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ORIGINAL ARTICLE

Regeneration of Iris through Somatic Embryogenesis of Various Explants

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ABSTRACT
Since there are many limitations in regeneration methods, applications of the new cellular and molecular technique are necessary to create economic characteristic in Iris flower. The application of molecular techniques itself requires the development of tissue culture system. The Somatic embryogenesis is one of the most appropriate skills of tissue culture which is superior to the other techniques. The aim of the current research includes regeneration of Iris through somatic embryogenesis of various explants. In addition, the laboratory experiment method is used. According to achieved results, the regeneration of Iris via various explants can be offered as an alternative method to traditional way of proliferation via bulb in farm, in which there is lots of risk for pollution.
Key words: *somatic embryogenesis, organogenesis, herbal growth regulators, auxin*

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INTRODUCTION

Iris is a plant from the family of Iridaceae. This plant family owns gramineous , rhizome or corm in their structure. Irises' leafs are usually ensiform, interwoven and nearly folded from the middle. It is inflorescence or single, bisexual flowers, arranged and sometime disarranged. Each flower contains three stamens and three integrated pinnules with three cell ovary. The Styles are typically divided, with lots of ovules, which are established. The fruit of these plants are capsule [1,2].

The irises are plants with years of ageing which include around 300 species and dispersed at north hemisphere. It consists of six Perianths that three inner components (petals) are in stand form and called oriflamme and the other three lower ones (leaflets) called lappet. The stamens are stick to the bottom of three female sex organ called petiole crests. The female style is also divided into three colorful flats, which each sprout is bended over one stamen; therefore, the stamen and female organ are close to each other. Regarding the hypogeal organs, there are two types of Irises, rhizome Irises and bulb Iris which are cultivated to be used as cut flower; typically the rhizome Irises are used in gardens [3].

Recently, there is a rush to apply the micro- propagation throughout the various gardening plants. There are modern methods for the producers of decorative plants and medical herbs which can meet the demands of global markets in next century. The tissue cultivation is an alternative method for commercial proliferation and applied to micro-propagation of various specious of medical and decorative herb [4].

The herbal tissue culture is a general term that is used as an instance for protoplast, cellular, tissue and herbal organ culture in the sterile condition of the glass. In other sense, the herbal tissue culture is a technique to facilitate the production and perfect proliferation of various tissues of herb. The significance of this method is illustration of herbal cells abilities (Tot-i-potency), in a better sense; all the living cells of the herb have the capacity to form a perfect plant [5].

Nowadays, many of the plants in worldwide scale are produced through the tissue culture and micro-propagation becomes an obligatory in modern agriculture. Nearly in 70 % of commercial experiments, the main aim is quick proliferation of plants. According to the sale statistics, the production of tissue culture is estimated for over one billion per year. Regarding the serious problems in Iranian flower industry, applying the various techniques of tissue culture has the positive effect in achievement of proper scale throughout the global markets. Therefore, the aim of the research is regeneration of irises through somatic embryogenesis of various explants [6]. In this respect, the proliferation through leaf and the other explants are studied in the current research, to evaluate the possibility to offer the intra-glass condition as an alternative method instead of bulb method in the farm.

Reviewing the Iris and its types:

The background of such beautiful flower is tied with the ancient history, since the ancient Greece planted Irises beside the tomb of their dead and drew its figures on the surface of recently passed on' tombstone. The Greek emperors use to decorate their crown with Irises flowers and granted the irises as a present to each other. In the Persian poem, there is lots of quotation about Irises and this is referred to its dignity among the Iranian. Every spring, the wild Irises cover the hillsides of the mountains in Iran and create a spectacular landscape [7].

Iris is used to be respected and remarked as a decorative and medical herb since the early times. Currently, the iris culture becomes an industry in world scale. So, the high commercial value and immense culture of this herb are completely tangible. After the Tulip, Lily and Gladiolus, Iris is the fourth bulbar flower which is exported annually. Reasonable price and low cost of culture makes it to gains a more proper position in Iranian flower market; if it is offered in seasons other than winter.

Regarding the Iris types, which owns rhizome, the most important ones are divided into 8 classes: 1. germanica 2. florentina 3. Variegate 4. Pumila 5. susiana 6. Kaempferi 7.Siberica 8.Graminea. The iris usually is cultivated in neutral or alkaline soil and hermetic context. The best soil for such plant is divided into two parts of sand, one part of garden soil and one part of peat. Iris needs to full sunlight and temperature between 12 to 23 c° and medium irrigation.

The Iris germanica L belongs to Iridaceae family, which covers more than 300 species. This herb is one of the prominent bulbar and decorative plants in moderate weather regions, and its species and types are raised properly all over the world. The Irises are the incredible garden plants [7].

Figure-1 the Iris germanica L has 120 cm height and 30 cm diameter. Its root can transpire in underground up to 10 cm. The *Iris germanica* is a rhizome herb blossomed in May.

Regeneration of Iris:

Overall, the propagation of this herb is carried out annually through non-sexual method by rhizome division at summer end. Therefore, the breeders of herbs need years to produce enough essential materials to experiment their fertilization ability, disease resistance and premature capability. Therefore, many years are needed to obtain a suitable mass for commercial purposes. An effective and fast reproduction system is required due to the slow rate of reproduction in natural systems and quick infection of virus-free parent materials [8]. One of the problems of asexual reproduction is to maintain the virus-free product. Today's, tissue culture techniques are used to produce commercial plants which are free of pathogens and to preserve rare and endangered plants. Reproduction of plants under laboratory conditions is a useful method to produce high quality herbal medicines [9]. Sterilized tissue culture of lily was fully described. Studies focused on reproduction rate, destruction of virus, embryo culture and

physiology of flowering initiation. In the Iris germanica (rhizomatous lily), misplaced foliage are developed from flower stem and new axillary buds which are alongside the foliage [8-10].
When embryo is created from non-sexual tissues and cells (which are haploid, diploid etc.), it will be called somatic embryos unlike the sexual embryo production that is created due to the insemination of the germ cell. Different names such as embryoid bodies, misplaced embryos, embroid have been given to somatic embryos in different scientific references and the process of its production have been called creation of misplaced fetus and creation of somatic embryo. Regeneration via somatic embryogenesis has many advantages including the possibility of single celled origin of the seedling, reducing the undesirable Shimmer phenomenon and producing many plants [11]. Plant growth regulators play significant role in callus formation and their effect on the regeneration of plants was investigated in the cultivation of the corn callus [12]. Generally, callus Induction and reproduction environment needs high levels of auxin (such as 2, 4 D). Usually, concentration range of 4.5-13.6 μM is suitable for formation of Embryogenic callus in the tissue culture of cereals. Based on the reports, 2 4 D auxin is a determining factor to induce callus in immature corn embryos [13].
Embryogenic calluses are usually transferred to hormone-free environments to become a complete plant [13]. One reason is that somatic embryos acquire the ability to become a complete plant. Although their fate is predetermined, in some studies auxin is known as a driving factor for growth and converting into a whole plant [14].
In general, regeneration and production of plants by tissue culture can be performed by using two methods:

1. Somatic embryogenesis: the first principle is polarization of plant cells in a way that all vacuoles are in one pole and all mitochondria are at the front pole. Branches are developed from the upper pole that is vacuole-gathering place and roots are developed from the lower pole. Embryogenesis can be direct or indirect. In the direct kind, embryogenesis is done without callus formation while in the indirect kind embryogenesis happens with callus formation.

2. Organogenesis: In this method, organogenesis develops in plant without embryogenesis. It should be noted that if an organ such as root or branch is created in the tissue culture, it will be misplaced since it did not developed from meristem.

MATERIAL AND METHODS

In the current study, some cultivates of the stem of *Iris germanica* L. containing the terminal bud and a few small leaves are provided, and cultivated in MS mediums. Some parts of stems and leaves of the reproduced plant are selected and transferred to mediums containing different growth regulators for regeneration. Among regenerated plants, several lines will be evaluated in terms of morphological and genetic diversity. Plants can be improved genetically through somatic diversities that are known as a logical process to induce genetic changes in order to create divergence and branching in plant species. Morphological and biochemical markers can be used to show genetic diversity in plants. However, they cannot be widely used, since they are very sensitive to environmental factors and have limited numbers of locus on plant genome. Hence, DNA markers with high capacity and unlimited numbers of locus can be used to investigate genetic diversity in plants. One of the popular markers is PCR based RAPD. This technique can be very useful in the recognition of somaclonal diversity in regenerated plants.

Test

First, some plants of *Iris germanica* L. and bulbs were collected and some bulbs were cultivated in pot in the laboratory in order to produce flowers and some others are cultivated directly in vitro on glasses. Onions and plant pieces first were washed by water and then were put in a fungicide such as Captain Benomyl 1 % for an hour. Next, they were disinfected under laminar with different disinfectants as follows:

- 70% alcohol
- Sodium hypochlorite 20% for 5, 10 and 15 minutes
- Leaching with distilled water

If above mentioned treatments can't control the pollution adequately, Mercuric chloride 1% will be used for 5, 10 or 15 minutes instead.
Then plant pieces were divided into smaller parts and were put in MS medium with different hormonal treatments.
In the somatic embryogenesis, tiny samples are assessed based on morphological characteristics, callus production percent and volume of produced callus based on numbers, callus color and callus phenolization rate. In the final regeneration, this is done based on the number of leaves, the length and

number of the shoot, underground organs formation time, the number of rhizomes and the produced bulbs number.
To induce callus from tiny samples treatments include:
(0.5-1.2 mg/l) 2, 4-D + (0.5 mg/l) kinetin
(0.5, 1, 2 mg/l) 2, 4- D + (0.5, 1 mg/l) BA
MS medium+ ((1mg/l) 2, 4- D+ (0.9 mg/l) NAA+ (0.1 mg/l) kinetin)
After induction of embryogenesis calluses, the aim is to regenerate branch from callus and hormonal treatments are:
(0.2, 0.5, 1 mg/l) BA+ (0.5, 1, 2 mg/l) NAA
In order to induce callogenesis, we must keep the plant tissues in darkness. After the formation of callus from plant tissue, our objective will be generation of foliage, and the callus will stay on cultivation environment with different density of BA along with NAA.

Evaluated Characteristic

Callus: callus volume- derived amount of callus in explants
Branch: number of generated branches from callus- branch length- number of tissues
This study is carried out, in form of factorial test with 5 variants and a random design; in every stage, depending on progress of work, we use different treatments, that we have described it in research method. In every variant, there is 3 explants. In this research, after reviewing earlier studies, we consider several hormone treatments and stem nodes explants, onion scales, and leaf pieces of our test. After placing our explants on cultivation environment including hormone treatments, petri dishes are also placed in incubators of tissue cultivation laboratory of laboratory complex of Islamic Azad University of Razi, Science and research branch of Tehran, and normal temperature (25+-1) light (16 hours of light and 8 hours of darkness) are taken into account .
Callus induction percentage, thickness of callus and its weight are measured two months after placing the explants in cultivation environment containing auxins. Gathered data are examined in factorial test with random-based design. We have designed charts in Excel, analyzed data using SPSS, and compared the means, using Duncan test.

RESULTS AND DISCUSSIONS

In present study, we have examined the possibility of *Iris Germanica* L cultivation, using tissue explant, stem, rizome. Due to some limits in *in vitro* regeneration method, using the new cellular and molecular techniques in order to generate economical specifications in lily plant is necessary.
Figure 1-5- d, shows different stages of germination of lily.

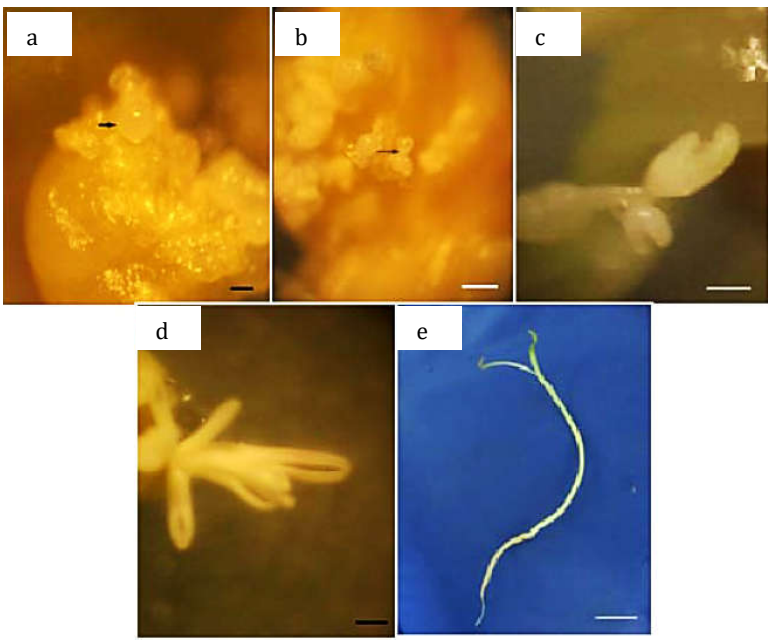

Figure-2 different growth stages of lily a) spherical stage b) heart-shaped stage with maintaining structure c) torpedo shape stage d), e) somatic embryogenesis

The result of callus induction and shoot culture show that after some weeks of first cultivation, in some explants and treatments, callus induction is observed. In addition, among all hormone treatments, we only observed callus induction and shoot culture, in hormone treatments A, B, C, D and E. Hormone treatments A, B, C, D and E, which are not capable of callus induction and shoot culture are a mixture of NAA auxin and BA cytokinin.

Based on results, we have observed callus formation from stem nodes in 57.4 percent of cases. Formation of callus in hormone treatment (0.5) BA+ (2.5) NAA has been in its maximum amount and in hormone treatment (0.5) BA+ (0.9) NAA in its minimum amount. Heydari *et al.,* [10] studied in vitro regeneration of *(Securigerasecuridaca)* through somatic embryogenesis. Different hormone treatments NAA and TDZ, in all explants, lead to callus induction and embryogenesis.

The examination of hormone treatment on the average size of generated callus from stem nodes showed that the maximum average size of callus is observed in hormone treatment (3)BA+(2.5)NAA and minimum average size of callus in hormone treatment (0.5)BA+(0.9)NAA . With the increase of BA hormone density, the amount of generated callus has not a significant increase. But in this measure, the average of callus size shows a significant increase. The reason could be the high capacity of BA hormone in generation of callus with maximum size. In addition, due to unnecessary increase of hormone pressure on the explant of stem node, callogenesis decreases. With the increase of BA, hormone NAA puts positive effect on callus genesis with maximum average size, in density (2/5).

Callogenesis from explants of onion scales showed that the percentage of callogenesis is 53. The examination of hormone treatment on the average size of callus genesis from onion scales showed that the maximum average of callus genesis is observed in hormone treatment (0.5)BA+(2.5)NAA and minimum average of callogenesisis observed in hormone treatment (0.5)BA+(0.9)NAA . The examination of hormone treatment on the average size of generated callus from the explant showed that the maximum average of callogenesis is observed in hormone treatment (3)BA+(2.5)NAA and minimum average size of callus in hormone treatment (0.5)BA+(0.9)NAA .

Callus genesis from leaf tissues showed that the percentage of callus genesis in this explant is 32/2. The examination of hormone treatment on callus genesis from the explant showed that the maximum average of callogenesis is observed in hormone treatment (0.5) BA+ (2.5) NAA and minimum average of callogenesis in hormone treatment (1) BA+ (1) NAA.

The examination of hormone treatment on the average size of generated callus from this explant showed that the maximum average of callogenesis is observed in hormone treatment (3)BA+(2.5)NAA and minimum average of callogenesisis observed in hormone treatment (0.5)BA+(0.9)NAA .

The result of statistical analysis of shoot culture from stem node explant showed that, the percentage of shoot formation in this explant is 43/2. The examination of hormone treatment on the average of shoot genesis in this explant showed that the highest average of generated branch number was observed in the hormone treatment of (3) BA+ (0.9) NAA and the least amount in (0.5) BA+ (0.9) NAA. Investigation of the effect of hormone treatment on the average of the length of formed branch in this explant showed that the most average of the generated branch length was in the hormone treatment of(0.5) BA+ (2.5) NAA and the least amount observed was in (0.5) BA+ (0.9) NAA.

The study of the effect of the hormone treatment on the number of produced leafs in the explant showed that the highest average number of produced leafs was in the hormone treatment of (0.5) BA+ (2.5) NAA and the least amount was for the hormone treatment of (0.5) BA+ (0.9) NAA.

The results obtained from shoot organogenesis of onion scales showed the 43/2 percent of branch formation in this explant. Investigation of the effect of hormone treatment on the average number of generated branches in this explant showed that the highest average of the formed branch number was in the hormone treatment of(0.5) BA+ (2.5) NAA and the least amount observed was in (0.5) BA+ (0.9) NAA.

Shoot organogenesis results of an explant of leaf pieces showed 43/2 percent of generated branch. Investigation of the effect of hormone treatment on the average number of generated branches in this explant showed that the highest average number of the generated branch was in the hormone treatment of (0.5) BA+ (2.5) NAA and the least amount observed was in (0.5) BA+ (0.9) NAA. Investigation of the effect of hormone treatment on the average of the length of generated branch in this explant showed that the highest average of the generated branch length was in the hormone treatment of (3) BA+ (2.5) NAA and the least amount observed was in (0.5) BA+ (0.9) NAA. Study of the effect of the hormone treatment on the number of produced leafs in the explant showed that the highest average number of produced leafs was in the hormone treatment of (0.5) BA+ (2.5) NAA and the least amount was in the hormone treatment (0.5) BA+ (0.9) NAA.

One of important factors that should be considered for generation of Somatic embryos is type and density of used hormones in the medium. In this study it was affirmed that (0.5) BA+ (2.5) NAA hormones are

effective for Callus generation. Devendra *et al*. [5] showed that hormones growth regulators of BAP and NAA are effective for callus induction of *Eclipta alba* L. in 2011. The results showed that different explants have not the same ability of callus generation. Merkel *et al*. in 1995 attributed this issue to the internal hormone levels of the explants and suggested that the levels of the endogenous hormones are correlated with the callus generation ability of explants.

CONCLUSION

Results showed that maximum amount of callus generation (57/4 percent) was observed in stem node explants in the least amount of BA density (0.5) and the highest density of NAA (2/5). In the same densities of the explants of onion scales and leaf pieces, the maximum callus was observed. In the explant of branch node with the least amount of BA hormone (0/5) we obtained the maximum branch length. Callus generation from the onion scales showed the maximum callus generation but callus generation from the leaf pieces conclude the least amount of generation. Based on results of the study, combination of BA and NAA in all three explants (onion scales, leaf pieces, stem nodes) showed the capability of callus generation, embryogenesis, and regeneration of Iris. Having diverse climates and wide geographical area, Iran is a habitat and source of many wild, decorative plant species such as Iris and Gladiolus. In addition, it is one of unique countries that contain plant genetic reserves and diverse species, in the world. Culturing and producing Iris is a global industry and has a high commercial value in Iran.
Generally, we can say that regeneration of Iris using different explants can be used as an alternative method for traditional way of proliferation through bulb in a field, exposed to contamination.

SUGGESTION

According to the results of the study, we suggest the following:

- Use of regeneration system for the bulbous plants in the practical condition.
- Investigation of the effective factors of practical use of somatic embryogenesis of herbal plants.
- More study on the effects of hormone treatments on the regeneration of plants.

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