Research & Reviews: Journal of Botanical Sciences

e-ISSN:2320-0189
p-ISSN:2347-2308

In Vitro Regeneration of Seven Strawberry (*Fragaria* × *ananassa* Duch.) Genotypes from Seed, Terminal Meristem, Terminal Bud, Leaf and Petiole Explants

Ahmad Moieni[1], Ashkan Tavizi[2], Mokhtar Jalali Javaran[2], Mehdi Mohebodini[3], Mohammad Hadi Eskandari[4] and Esmaeil Ebrahimie[5]*

[1]Institute of Biotechnology, Shiraz University, Iran and ALHSB Health Research Center, Shiraz, Iran

[2]Department of Biotechnology, Tarbiat Modares University, Tehran, Iran

[3]Department of Horticulture, University of Mohaghegh Ardabili, Ardabil, Iran

[4]Department of Nutrient Industry, Shiraz University, Shiraz, Iran

[5]Department of Genetics, School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, Australia

Research Article

Received: 30/07/2018
Accepted: 05/09/2018
Published: 10/09/2018

***For Correspondence**

Esmaeil Ebrahimie, Department of Genetics, School of Life Science, The University of Adelaide, Adelaide, Australia. Tel: +618 8313 2522.

E-mail: esmaeil.ebrahimie@adelaide.edu.au

Keywords: *Fragaria* × *ananassa Duch,* Tissue culture, Explant, Plant growth regulators, Direct regeneration, Seed germination, Mass production.

ABSTRACT

Strawberry is one of the most important fruits because of its distinctive flavour and being rich in vitamins A and C. World population increase, high demand for strawberry, using more farms and greenhouses for cultivating it as well as time-consuming, and conventional methods of modification make using tissue culture for producing plants which are healthy, uncontaminated a necessity. In this research, Alpine and Sarian are used as cultivars in order to solve the problem of germination in strawberry seeds and the highest proportion of germination was observed in ½ MS+2 mg l^{-1} GA_3 +Sulfuric acid 36 N and +½ MS 2 mg l^{-1} GA_3 + $H_2O_2$100%. Afterwards, seven cultivars, Alpine, Camarosa, Gaviota, Pajaro, Paros, Sarian and Selva were used to improve the tissue culture of strawberry. In the experiment carried out for culturing terminal meristem, each cultivar responded perfectly to T12, T4, T2, T1, T3, T14 and T5, respectively. Next, the experiment of regeneration of strawberry from terminal bud was carried out in which each cultivar had the best regeneration rate in B5, B14, B1, B2, B3, B4 and B12. The experiment of bud culture optimization was devised based on the results of final stem culture where the highest amount of production of strawberry plantlets were achieved in 03, 05, 02, 08, 09, 01 and 04. Finally, a complementary experiment to direct regeneration was done on explants of leaves and petioles in which the highest proportion of regeneration was observed in LP24, LP2, LP6, LP13, LP5, LP11 and LP18.

INTRODUCTION

A hybrid of two American species, *F. virginiana* Duch. And *F. chiloensis* Duch, were used in this research which is called *Fragaria* x *ananassa*. The global production of strawberry has doubled in the past 20 years at 4.2 million tons [1]. Second green revolution uses gene modification and biotechnology to improve the production of crop as well as its quality and plant tissue culture technique plays a vital role in it. Although those working with this technique may still have concerns about its details, more researchers are getting involved in its application in basic aspects of cell differentiation and proliferation as well as improving crop quality. Strawberry is rich in vitamins C, A, anthocyanin and useful amino acids which makes it a medicinal and anticancer compound [2,3]. Strawberry is also a good choice for production of pharmaceutical and recombinant proteins as oral vaccine development due to rapid production of a large number of transgenic plants by runners [4,5]. Plant tissue culture is defined as growing plants cells, tissues or organs through their isolation from the mother plant in an artificial media [6]. Plant tissue culture techniques can be used to obtain complete plants from variety of explants via direct or indirect morphogenesis as well as somatic embryogenesis. In direct regeneration, shoots from explants are produced without passing through callus phase whereas in indirect regeneration this is done through induction of shoots through callus phase. Organ culture refers to obtaining an organized form of growth continually [6]. The vastly used organs for micropropagation are meristem, shoot, embryo and isolated root cultures. Other types of

e-ISSN:2320-0189
p-ISSN:2347-2308

culture such as callus, suspension or cell cultures, and protoplast cultures are considered unorganized tissue cultures. Strawberries are among the most favorite fruits because of their flavor, characteristic aroma and, more importantly, their healthful qualities [7]. It is one of mostly distributed and cultivated fruits in the world [8]. But there are several common problems and prospect in strawberry tissue culture. Bacterial contamination in both initiation and multiplication stages which was observed in the form of a whitish exudate of bacteria around the base of buds and the presence of phenolic compounds which caused death of explants are among the most important problems in tissue culture. Using explants taken from mature trees was also problematic since they were different from juvenile leaves and behaved differently in the media. Hyperhydricity was another issue; that is the response of plant tissue to stressful conditions in form of elongated, wrinkled and translucent leaves, shoots with shorter internodes, reduced number of shoots. Shoot tip necrosis, which is sometimes linked to hyperhydricity, was observed in shoot cultures mainly due to calcium deficiency in plants which results in poor root growth and in the blackening and curling of the margins of apical leaves [9]. As far as illumination is concerned, three elements must be taken into account: intensity, length of the daily exposure period, and quality. It is important to know that tissue cultures do not need as much light as whole plants since photosynthesis is not equally necessary for tissue cultures [10]. Through the tissue culture cycle many somaclonal variations have been made which can be very useful in agriculture. However, they can cause major problems if they result in producing undesirable plant off-types. Loss of large number of micro-propagated plants transferred from *in vitro* conditions to greenhouse or field environment is another difficulty in this research. Relatively lower humidity, too much light and septic environment in these media is stressful to *in vitro* cultured plants [11]. Furthermore the *in vitro* germination of the strawberry plant achenes constitutes a necessary stage in the production of sterile seedlings essential to any process in biotechnology as genetic transformation. However, the germination of achenes is often poor [12].

Simple and efficient method for regeneration is required for cultivars improvement in strawberry. In the present study, *in vitro* regeneration of seven strawberry (*Fragaria* × *ananassa* Duch.) cultivars from different explants was investigated.

MATERIALS AND METHODS

Plant Material

The seven genotypes were used in this project: Paros, Pajaro, Selva, Gaviota, Camarosa, Alpine and Sarian Hybrid (Sarian F1 and Alpine seeds bought from Johnny's Selected Seeds Company and other cultivar's runner obtained from agriculture faculty of Trabiat Modares University).

Hastening strawberry seed germination by using hydrogen peroxide and sulfuric acid treatments

Two separate factorial experiments based on completely randomized design with five replications were established, for breaking seeds dormancy. In the first experiment, the factors included pre-treatment of gibberellic acid (GA_3)) 1 hour immersion) in 6 concentrations (0, 1, 2, 4, 6, 8 mg l^{-1}), the two cultivars of Alpine and Sarian F1 seeds, and the use of sulfuric acid in which the seeds were immersed for 30 seconds in 7 concentrations (0, 1, 3, 12, 18, 24, and 26 N). The second experiment was the pre-treatment of GA_3 and seeds' immersion for one hour in 6 concentrations (concentrations of 0, 1, 2, 4, 6, 8 mg l^{-1}). The second factor was performed in the two cultivars of Alpine and Sarian F1 and the third one was done by the use of hydrogen peroxide in six concentrations with percentages of 0, 10, 30, 50, 70, and 100. The seeds were kept in fridge in ± 4°C temperature for a month. They were washed with washing liquid (common dishwashing liquid) for 4 minutes for disinfection of the seeds. After rinsing, they were shaken three times in a falcon with hypochlorite sodium 1% and five drops of Tween 20 (five minutes in each time). In this research, the two commercial materials, sulfuric acid from Razi petro chemistry and Hydrogen Peroxide from Degussa Company and GA_3, on eliminating dormancy in strawberry seeds were investigated. After treating the seeds, in the, ½ MS (Merk company) was performed including 15 gr l^{-1} of Sucrose and 5 gr l^{-1} of agar (pH=5.7). The produced plants were sub-cultured every four weeks. In order to obtain runners, when the seedlings of strawberry seed germination developed their roots, they were transported to the soil which contained two volumes of pit and one volume of perlite. They were irrigated once a day by hydroponics.

In vitro culture of terminal meristem

On each cultivar, experiment was independently established in the form of factorial experiments based on completely randomized design with 10 replications. The studied factors were MS and Boxus medium and growth hormones (BAP and BA cytokinins, IAA, IBA, and NAA auxins, and GA_3 hormone) [13,14]. The following factors were studied: medium in two forms: solid (M_1) and liquid (M_2), and different concentrations of plant growth regulators **(Table A1)**. Then, in order to know the best cultivar for slicing, interactions on different cultivars were studied. The number of normal and abnormal plantlets and their creation on the explants was calculated. When the materials were supplied, their redundant parts (leaves, roots, and flakes) were removed by the use of scalpel. In order to sterilize the surface of the plantlets at the end of runner, the following steps were taken: washing four minutes with the use of five drops of washing liquid, rinsing, 20 minutes, NaCLO 1% + Tween 20 and finally, rinsing three times with sterile distilled water. After removing the redundant parts of explants prepared for meristem culture, on each cultivar, plantlets were cultured in experimental tubes (with a 2 cm in diameter and 20 cm high, 20 ml in each tube) which contained solid medium and also in glass bottle (with a 6 cm in diameter and 8 cm high, containing 50 ml liquid medium in each bottle and with bridge paper). After the rooting of the plantlets of culture (greenhouse runners and inside-the-glass plantlets), some of them were transported to the soil. To this end, the steps in breaking dormancy of strawberry seeds were followed [12].

e-ISSN:2320-0189
p-ISSN:2347-2308

Table 1. Grouping interaction effects of cultivar and solid and liquid media for regeneration of abnormal and normal strawberry plantlets from terminal meristem, using Tukey's test with 0.05 probability level.

solid and liquid media	cultivar	abnormal plantlets	normal plantlets
solid	Camarosa	2.207143 [a]	1.621429 [a]
liquid		2.085714 [a]	1.592857 [a]
solid	Selva	1.828571 [a]	1.664286 [b]
liquid		1.928571 [a]	1.842857 [a]
solid	Pajaro	1.85 [a]	1.85 [a]
liquid		1.592857 [b]	1.485714 [b]
solid	Paros	1.914286 [a]	1.55 [a]
liquid		2.057143 [a]	1.785714 [a]
solid	Gaviota	1.771429 [a]	1.957143 [a]
liquid		1.864286 [a]	1.921429 [a]
solid	Alpine	2.028571 [a]	1.685714 [a]
liquid		2.035714 [a]	1.642857 [a]
solid	Sarian	1.8 [b]	1.935714 [a]
liquid		2.185714 [a]	1.792857 [a]
Means with similar letters are not significantly different at p<0.05.			

In vitro culture of terminal bud

The terminal bud in the bottom of each plantlet under one or two flakes with a height of about 3 mm was cut by scalpel for sterilizing **(Figure A1)**. It followed the same steps as described in *in vitro* culture of terminal meristem. Seven completely randomized design independently established with fourteen treatments and 10 replications. The traits that were studied included the number of normal and abnormal plantlets as well as callus formation in explants. The treatments included different tissue culture medium in fourteen level B1-B14 **(Table A1).** The plantlets were cultured in glass bottles containing 150 ml solid medium. The plantlets were evaluated after a month.

Optimization of in vitro culture of terminal buds

In order to optimization of *in vitro* culture of terminal buds, seven experiments were established separately on each cultivar based on completely randomized design with five replications. The type of medium in treatments (01-010) included 5 types of cytokinins in two levels and an auxin in one level **(Table A1)**. The studied trait was the number of adventitious buds in each explant. In this stage, the optimization of terminal bud culture in *in vitro* condition was investigated. The first stage of culture of terminal bud was conducted like the one described in *in vitro* culture of terminal bud. It was cultured in the best medium conditions according to result of statistical analyses. After a month, in order to do the first sub-culture, the explants were transferred to a new experiment medium. The conditions of the culture media were same as the one described in section strawberry seed germination. After 40 days, the cultures were evaluated and in order to producing complete plant, each adventitious bud was transferred to a complete, hormone-free MS medium. Finally, some of them were transferred to soil following the procedure explained in breaking dormancy of strawberry seeds.

In vitro regeneration of Fragaria × ananassa Duch. plantlets by leaf and petiole explants

The factorial experiment on each type of explant was established independently based on completely randomized design with five replications. Normal Plantlets produced in each explant were evaluated. The studied factors were as follows: Type of explants (leaf and petiole), and amount and type of medium in 24 level (LP1-LP24) including BAP in 2 and NAA, IBA, IAA in four level **(Table A1)**. This research had the following aim: direct *in vitro* regeneration of strawberry. Leaves and petioles of *Fragaria ananassa* were taken from *in vitro* plants which were the result of seed germination and meristem tissue culture and terminal buds. Samples and cultures were maintained according to the steps described breaking dormancy of strawberry seeds. The experiment on each cultivar was conducted independently. The Plantlets were selected After one month. for rooting, the plantlets were transferred to a hormone-free, basic MS medium.

Statistical Analysis

Normality tests for data were assessed using Minitab 16 (MINITAB Inc., PA, USA www.minitab.com) and SPSS 18, Minitab 16 and SAS 9.1 were used for conducting statistical analyses of the experiments. The P value of 5% was used as the criterion for comparing variances and means of treatments. The diagram related to the data was drawn using Microsoft Excel 2010.

RESULTS AND DISCUSSION

Breaking Dormancy of Strawberry Seeds

The analysis of variance showed that there was a significant difference between concentrations of sulfuric acid and GA_3 in the breaking seeds dormancy experiment ($p<0.05$), while there was no significant difference among the cultivar of strawberries used in this study in terms of the application of sulfuric acid and GA_3. Similar results were observed between Alpine and Sarian

e-ISSN:2320-0189
p-ISSN:2347-2308

in the experiment of GA_3 and hydrogen peroxide as treatments. The results showed that the germination percentage significantly depends on the applied treatments ($p<0.05$). The controlled seeds of Alpine and Sarian F1 in the ½ MS medium did not show any change up to the end of the experiment period. The germination percentage differed in the strawberry seed from zero to one hundred percent depending on the applied treatments. The interaction between cultivar, GA_3 with 2 mg l^{-1} concentration and sulfuric acid 36 N, and the interaction between cultivar, GA_3 with 2 mg l^{-1} and hydrogen peroxide led to 100% **seed germination** in both the first and second experiment **(Tables A2(a) and A2(b))**. No significant difference between the cultivars and chemical treatments indicates that germination of the seeds directly depends on the amount of sulfuric acid, GA_3, and hydrogen peroxide, and there is no significant difference between different cultivars of strawberry **(Figure 1)**. According to the results in the equal conditions for the conducted studies, GA_3 induced physiological activities of seeds, and sulfuric acid and hydrogen peroxide were useful for chemical scratching which leads to weakening of the seed coat and increasing seed coats permeability **(Figure A2)**. Therefore, in order to have effective germination, GA_3, sulfuric acid and hydrogen peroxide can be used to soften the seed coat of strawberry and leads to water and oxygen penetration and breaking seeds dormancy. Due to the fact that there was no significant difference between different cultivars of strawberry, the best treatment can be used for the seed germination of other strawberry cultivars.

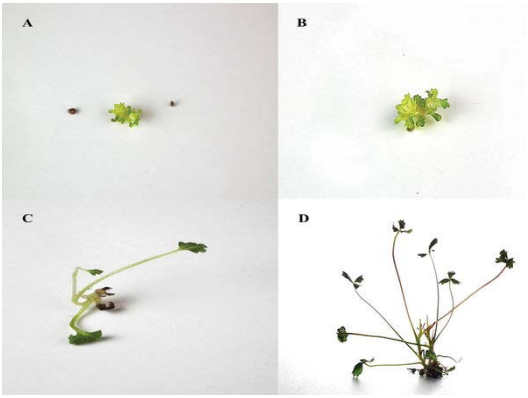

Figure 1. Strawberry seedling which is the result of seed germination by hydrogen peroxide and sulfuric acid (A-D).

Strawberry Proliferation from Terminal Meristem Culture

Analysis of variance for regenerated abnormal and normal strawberry plantlets indicated a significant three-way interaction ($p<0.05$). According to these results, the production of normal and abnormal plantlets in each cultivar depends on different concentrations of plant growth regulator treatments. However, Selva and Pajaro had the highest regeneration of normal plantlets in liquid and solid medium, respectively. But generally, the type of medium had no considerable effect on the efficiency of the production of normal and abnormal plantlets **(Table 1 and Figure A3)**. The best medium for the high regeneration of normal plantlets and low regeneration of abnormal plantlets was varied in different cultivars ($p<0.05$) **(Figures A3 and A4)**. Based on the results of **(Table A3)**, each one of the seven studied cultivars had the maximum and minimum amount of production of normal and abnormal plantlets in a particular hormone treatment **(Figure A5)**.

Now-a-days the culture of meristem is an important technique for the production of disease-free plants. In this part of the experiments, three important factors in meristem culture (cultivar of plant, type of medium and concentration of plant growth regulators) were investigated in order to achieve a replicable protocol for Strawberry tissue culture. Generally, according to the findings of this research; it is possible to produce normal plants from meristem culture of strawberry. This is important due to increasing demand for the culture of this plant.

In vitro culture of terminal bud of strawberry

Based on the results of ANOVA, there was significant difference between the cultivars in the production of the maximum amount of normal plantlets or the minimum amount of abnormal plantlets ($p<0.05$) **(Table A4)**. The maximum production of normal plantlets of seven studied cultivars occurred in different levels of various medium compounds; this was also happened for the highest amount of callus production. The highest amounts of callus production have been highlighted in **Table A4**. In this Table, levels of the medium are the only effective compounds for stimulating callus production. According to the **Table A4**. In most of the levels of hormone compounds, the amount of production is equal to zero. The results showed that strawberry proliferation was possible by the use of the terminal bud culture. In a short period, a considerable amount of normal plantlets was produced, faster than the culture of terminal meristem **(Figure 2)**.

e-ISSN:2320-0189
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Figure 2. *In vitro* culture of terminal bud of Fragaria × ananassa. Regeneration of the terminal bud culture of strawberry (A-E). Callus formation from the terminal bud culture (F-G). Initial regeneration of strawberry (H). Strawberry plantlet obtained from terminal bud culture (I)Transferring regenerated plants to the soil (J).

Optimizing in vitro culture of terminal buds of strawberry

The results obtained from ANOVA demonstrated the significant effect of the medium and the interaction of the medium and cultivars ($p<0.05$, data not shown). According to the obtained results, there was no significant difference among the cultivars in the highest efficiency of adventitious bud production **(Table 2)**. The highest adventitious bud production was obtained in cultivar Selva and medium 04 **(Table 2 and Figure 3)**.

Table 2. Grouping interaction effects of cultivar and medium type for regeneration of strawberry adventitious buds, using Tukey's test with 0.05 probability level.

Cultivar	Medium type	Normal plantlets	Cultivar	Medium type	Normal plantlets
Camarosa	01	4.45 ^{b}	Gaviota	01	3.3 ^{c}
	02	6.85 ^{b}		**02**	**37.45** ^{a}
	03	7.65 ^{b}		03	8.3 ^{b}
	04	8 ^{b}		04	6.75 ^{bc}
	05	**40.4** ^{a}		05	4.35 ^{bc}
	06	4.4 ^{b}		06	6.7 ^{bc}
	07	6.25 ^{b}		07	4.3 ^{bc}
	08	6.85 ^{b}		08	5.6 ^{bc}
	09	6.4 ^{b}		09	4.6 ^{bc}
	010	5.9 ^{b}		010	5.7 ^{bc}
Selva	01	6.05 ^{b}	Alpine	01	4.35 ^{bc}
	02	8.5 ^{b}		02	6.3 ^{bc}
	03	8.3 ^{b}		**03**	**40.95** ^{a}
	04	**41.35** ^{a}		04	6.45 ^{bc}
	05	4.1 ^{b}		05	3.75 ^{c}
	06	5.6 ^{b}		06	6.55 ^{bc}
	07	5.1 ^{b}		07	5.95 ^{bc}
	08	4.8 ^{b}		08	9.45 ^{b}
	09	5.35 ^{b}		09	7.1 ^{bc}
	010	5 ^{b}		010	4.05 ^{c}
Pajaro	01	5 ^{bc}	Sarian	**01**	**36.2** ^{a}
	02	8.45 ^{b}		02	9.35 ^{b}
	03	5.45 ^{bc}		03	7.5 ^{bc}
	04	4.55 ^{c}		04	5.8 ^{bcd}
	05	4.15 ^{c}		05	3.55 ^{d}
	06	4.2 ^{c}		06	5.45 ^{cd}
	07	5.95 ^{bc}		07	5.75 ^{bcd}
	08	**38.8** ^{a}		08	4.6 ^{cd}
	09	4.95 ^{bc}		09	6 ^{bcd}
	010	6.75 ^{bc}		010	5.5 ^{cd}

e-ISSN:2320-0189
p-ISSN:2347-2308

Paros	01	6.7 [b]	--	--	--
	02	5.25 [b]	--	--	--
	03	4.05 [b]	--	--	--
	04	5.8 [b]	--	--	--
	05	6.35 [b]	--	--	--
	06	4.9 [b]	--	--	--
	07	8.25 [b]	--	--	--
	08	3.75 [b]	--	--	--
	09	**37.15 [a]**	--	--	--
	010	4.55 [b]	--	--	--
Means with similar letters are not significantly different at p<0.05.					

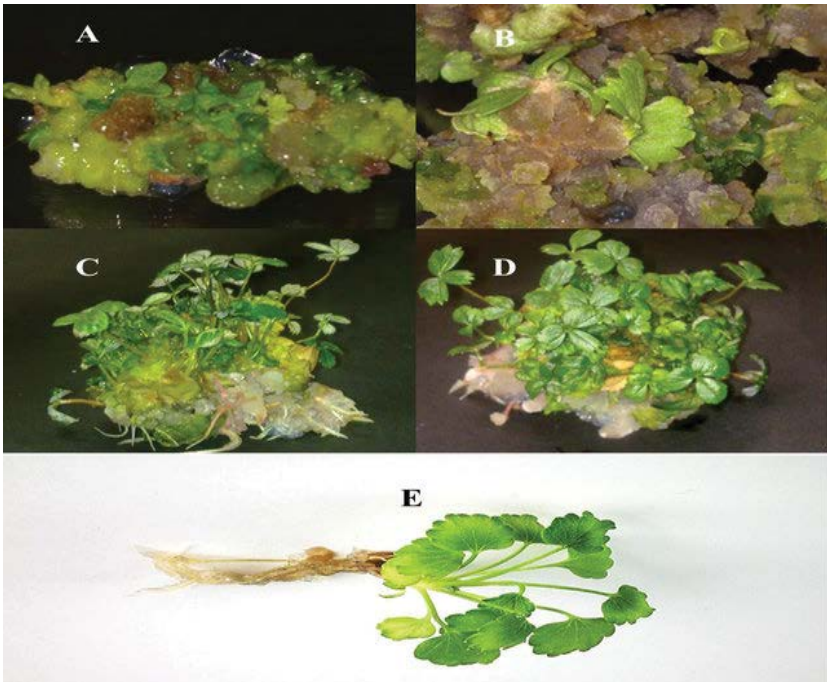

Figure 3. Optimization of *in vitro* culture of terminal buds of strawberry. Adventitious buds which is the result of optimization of culture of terminal buds of strawberry (A and B). The proliferation of terminal bud culture and the mass production of adventitious buds (C and D). Complete plantlet of strawberry after the transfer of an adventitious bud to hormone-free MS medium (E).

In vitro regeneration of strawberry (Fragaria × ananassa Duch.) plantlets by leaf and petiole explants

The analysis of variance results showed the best medium for the production of the maximum amount of normal plantlet is different for each cultivar (data not shown). Based on the results, the highest amount of normal plantlet production of the seven studied cultivars in different medium with different concentrations of plant growth regulators was different for each cultivar **(Table A5)**. The slicing of interaction effect of medium and explants showed that in the production of normal plantlets, except for LP11, LP21, and LP24 that had the maximum production in leaf petiole and leaf, respectively; all other media did not show any significant difference in different explant types. According to the analysis of variance, there was no significant interaction between types of explants. In general, the type of explants did not have any considerable effect on the efficiency of the production of normal plantlets **(Table A6)**. The findings of this study show more than 60% *in vitro* regeneration of the complete plant for leaf and petiole explants in some cultivars **(Table 3 and Figure 4)**. In gene transformation, the high percentage of regeneration plays an important role. Also, in the transformation of the target gene to a single-cell, the type of explants is essential to prevent the production of chimer plant. As mentioned above, this is an important finding for proliferation and gene transformation in strawberry [15].

Table 3. The introduction of the best type of medium for direct regeneration in strawberry's leaf and petiole explant cultivars.

Cultivar	Average percentage of direct regeneration in leaf explants	Average percentage of direct regeneration in direct regeneration in petiole explants	Best type of medium
Camarosa	74	68	LP24
Selva	74	68	LP18
Pajaro	76	70	LP13
Paros	84	80	LP5
Gaviota	94	84	LP6
Alpine	88	82	LP2
Sarian	92	84	LP11

e-ISSN:2320-0189
p-ISSN:2347-2308

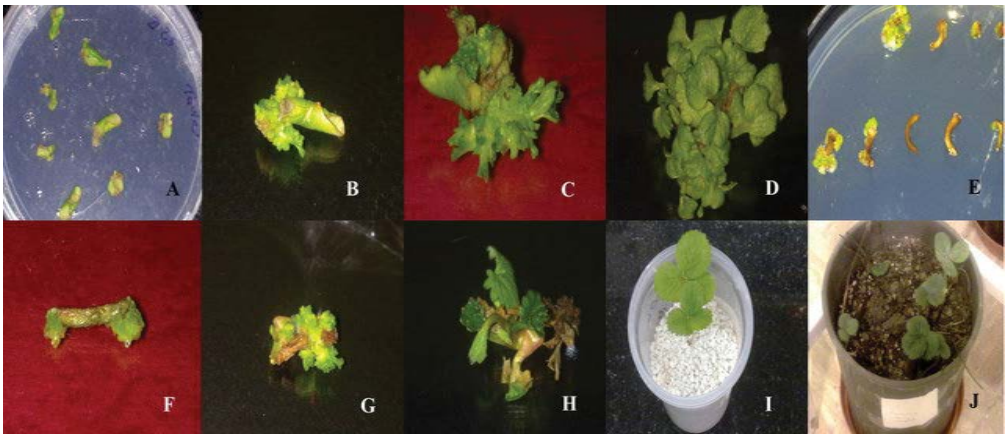

Figure 4. Regeneration of Strawberry *in vitro* plantlets by leaf and petiole tissue culture. Regeneration of complete strawberry plant as a result of in vitro culture of leaf explants (A-D). Regeneration of complete strawberry plant as a result of in vitro culture of petiole explants (E-H). The transfer of the strawberry plantlets which have been regenerated from leaf and petiole to perlite (I). Transferring of strawberry plants from growth chamber to vase (J).

CONCLUSION

According to our findings to achieve effective germination, GA_3, sulfuric acid and hydrogen peroxide can be used to soften the coat seed of strawberry seeds to absorb water and oxygen into them and breaks their dormancy. Now-a-days, more attention has been paid to callus-free (direct) pathways to speed up tissue culture and transformation process [16,17]. Generally, using cell, tissue, and organ culture of strawberry (*Fragaria* × *ananassa* Duch.) on a medium under the *in vitro* condition and without micro-organisms can lead to the production of pollution-free plants. The obtained results indicate that by the use of the terminal bud culture of strawberry, their proliferation would be possible. After only three plantlets with each having 30 days interval from the other one, there was a considerable amount of normal plantlet which grew faster than the culture of terminal bud.

Also, the results showed that the terminal bud culture was a very good way for strawberry proliferation. Using this method can save a lot of time and expenses. Leaves and petioles can be created for incubation and gene transformation [18-21].

Moreover, this research was intended to study the strawberry tissue culture under limiting growth condition in terms of hormones, compounds, and minerals. In the issue of gene transformation, apart from the importance of producing virus-free plants, the transformation of the target gene to single-cell and direct regeneration are vital issues [6]. Leaf and petiole can be separately introduced as suitable explants for regeneration by the use of leaf discs and direct regeneration over the selection medium. It is hoped that the findings of this study have a significant effect on achieving the purposes related to strawberry.

The type of explants is essential in the transformation of the target gene to a single-cell because it prevents the production of chimer plant. Findings of this research show that the high percentage of regeneration through *in vitro* culture of explants, the leaf and petiole which are the result of seed culture, meristem, and terminal bud can enhance the probability of successful gene transformation [15]. In comparison with other references and reports which have been made so far, the findings of this study show (60%) *in vitro* regeneration of the complete plant for the leaf and petiole explants of some used cultivars **(Figures A6 and A7)**.

ACKNOWLEDGEMENT

This study was supported by a grant from Shiraz University and Tarbiat Modares University of Iran.

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e-ISSN:2320-0189
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