirements that were necessary for the establishment and rooting of this plant.

The reaction of six different Begonia's petiole in one medium with different composition of Benzyl adenine and naphthalene acetic acid was studied. These reactions were different for regeneration in different concentrations of hormones (17).

Ring et al. (26) placed the leaves, petioles, and peduncles of several begonia species in a medium and reported that the presence of a type of cytokine, adenine and auxin was necessary for the formation of the maximum number of inappropriate shoots, especially on the peduncle and petiole. Flower buds formed on the leaf and the peduncle but did not form on the petiole, and the flowers were all male.

Takayama et al. (31) reported the physical and chemical growth factor of *B. hiemalis* in suspended cells cultivation. In this experiment, differentiated buds grew rapidly, and rootless mass seedlings were obtained. These Seedlings were divided into single shoots and transferred to Agar medium. With this method, 10¹⁴ or more seedlings can be obtained in one year from a 7 × 7 mm leaf micro-sample.

In a study, the petiole's regeneration efficiency of 17 different species of Begonia on different medium with different hormonal composition was investigated. In this experiment, it was suggested that the efficiency of the shoot and root formation from petiole depends on the combination of growth regulator, the composition of growth medium, and selection of species and maternal treatment (33).

The different medium includes salts with different concentrations along with appropriate amounts of vitamins, amino acids, organic additives (casein hydrolyzed, etc.), different growth regulators, carbohydrate sources, and solids materials can be used for micro-propagation of begonia (23, 18, 29, 14, 25, 5).

In an experiment, a rhizome begonia specimen was infused on an MS medium containing 5 mg of benzyl adenine and 1 mg of naphthalene acetic acid and was inflated after 3 to 4 weeks. After 14 weeks, the shoots were transferred to a medium containing 2 mg/L indole butyric acid for rooting. This method was used by Debergh and Maene to produce roots in species that hardly formed roots (23, 13).

In another study, the effects of 0-10 mg/L naphthalic acetic acid and 0-1 mg/L benzyl adenine on the regeneration of begonia petiole were compared with each other. Three weeks after applying 0.1 of naphthalene acetic acid and 0.1 of benzyl adenine the shoots were formed. And after 5 weeks the shoots and roots were formed. However, after applying the concentration of 10 mg/L naphthalene acetic acid, the roots and callus were formed (20).

In a report, the effects of naphthalene acetic acid and benzyl adenine on the regeneration of Begonia's (*B. cheimantha*) petiole micro-samples were compared with each other. When these two regulators do not exist in the medium or when the concentration of naphthalene acetic acid is at the lowest level, then the number of micro-samples that was able to stay alive increase dramatically and only a few of them survived; in order to increase alive micro-samples, the concentration of benzyl adenine must be high. Auxin is the best growth regulator to make the micro-samples survive. 100% of micro-samples can survive in the concentrations of 0.1 to 1 mg/L. The formation of shoots is highly dependent on cytokinin. It should be noted that auxin has only had a transforming effect on the cytokine effect. Therefore, regeneration of shoots through benzyl adenine alone is not possible, but it is possible to do it with the help of naphthalene acetic acid (14).

The formation of shoots and roots in *B. hiemalis* leaves depends on the concentration of hormones in the growth medium. Both auxin and cytokinin should be used at an optimal concentration. The amount of auxin should be much higher than the amount of cytokine. In begonia, the limbs were formed without hormones.

In the case of *B. cheimantha*, the amount of cytokinin should be higher than the auxin to form shoots (6, 14, 5, 19).

In case of *Begonia* (*B. evansiana*), the buds were not formed when benzyl adenine was used alone, but when adenine and phenylacetic acid were used together in a growth medium, then many buds were formed (26 and 28).

If cytokinins used alone, it is unexpected to be inactive. Cytokinin are known to intensify bud formation of *Begonia*, but when the auxin is added to the medium without cytokinins, then the auxin intensification effect can be observed. The effect of auxins has been studied more in combination with cytokinins. Another compound that produces buds is adenine, which its effect in bud formation is the same as cytokinin. In the case of peduncle cultivation, there was no sign of bud formation, except when adenine was added to the medium with auxin and cytokinin (30). Auxin indole acetic acid and phenylacetic acid increase bud formation of the plant. The amount of indole acetic acid needed to form flower bud in cultivation is much greater than indole acetic acid needed for creating vegetative buds. These unexpected results might be due to the existence of a very small amount of auxin in the peduncle. Auxin may have an indirect effect because auxin is not capable of increasing the amount of flowering, but it can keep the micro-samples alive. With more differentiating, the meristic mode is obtained (26).

In another study, the 0-10 mg/L Benzyl Amino-Purine and 0-10 mg/L naphthalene acetic acid were used. The reaction of six different species of *Begonia* in term of root, callus, shoots, and seedlings formation was different (9).

The effect of naphthalene acetic acid and kinetin on the differentiation of *Begonia's* (*B. hiemalis*) petiole was investigated. The suitable amount of naphthalene acetic acid and Kinetin for this differentiation activity was 0.1-1 mg/L. In comparison conducted between quinidine and benzyl adenine, adding 0.3 mg/L of benzyl adenine and 0.1 mg/L of Kinetin caused the well-formation of the buds, while roots were formed in a wide range of benzyl adenine and kinetin concentrations (14 & 31). In the other experiment, the highest number of shoots was obtained with 1 mg/L naphthalene acid and 10 mg/L kinetin (25).

In the case of *Begonia's* petiole cultivation in Murashige and Skoog medium containing 0.1 mg/L of naphthalene acetic acid and 0.1 mg/L benzyl adenine, it was observed that misplaced buds were formed after eight weeks. When the leaves of long shoots were cultivated in the same medium with one mg/L naphthalene acetic acid and 0.01 mg/L Benzyl Amino-Purine, the number of seedlings and sprouts was less than when an adult petiole was used. After six weeks, the regeneration of callus from in-vitro leaves in Murashige and Skoog medium 0.75 mg/L of 24.D and 0.001 mg/L Benzyl Amino-Purine was carried out. In order to create seedlings, the obtained calluses were transformed into a new medium containing kinetin and naphthalene acetic (7).

The proliferation of *Begonia's* shoots was carried out with the cultivation of leaf, petiole, peduncle, flower bud and epidermal layer with small colonies micro-samples (8, 24, 21, 10 & 26). The composition of the growth medium, the tissue type of the main organ, the number, the time of the getting the micro-samples, and the environmental conditions affect the shoots and roots regeneration (26, 32). The shoots formation is highly dependent on cytokinin (25).

Materials and Methods

Establishment, medium, and treatments

First, the pots containing cuttings of *Begonia* (*B. lucerna* Hort) were purchased and grown in the greenhouse under appropriate light (the indirect light of the sun that some of it was absorbed), temperature (average greenhouse temperature between 26 ± 3 °C in day and 16 ± 3 °C at nights), humidity (75 ± 5 %) and nutrition (chemical fertilizers of nitrogen, phosphorus, and potassium were grown at a concentration of 500 parts per million and 2 g/L micro-fertilizers per every 20 days) conditions. Then, more plant cuttings were taken from these pots and were planted in plastic pots containing a special soil mixture. This mixture includes one-third of the clay soil, one-third of the sand and one-third of the manure were planted and irrigated every five days. After root created and the plant started to grow, it was fed with nutrient solution (2 g/l) once a week. The plants were kept in these conditions for about two months. Then the leaves (mature) that completed their growth were used as a micro-sample.

Half an hour to one hour before working under the air flow device, the surface of the machine was disinfected with 70% ethylic alcohol, and then the ultraviolet and air venting device and ultra-violet light bulb were turned on. Containers containing the medium were disinfected for 15 minutes in an autoclave at 121 °C and steam pressure of 1.5 kg / cm². After this, the containers with medium were cooled down in the air flow device under ultraviolet light. In cases that these containers are not used immediately, they would be kept in the refrigerator (at 4-5 °C) until the time of cultivation. After cultivating the micro-samples, the glasses were sealed with paraffin strips. Before starting the treatment of leaf micro-samples with disinfection solution, these micro-samples were kept inside a one-liter Beaker for 10 minutes along with a few drops of dishwashing liquid and then were washed for half an hour under the running water. After surface disinfection (one minute in 70%

alcohol solution, then 15 minutes in 10% chloroxate solution) and three times rinsing with sterilized distilled water, the leaf samples (mature leaves) were transferred on the pre-disinfected ceramic tile with the help of a sterile pence that was disinfected with fire. Then the margins of the samples were separated with a 3 mm laboratory sterilized knife, and the remaining micro-samples were divided into 7×7 mm dimensions and placed on the Murashigeand Skoog growth medium, which contains various hormonal treatments of benzyl adenine and naphthalene acetic acid. Afterward, the cultivated samples were under a light with 1500 lux intensity for 16 days. This light was created from two fluorescent lamps. During the experiment, the average temperature was $25 \pm 3^\circ\text{C}$.

The basic growth mediums that were used for creating roots and shoots are Murashigeand Skoog medium (25) with 8 g/lagar, 30 g/l sucrose and vitamins (nicotinic acid, pyridoxine, thiamine, and glycine). Plant growth regulators used in this medium for preliminary stages of shoot creation experiments include 0, 1, 2, 3 and 4 mg/l of benzyl adenine and 0, 0.1, 0.2, 0.3, 0.4 And 0.5 mg/l of naphthalene acetic acid. According to the results of preliminary experiments, concentrations of 0, 0.5 and 1 mg/l of benzyl adenine and 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l of naphthalene acetic acid were selected for the second stage of the experiment. Then, based on the results of two previous experiments, concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l of benzyl adenine and 0, 0.2, 0.4 and 0.6 mg/l of naphthalene acetic acid and 8 g/l agar were selected. The concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l of benzyl adenine and 0, 0.2, 0.4 and 0.6 mg/l naphthalene acetic acid were selected as the growth naphthalene acetic acid for shoot creation experiments. After the roots of the shoots were formed inside the growth medium, the samples were transformed to the main medium containing one-third of the sandy clay soil, one-third of the sand and one-third of the moos pit.

The proliferation of the shoots and rooting

The shoots that were reproduced from cultivating the leaf micro-samples, were cultivated on the best environment for increasing the shoots; the obtained shoots were cultivated again, and this procedure continued until four cultivations for four weeks in order to assess the shoot creation ability of micro-samples. In order to create the roots for the obtained shoots, the basic Murashigeand Skoog medium with various hormonal treatments were used. After cultivating the micro-samples on the growth medium, these samples were placed under a light condition with 1500 lux intensity for 16 hours.

Transfer to soil and adaptation

After eight weeks, the rooted shoots were transferred to pots containing disinfected soil mixtures such as moss pit, quartz sand and soil mixtures (one-third of loam soil, one-third of sand, one-third of the pit) under disinfected conditions. Each pot was placed in a sealed plastic bag for 24 hours. Then the pot containing the plants was placed in plastic boxes with 2airflow regulating valves. In order to irrigate the pots that contained moss pit and quartz sand was performed using a solution that its concentration is one-eighth Murashigeand Skoog solution. The irrigation of the pots containing a mixture of soil can be carried out with normal water. Each of these irrigations is continued for one week. All of the solutions used for irrigation had 1% benomyl antifungal. After opening the valves, the plants were adapted to the outside environment. After transferring the rooted seedlings to the pots, the seedlings were exposed to 1500 lux light condition for 16 hours. The reaction of leaf micro-samples was recorded in two preliminary experiments and the number of shoots, rooting percentage and shoots formation in four cultivations were counted. Experiments were conducted in a completely randomized design with 6 replications. Data were analyzed statistically and the means were compared using Duncan's new multiple range test (DNMRT).

Results

The shoots proliferation from leaf micro-samples

All these stage observations were performed four weeks after planting micro-samples in the above growth medium. These medium contain 1 mg/l benzyl adenine and 0.2 mg/l naphthalene acetic acid had the highest percentage of survived micro-samples and did not produce root and callus. In combination treatment, if the concentration of benzyl adenine increases to more than 1 mg/l, then the micro-samples would burn out. In a combination treatment with zero mg/l benzyl adenine, the concentration of naphthalene acetic acid increases up to 0.4 mg/l, then the average percentage of healthy micro-samples would be increased; however, when this concentration increases to more than 0.4 mg/l, the percentage of healthy micro-samples would be decreased. In the former, the micro-samples were able to survive, but could not produce roots and callus. In the treatment combination with one mg/l benzyl adenine, if the concentration of naphthalene acetic acid increases up to 0.2 mg/l, then the average percentage of healthy micro-samples would be increased. However, if the concentration of naphthalene acetic acid increases more than this amount, then the percentage of healthy micro-samples would be decreased (Table 1).

Table 1: Comparison of the mean percentage of survived micro-samples of Begonia after four weeks with 30 growth regulator for the proliferation of the shoots

benzyl adenine (mg/l)	naphthalene acetic acid (mg/l)						Mean
	0	0.1	0.2	0.3	0.4	0.5	
0	0 ^d	0 ^d	50 ^{abc}	50 ^{abc}	67 ^{ab}	50 ^{abc}	36 ^A
1	0 ^d	67 ^{ab}	83 ^a	33 ^{bcd}	33 ^{bcd}	0 ^d	36 ^A
2	0 ^d	33 ^{bcd}	50 ^{abc}	0 ^d	0 ^d	0 ^d	14 ^B
3	0 ^d	17 ^{cd}	0 ^d	0 ^d	0 ^d	0 ^d	3 ^B
4	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^D
Mean	0 ^D	24 ^{AB}	37 ^A	17 ^B	20 ^B	10 ^{BC}	

The means with similar letters (small letters for percentages and capital letters for the mean of rows and columns) were not significantly different at the 5% probability level of Duncan's multiple range tests.

Hormonal treatments of 0.5 mg/l benzyl adenine and 0.2 mg/l naphthalene acetic acid had a significant difference with other treatments used for regeneration of shoots (Table 2). In the concentration of 0 and 1 mg/l benzyl adenine, there was no shoots regeneration, but at a concentration of 0.5 mg/l, when the concentration of naphthalene acetic acid increased to more than 0.2 mg/l, the regeneration of shoots decreased. Shoots that were produced after four weeks are shown in Fig. 1.

Table 2: Comparison of the average percentage of micro-samples that produced shoots after four weeks being under 18 different growth regulating treatments.

benzyl adenine (mg/l)	naphthalene acetic acid (mg/l)						Mean
	0	0.1	0.2	0.3	0.4	0.5	
0	0 ^c	0 ^c	0 ^c	0 ^c	0 ^c	0 ^c	0 ^C
0.5	0 ^c	17 ^{bc}	67 ^a	33 ^b	0 ^c	0 ^c	36 ^A
1	0 ^c	0 ^c	0 ^c	0 ^c	0 ^c	0 ^c	0 ^C
Mean	0 ^C	6 ^B	22 ^A	11 ^{AB}	0 ^C	0 ^C	

The means with similar letters (small letters for percentages and capital letters for the mean of rows and columns) were not significantly different at the 5% probability level of Duncan's multiple range tests.



Fig. 1 Shoots produced after four weeks

0.6 mg/l benzyl adenine and 0.2 mg/l naphthalene acetic acid were the best hormonal treatments for shoots, which had a significant difference with other treatments at 5% level (Table 3). If the concentration of benzyl adenine is reduced to 0.5 mg/l (less than 0.6 mg/l), then the amount of shoot production would be decreased. When its concentration is increased to more than 0.6 mg/l, the amount of shoot production would be decreased as well.

Table 3-Comparison of the mean number of micro-samples that produced shoots (after four weeks in growth medium)

naphthalene acetic acid (mg/l)	benzyl adenine (mg/l)									
	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	mean
0	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^B
0.2	0 ^d	0 ^d	0 ^d	33 ^c	33 ^c	67 ^b	100 ^a	33 ^c	0 ^d	30 ^A
0.4	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^B
0.6	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^d	0 ^B
mean	0 ^C	0 ^C	0 ^C	8 ^{BC}	8 ^{BC}	17 ^{AB}	25 ^A	8 ^{BC}	0 ^C	

The means with similar letters were not significantly different at the 5% probability level of Duncan's multiple range tests.

In under cultivation stage, the shoots proliferation was from the best shoots proliferation hormonal treatment at the deposition stage, including 0.6 mg/l benzyl adenine and 0.2 mg/l naphthalene acetic acid, which was cultivated four times at the same medium and the result of each cultivation were evaluated 4 weeks after the cultivation.

In the second cultivation at the 1% significant level, there was a significant difference between this treatment and other treatments. In second, third and fourth cultivations the number of shoots decreased respectively (table 4). Shoots that were 0.7 to 1.5 cm long were evaluated.

Table 4: Comparison between the average percentages of shoots produced in four cultivations

cultivations	At 1% level
First	7.86 b
Second	15 a
Third	10.57 b
Fourth	6.71 B

The means with similar letters were not significantly different at the 1% probability level of Duncan's multiple range tests.

Rooting, transfer to soil and adaptation

Compared to other treatments (table 5), more rooted micro-samples (Fig. 2) were created eight weeks after cultivation on Murashigeand Skoog medium with 0.1 mg/l benzyl adenine and 0.6 mg/l naphthalene acetic acid. The growth of micro-samples in soil mixtures and moss pit were better than quartz sand.

Table 5: Comparisons between the average percentage of rooted shoots after eight weeks in 16 plant growth regulator treatments

BA (mg/l)	naphthalene acetic acid (mg/l)				
	0	0.2	0.4	0.6	mean
0	0 ^c	33 ^{abc}	50 ^{ab}	50 ^{ab}	33 A
0.1	50 ^{ab}	0 ^c	33 ^{abc}	67 ^a	38 A
0.2	0 ^c	0 ^c	17 ^{bc}	33 ^{abc}	13 B
0.3	0 ^c	0 ^c	17 ^{bc}	0 ^c	4 B
mean	12 B	8 B	29 AB	38 A	

The means with similar letters (small letters for percentages and capital letters for the mean of rows and columns) were not significantly different at the 5% probability level of Duncan's multiple range tests.



Fig. 2. Rooted plants on the Murashige and Skoog medium with 0.1 mg / L BA and 0.6 mg / L NAA after eight weeks.

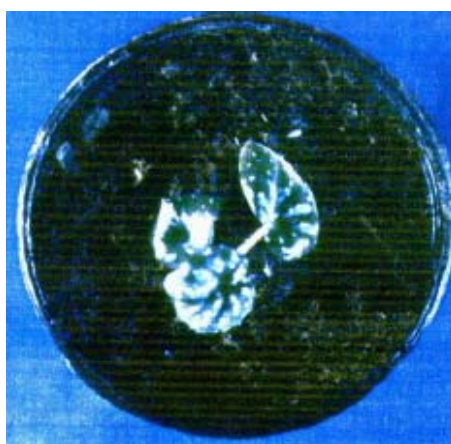


Fig. 3: The adapted Begonia plant with the soil mixture, two weeks after the transfer.

Discussion

The shoots proliferation from leaf micro-samples

The results of experiments indicated that the reduction of benzyl adenine and an increase of naphthalene acetic acid has a significant effect on the creation of roots and callus in leaf micro-samples. In this study, by increasing the concentration of benzyl adenine and naphthalene acetic acid, the number of survived micro-samples was low. In treatments containing zero benzyl adenine and different level of naphthalene acetic acid, the roots and callus were created in micro-samples. When the benzyl adenine was added to the medium, the production of the root and callus decreased and shoots creation was stimulated. By reducing the concentration of benzyl adenine, more fresh and healthy micro-samples can be obtained, and micro-samples were stimulated for shoot proliferation. Generally, auxin was essential for micro-samples survival, it was also necessary for the formation of root and callus. In naphthalene acetic acid treatments in which the micro-samples produced root and callus, the shoots were not created. In order to form the shoots, both auxin and cytokinin are needed in an optimal concentration in the growth medium. The results of the study were consistent with the results of researches conducted on other species of Begonia (14, 17, 20, 24 and 26).

Skog et al. (30) reported that when cytokinin alone is used in the medium for root or shoot creation (*B. hiemalis* Fotsch), it became inactive; but, when auxin was added to the medium, depending on the concentration of both hormones, there was observed an increasing effect on the root or shoot creation. In this study, it has been shown that the formation of shoots is highly dependent on cytokinin. The results of this research are consistent with other researches (20 and 26).

Cultivation

By increasing the number of cultivation, shoots proliferation and the length of the branches, the amount of shoot cultivation was reduced. This was caused by the amount of internal growth regulator and food storage in the tissues. In this experiment, branches with a length of 0.7 to 1.5 centimeters long were evaluated. The time needed for branch growth and reaching to 0.7 cm or longer was increasing, which was an indication of nutrients reduction in the tissues and growth regulators. Similar results were obtained for miniature roses by Salavi and Khoskooy (27) and for lemon by Reza zadeh (3). It shows that by increasing the number of cultivations, the

number of branches and the length of these branches would be decreased. Also, the amount of time needed to reach the desired length of the branch would be increased.

Rooting

In preliminary experiments, leaf micro-samples were able to create roots after three weeks. However, even after eight weeks, the shoots were not able to produce any root. Since the photosynthesis is performed in leaf micro-samples, the amount of carbohydrate in the leaf tissue is higher than the stem tissue, and the amount of cytokinin in the stem tissue is higher than the leaf. It was reported that carbohydrates could increase the effect of auxins. Also, an optimal concentration is needed for both auxin and cytokinin to create roots in micro-samples (15, 20, 31).

Adaption

The growth of seedlings that were planted into soil mixtures (1.3 volumes) and pits were 80% more than other growth mediums, which is due to the usage of nutrients and humidity of the surrounding environment. Seedlings with a higher number of roots or longer roots were more easily adapted compared to others, which was consistent with the results obtained by Mickelsen and Sink (20) on *B. hiemalis*Fotsch.

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