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Studies on *in vitro* propagation of pomegranate (*P. Granatum*)

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ABSTRACT

An attempt was made to provide an outline on overall protocol for the *in vitro* propagation of *P. Granatum*. Surface sterilization of the explants collected from the greenhouse were done after being washed with water, anti oxidizing agent, fungicides like bavistin for a respectable amount of time. Initiation of the sterilized explants was done using MS Medium with 2 mg/l of BAP. They were then incubated and left for a week under the mentioned conditions. Multiplication of explants is done by using MS medium with 2 mg/l of BAP. They were again incubated under the mentioned conditions. The explants are then selected for the next step on the basis of height, color, strength of the culture. Rooting is done in an MS medium containing 0.5 mg/l of IAA and 0.5 mg/l of IBA. Rooting should result in strong, diverse roots. Hardening is done in the greenhouse in two stages primary and secondary hardening. Primary hardening involves leaving the rooted cultures in sterile coco pit mixture for one month in 100% humidity and under diffused sunlight. Secondary hardening involves transferring the explants to soil and under maximum sunlight for two months. The plants are then dispatched to the respective customers or farmers.

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KEYWORDS

In vitro propagation;
Hardening;
Explants;
Punica granatum.

INTRODUCTION

Plant Tissue Culture, which can be more aptly termed as micropropagation, is a field of great diversity and a great potential. Plant tissue culture or micropropagation deals with the rapid multiplication of stock plant materials to produce a large number of offsprings using the various techniques of plant tissue culture. It plays a major role in fields such as botany, chemistry, hybrid development, food science etc^[1,2].

The Austrian Botanist Gottlieb Haberlandt (1854-1945), who pointed out that one could successfully cultivate artificial embryos from vegetative cells did the first

attempt at micropropagation^[3]. His views lead to various significant discoveries in biology and gave a foundation to the concept of Totipotency.

The principles of micropropagation revolve around the concept of totipotency. Totipotency is the ability of a cell, such as an egg, to give rise to unlike cells and thus to develop into or generate a new organism or part^[4-6]. Hence, with this concept and others as well, the studies on plant tissue culture have had major breakthroughs.

P.R.White carried out the first successful prolonged *In vitro* cultivation of plant tissues and organs-and their proliferation and differentiation in 1934 on tomato roots.

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Also in the same period, another major breakthrough was the production of tobacco and carrot calluses by R.J.Gautheret and P.Nobecourt in the year 1939. Growth of shoot tips and meristems by E Ball in 1946^[7].

Soon an array of various techniques and procedures became available for the *In vitro* cultivation of plants^[8-12].

MATERIALS AND METHODS

Protocol for surface sterilization of pomegranate shoot explants

The following are the steps involved in the surface sterilization of pomegranate explants.

- Explants are carefully selected from fully-grown plants that are present in the green house. This involves careful selection of newly formed shoot tips. These are carefully cut and stored in a bottle, care should be taken no to the damage the axillary buds that are formed, as these will help in development of new shoots.
- Then wash the explants with tap water. This is done in order to remove dust and other surface contaminants that can damage the plant while growing *in vitro*.
- The explants are then washed for a defined period with 1% Bavistin solution, a fungicide, to remove microbial contaminants. The solution is prepared by adding 0.5g of bavistin in a small amount of water, just enough to fully dissolve it. The explants are washed thoroughly by the continuous shaking of the bottle. The bottle is sealed to prevent the solution from leaking.
- The bavistin solution is discarded and the bottle containing the explants is rinsed thoroughly with sterile water twice to remove any trace of the fungicide solution.
- The next step is to wash the explants with 0.15% mercuric chloride solution. The solution again acts as an anti microbial agent and removes microbial contaminants from the explants. The solution is prepared by dissolving 0.15g of mercuric chloride in sterile water. The explants are then dipped into the solution and the bottle is sealed. The bottle is thoroughly shaken for a defined period.
- The mercuric chloride solution is discarded and the bottle containing the explants is washed thoroughly

with sterile water twice to remove any trace of the chemical.

- The explants are then washed with antioxidant solution. The solution is prepared by dissolving 0.1g of chemical in sterile water. The plants are then added to the solution and rinsed with it for one minute^[13-16].

Initiation of pomegranate explants using apical shoot tips

Apical shoot tips were obtained from the nursery, sterilized by Treatment D, inoculated on plain MS media and a mixture of different concentrations of growth hormones with MS media, and incubated under standard conditions^[17-19].

- Temperature : 23-25°C
- Humidity : 50-60%
- Light Intensity: 2000-2500 lux
- Photoperiod : 16:8

The following are the combinations of media and growth hormones concentrations.

Media A: MS Media with minute amount of growth hormones; Media B: MS+0.5 mg/l BAP; Media C: MS+1.0 mg/l BAP; Media D: MS+1.5 mg/l BAP; Media E: MS+2.0 mg/l BAP

In vitro multiplication of *P.Granatum* explants

In Vitro derived shoots of pomegranate obtained from initiation cultures were inoculated on MS media of different strengths and different concentrations of growth hormones. The cultures were incubated under the following conditions.

- Temperature: 23-25 °C.
- Humidity: 50-60%.
- Light Intensity: 200-2500 lux.
- Photoperiod: 16:8

The following are the combinations of media and growth hormones concentrations^[20-22].

MS Media with minute amount of growth hormones; MS+0.2 mg/l BAP; MS+0.5 mg/l BAP; MS+1.0 mg/l BAP; MS+2.0 mg/l BAP

In vitro rooting of pomegranate

In vitro derived shoots of pomegranate obtained from initiation cultures were inoculated on MS media of different strengths and different concentrations of growth hormones. The cultures were incubated under the following conditions^[23-26].

- Temperature: 23-25 °C.

- Humidity: 50-60%.
- Light Intensity: 200-2500 lux.
- Photoperiod: 16:8

The following are the combinations of media and growth hormones concentrations.

Media A: MS Media with no added growth hormones; **Media B:** MS+0.01 mg/l IBA; **Media C:** MS+0.01 mg/l IAA; **Media D:** MS+0.5 mg/l IBA; **Media E:** MS+0.5 mg/l IAA; **Media F:** MS+1.0 mg/l IBA; **Media G:** MS+0.5 mg/l IAA + 0.5 mg/l IBA.

Acclimatization and field establishment

Rooted plantlets grown invitro were washed thoroughly to remove the adhering gel, transplanted to cavity trays. The plants are initially washed in bavistin which acts as an antifungal agent. The cavity trays containing 98 net pots are filled to the brim and the rooted cultures are firmly placed into the net pots. They are placed initially in the lower bench in a condition of 100% humidity for about 10-15 days and then transferred to the upper bench where the conditions are 70%-80% humidity and an optimum temperature of about 28-30°C^[27-30].

After about a month the plants will be transferred to pots containing soil and a mixture of manure, compost, red soil, etc that will help the plants is acclimatized to the natural environment^[31-33].

RESULTS

The data indicates that Treatment E was found to be the best treatment for achieving maximum surface sterilization for pomegranate shoot tips. This treatment included the following steps:

1. Wash the explants for ten minutes under tap water.
2. Wash with bavistin (0.1% w/v) solution for thirty minutes.
3. Rinse with sterile water twice.
4. Wash with Mercuric Chloride (0.15% w/v) solution for seven minutes.
5. Rinse with sterile water twice.
6. Wash with antioxidant solution for one minute.

Initiation of pomegranate explants using apical shoot tips

The following are the steps to be carried out for the initiation of pomegranate culture.

Explants are obtained from the nursery and sterilized using treatment D.

In vitro multiplication of *P.Granatum* explants

Thus the data signifies that MS medium containing higher concentration of BAP are better suited for multiple shoot formation. Hence Media E was the preferred media for the multiplication of pomegranate cultures. Hence the following steps are to be carried out for the multiplication of pomegranate cultures.

- Obtain initiation cultures from the growth room and under sterile conditions remove all dead matter, gel, etc from the culture.
- Inoculate the freshly cut cultures onto a medium containing a combination of MS media and 2 mg/l of BAP growth hormone.
- The cultures were then incubated at 23°C at 2000-2500 lux light intensity and at 50 to 60% humidity for one week.

Different media trials for multiplication were carried out. Of these it was found that Media E which was a combination of MS media and 2mg/l of BAP was the most effective in multiplication of pomegranate cultures. A very high percentage of shoot multiplication was observed in the basal region of the plant and maximum percentage of shoot proliferation was seen in the apical region of the plant.

Shoot Multiplication and shoot proliferation was seen in Media D. The plants however were not tall, they were found to be short. Maximum shoot multiplication was observed in Media C.

Media B showed a good multirate but the plants formed were not very healthy. Media A showed bad response with maximum dead leaves and no shoot multiplication. No growth was seen in the middle region of the plant. At lower concentration of BAP (0.1 mg/l) the rate of shoot multiplication declined.

In vitro rooting of pomegranate

Thus the above data signifies that MS medium containing higher concentration of auxins are better suited for root formation. Hence Media E was the preferred media for the rooting of pomegranate cultures. Hence the following steps are to be carried out for the rooting of pomegranate cultures.

- Obtain initiation cultures from the growth room and

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under sterile conditions remove all dead matter, gel, etc from the culture.

- Inoculate the freshly cut cultures onto a medium containing a combination of MS media and 0.5 mg/l of IAA along with 0.5 mg/l of IBA growth hormones.
- The cultures were then incubated at 23°C at 2000-2500 lux light intensity and at 50 to 60% humidity for one week.

DISCUSSION

The study conducted has shown that it is possible to manipulate various growth factors of *Punica Granatum* species by alternating the growth hormone concentrations in the media.

Cytokinins such as BAP were used in different concentrations for initiation as well as multiplication of shoots in the cultures.

The maximum initiation for the pomegranate cultures was observed in MS media supplemented with 2mg/l of BAP. In the case of multiplication the medium used was a combination of MS medium supplemented with 2mg/l of BAP. This medium had a very good multiplication rate of 1:6, which means that there were 6 cultures produced for one culture inoculated. It was also observed that declining cytokinin concentration resulted in lesser multiplication rate.

In the case of rooting the medium selected was MS media supplemented with both IAA and IBA at 0.5 mg/l. These results highlighted the importance of auxins in the rooting process of the pomegranate culture. The roots produced by this medium were healthy, highly branched, thick and long. All these are essential for the plant to be transferred to the hardening stage. The declining concentration of auxins showed a drop in the quantity as well as quality of rooting among the cultures.

In Greenhouse after 4-5 weeks of transfer of plantlets to cocopits it was observed that Plants survived well in washed cocopit with 80% survival rate of plants and less mortality. Thus it was proved washed cocopit suits better for the survival of pomegranate in greenhouse.

Hence it is evident that plants propagated through tissue culture have many advantages over plants cultivated in the traditional method. The major advantage in

In Vitro propagation is the lesser amount of time taken to obtain results and the greater yield obtained from the plants. As discussed earlier if one plant gives rise to 6 more plants then definitely the outcome will be very high when cultivating with *In Vitro* techniques.

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