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Shoot Micropropagation and Microtuberization in Potato (*Solanum tuberosum* L.) By the Semi-Continuous Bioreactor

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Abstract: The aim of this investigation is introducing the use of continuous and semi-continuous bioreactors and their functions at shoot multiplication and microtuberization of potato. The study shows that the explants have several nodes and when they are suspended continuously under the liquid culture medium, the shoot micropropagations and microtuberization was inhibited. The surfaces of the explants were formed callus and subsequently, they were died. However, in the semicontinuous bioreactor, with the periodical pumping of the nutritional medium the explants aren't continuously suspended under the nutritional solution. The shoot micropropagation, the leaf growth and, the root formation are suitable. In the microtuber inductive medium, the numerous of the tubers are induced. They were sessile tubers. The dormancy of the tubers are long-term, spourting after 3-4 months in the room conditions.

Key words: *Solanum tuberosum* L., micropropagation, microtuberization, semi-continuous bioreactor

INTRODUCTION

Microtuberization of potato has been one of the successful methods of increasing this plant *in vitro* conditions (Yu *et al*., 2000). The main problem in the extent of the producing microtubers by the common method of Flask is the low yield of the tuber production (1-1.5 tuber per plant) and their small size that restrains the direct transplantation of microtubers to field (Fujino *et al*., 1995). Thus, the necessary condition for *in vitro* microtuberization of potato has been studied deeply and their propagation efficiency has been improved. Recently, large-scale production has been examined on some plants especially on potato, because these plants are economically suitable (Akita and Takayam, 1994a). To increase the quality and the number of microtubers per each plant, different methods such as, changing the components of the culture medium, increasing the amount of sucrose and adding the plant growth hormones (Khuri and Hoorby, 1995, 1996; Leclercy and Donnelly, 1995; Seabrook and Coleman, 1993), manipulation culture conditions such as, temperature, illumination and photoperiod (Avila *et al*., 1998) and using from liquid culture in flask on shaker or bioreactors (Banfalvi *et al*., 1997; Gopal *et al*., 1998) were used. The increased microtubers of potato through *In vitro* method was considered by their storage, easy transport and distribution. Liquid culture for mass and automatic multiplication and price decrease is necessary (Bhojwani and Razdan, 1992). Common methods for producing microtubers through using liquid culture on shaker will produce limited amount of microtubers. In this case, bioreactors are used in laboratory scale which can provide many microtubers.

The advantages of bioreactors are as following (Xu *et al*., 1998):

- A great number of plants can be produced.
- Plant cultivation, transferring, storage and harvesting will be more suitable, considering experience and time.
- The explants are always or periodical and timing in contact with culture medium that this factors, stimulates nutritious material absorption which resulted growth stimulation.
- Effective method for providing oxygen will be more likely which in turn stimulates growth and finally causes the accessibility of suitable biomass.
- The explants are always moving in bioreactors and this matter in turn eliminates the apical dominance which at least make vegetative buds produce many shoots.

Many researchers have explained many methods for shoot micropropagation (Harvey *et al*., 1992), mass propagation of tubers (Kefi *et al*., 1998) and the potential

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use for producing seed tubers. Akito and Takayam (1994b) by using 2000 mL fermontars and two-staged culture could produce approximately 223 micro tubers and through using the semicontinuoes 1000 mL bioreactors could produce 502 micro tubers, which total fresh weights were 888.8 g. Huslscher *et al.* (1996) were propagated shoots and induced microtubers to produce 1653 microtubers with total weight of 1420 g by the fermantors of 10000 mL. Jimenez *et al.* (2000) through using Semicontinuos bioreactors, emphasized the improved production of potato shoots and microtubers through temporary immersion system. These researchers were believed that temporary immersion is a valuable method for producing microtubers. This method can induce more tubers and produce bigger and heavier tubers (Takayama and Akita, 1994). Therefore, this method of culturing in bioreactors, especially semicontinuos bioreactors, is valuable for studying the physiology and creation of microtubers, because this method makes possible the mass production of inducing tissues of tubers and microtubers under laboratory condition.

This main aim of this study was increasing microtuberization, through using planned and designed bioreactors under laboratory scale.

MATERIALS AND METHODS

Culture medium: This study was conducted on 2002 at research laboratory of plant physiology of Islamic Azad University of Damghan, Iran. The culture medium were included MS Salts medium containing organic materials like Thiamin-HCl (0.4 mg L^{-1}), Myo-Inositol (100 mg L^{-1}), pyridoaxin-HCl (0.5 mg L^{-1}), Nicotinic Acid (0.5 mg L^{-1}), Glycin (2 mg L^{-1}) and sucrose was used. The meristems were cultured onto a semi-solid agar (4 g L^{-1}). Then, the nodal explants raised from meristems were fragmented to the single node explants and then, were culture onto a solid condition including agar 7 g L^{-1} and were incubated at 25°C under 16 h photoperiod.

The semi-continuous bioreactor structure: This bioreactor is composed of two connected glassy vessels containers (Fig. 1 and 2). The lower chamber with the capacity of 1000 mL stores the nutrient solution and the upper chamber with maximal capacity of 150 mL is growth room. A tube relates two chambers in away that provide a bilateral relation. The nutrient solution by using pressed sterile air is transferred from lower chamber to the upper growth chamber. The speed of this transferring is 750 mL min^{-1}. The time of returning is controllable. In this bioreactors, the nutrient solution is pumped cyclically to the growth chamber and return to the store chamber.

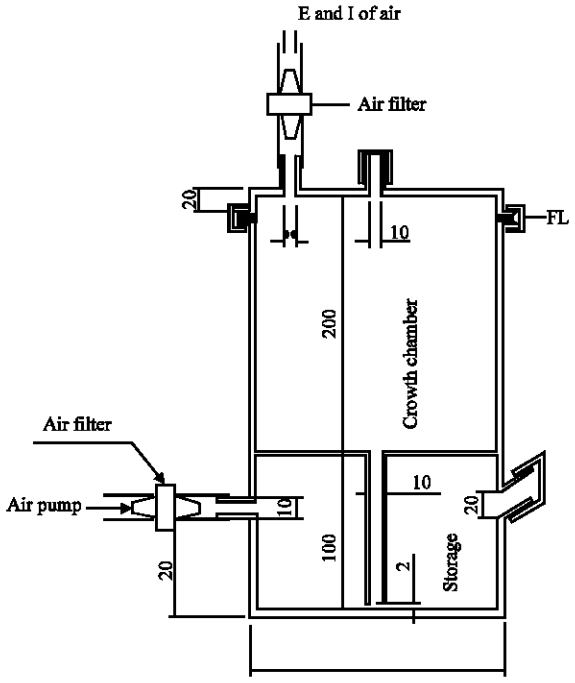

Fig. 1: Semicontinuous bioreactor

The designed electronic circuit for switching the pump on or off. Since the air pump under suitable condition should be on cyclically and for a certain period and then, should be switched off. It was necessary to design an electronic circuit for witching the pump on and off in suitable time schedule and computing measuring the right time for keeping the pump on. So, a circuit was designed which included a 24 h chronometer with controlling keys for 10 min, a 60 sec micro timer, a conductor and a very small fuse, with connecting this circuit to electricity. The 24 h timer will switch on the 60 sec timer. Then, electricity is transferred through micro timer to conductor and the conductor switches the pump on. After a while for example, 20 sec, the micro timer is switched off and the conductor cuts all the electricity power and switcher the pump off. This process goes on repeatedly.

Plant culture: The tubers of potato (*Solanum tubersum* var. Marfona) were planted in sterile soil the produced plants. After shoots growth, through sterling the nodal explants were deeped with 2.5% NaOCl solution and some drops of detergent for 15 min and followed by several times rinsing with distilled water. By stereo microscope under laminar airflow, the meristems were excised from these sections. The meristems were cultured under semi-solid MS container 0.1 mg L^{-1} NAA and 0.2.5^{1} GA_3 and sucrose 2% in temperature of 25°C and a photoperiod of

Fig. 2: The formation of callus and necrosis of the explants in continuous bioreactor

16 h (with the intensity of 3000 Lux) and 8 h darkness. After one month, the thin and weak explants were transferred in test tubes (length 15 cm and thickness of 3 cm) which included MS solid medium container sucrose, 0.5 mg L^{-1} GA_3. 0.4 mg L^{-1} NAA with the previous photoperiod was used (Kefi *et al*., 1998). After 3 or 4 weeks, plants with average length of 13 cm and 13-14 nodes were produced of these explants. Potato microtubers were induced by the two-step culture method. In step A, the 15 stocks plants with of 3-4 nodes were inoculated in the bioreactor for growth and multiplication of plantlets (MS includes sucrose 8%, 0.4 mg L^{-1} GA_3, 0.5 mg L^{-1} BAP). After three weeks, the medium was replaced with a new one to proceed to step B for microtuber induction (MS including 8% sucrose, 10 mg L^{-1} BAP) at the darkness.

RESULTS AND DISCUSSION

The growth of the axillary buds, shoot micropropagation and microtuberization are inhibited in the explants having the single-node or the several nodes that are suspend continuously under the liquid culture medium in the continuous bioreactor. In the surface of the explants is occurred calligenesis and them are swollen.

In the semi-continuous bioreactor, the shoot formations and the number of them are influenced by increasing the period of nutrient solution pumping from the store chamber to the growth chamber (the first stage). The percentage of the shoot formations was highly decreased by the increase period (Fig. 3). The best length growth of shoots (Fig. 4), the leaves surface growth and the shoots number are happened in the repeated periods of 20 min and the retention time of 40 sec of the nutrient solution in the growth chamber. The diameter growth of the shoots and the leaves was better than their on Shaker and many the white adventitious roots were formed from the nodal segments and with positive geotropism will grow downward.

In induction and growth stage of microtubers (the second stage), the culture medium of shoots was eliminated and the inductive medium was replaced for it and the bioreactor was transferred to darkness and the temperature of 25°C. During the first 4 or 5 days of the first week in inductive condition, many of the buds produced stolons during the darkness period. The color of the leaves is chlorosis. The growth of standing white shoots during the whole the inductive period (up to end of tenth week) continues. During sixth and tenth week in the inductive condition, the total fresh weight, the fresh weight and the dry weight of shoots are increased (Fig. 5).

The mainly part of the weight changes is the result of forming the white shoots with stolons and microtubers formation. The microtuber formation was occurred within the end of the first week to the end of second week after induction and until the end of tenth week, their number remains fixed (Fig. 6).

The investigation of the change of the fresh and dry weights of microtubers during the second, sixth and tenth week after the induction (Fig. 7), indicated the increasing weight of tubers. During the second week after induction, the mainly number of tubers weight almost 10 mg and a number of tubers weigh maximally 110 mg. During the sixth week, the tuber weights was increased. During the tenth week, in all, the weight of them was increased up to 320 mg. The interesting point is the irregularity in the growth of microtubers.

The microtubers were formed at on the whole surface of growth container (Fig. 8). Some of these microtubers were sessile tubers and some of them were formed as connected side by side to the white stolons and some were produced as the enlargement of the subapical region of the tip stolon. The sessile microtubers are always bigger than other they are. The smallest tubers were produced from the subapical of the stolon. From the average of 532±95.78 (n = 3) buds, only 105 microtubers were meanly produced.

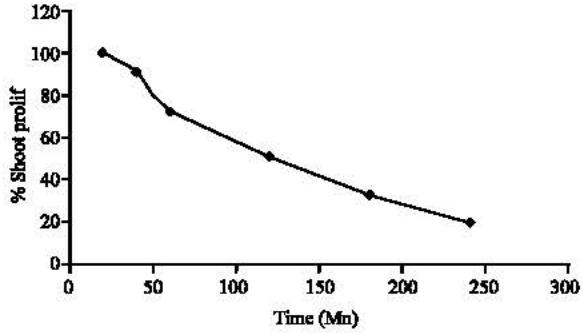

Fig. 3: The percent of shoot proliferation with the increase at periods of the nutrition solution pumping in semicontinuous bioreactor

Fig. 4: The growth of numerous shoots produced from the explants

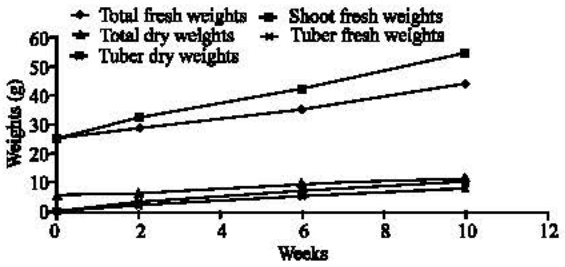

Fig. 5: The changes of fresh weights, total dry weights of shoots and microtubers between second weeks to tenth week in the microtuber inductive conditions

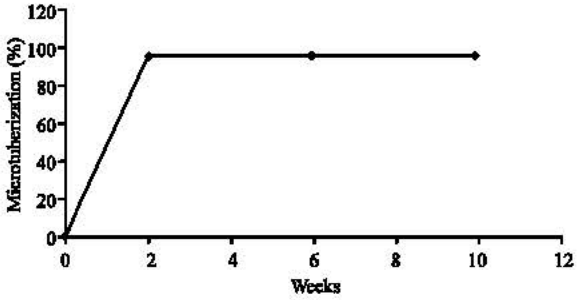

Fig. 6: The period and percent of microtuberization between second weeks to tenth week in the microtuber inductive conditions

The microtubers are white at the beginning and after being taken out of bioreactors their color is changed to yellow and brown. On the surface of microtubers lots of the lenticles can be seen and the microtubers are majorly spherical and a few of them are stretched and oval (Fig. 9).

Between two or three of the buds (eyes) were presented on the microtubers. Microtubers in the temperature of 25°C of the room condition and in the darkness and after one week were desiccated in different degrees (Fig. 10). This subject was indicated that there are linear relation between the early fresh weight and the weight of disccated microtubers during the first week of the maintenance in the room condition.

The desiccation rate of microtubers can be accounted through the following formula:

$$\frac{\text{the weight of microtubes after taking care in the room condition after a week}}{\text{the early fresh weight of microtubes}}$$

The subject indicates that the range of this changes was 0.5-0.95; this means that the microtuber weights was decreased between 5 and 46% and as the meanly the weight loss was about 27% (Fig. 11). The microtubers that during the first week lost more than 40% of their weight (12% of the total microtubers) were not capable of sprouting. The relation between the early weight of microtubers and the size of sprouts is non-linear that indicating the growth reduction and suspension of microtubers when they reach the size of 7-8 mm (Fig. 12). The equation linear of this relation follows $Y\text{-}0.86X^{2.18}$.

Microtubers have long-dormancy and only after 3 or 4 month, the sprouts of microtubers are activated and produce small branches (Fig. 13). The smaller microtubers that have less nutrient storage will bud lately and live for shorter time out of soil.

The obtained results related to the behavior of the explants in the continuous and semi-continuous bioreactors are similar to the results obtained by other

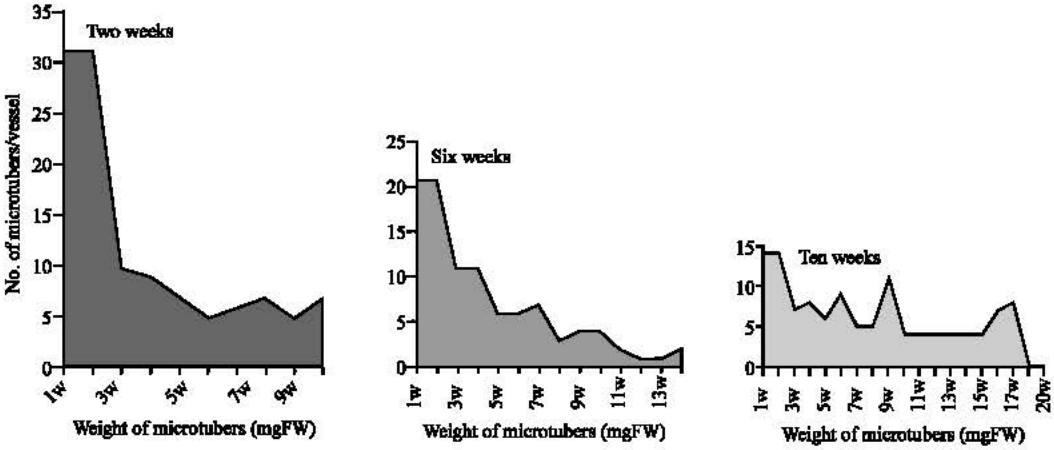

Fig. 7: The fresh weight changes between second week to tenth week in the microtuber inductive conditions

Fig. 8: The microtuber formation in continuous bioreactor

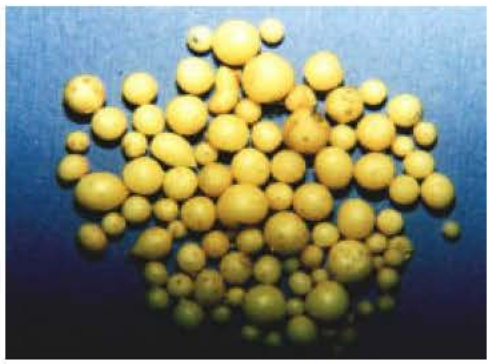

Fig. 9: The formed microtubers in continuous bioreactor

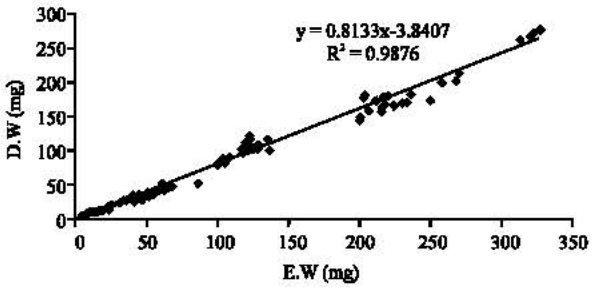

Fig. 10: The relation of early fresh weight with weights of desiccated microtubers

researchers (Xu *et al*., 1998). It seems that the growth of shoots is strongly under influence of the different conditions such as species reactions and culture conditions. Even long-immersion of the explants in the semi-continuous has a limiting influence on the growth of them the importance of aeration and the gaseous phase were shown in potato cultured in airlift bioreactors. Induction of tubers was inhibited under continuously submerged conditions. Jimenez *et al*. (2000) have emphasized that plants cultured in temporary immersing systems have shown better growth. The length of stems is almost 3 times longer than plants cultured in the solid media. In temporary immersing systems, plants have the greatest number of the nodes in each plant. Plants have bigger leaves, longer nodes. In the explant, there is no hyperhydricity during the culture period. These results emphasize the importance of the gaseous phase in the bioreactors for specific developmental phases.

Microtuberization in the inducing medium was begun during the first week and finishes during the second

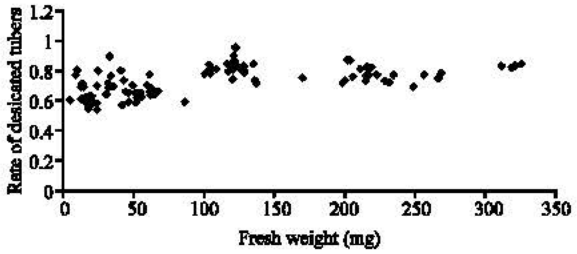

Fig. 11: The relation of fresh weights with rate desiccation microtubers

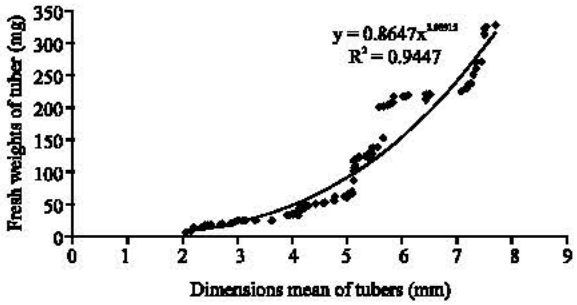

Fig. 12: The relation of fresh weight with microtubers dimensions

Fig. 13: The microtubers are sprouted after 3 until 4 months

week which is similar to Akita and Takayam (1994b) and Jimenez *et al.* (2002) results and are confirmable with the ontogenic-histological observations. (The unpublished results). The research of Jimene *et al.* (2002) using semi-continuous fermentors has shown that inducing of tubers during two weeks is observed and their number is not increased after this period. So, it seems that tubers induction during the first two week, in inducing medium finishes. This means that the inducing tissues of tubers and the growth condition of tubers must be provided during this period. In contrast, the total weight of tubers increaser stably during the ten weeks of culture period, meanwhile, the total weight of culture, due to branch growth, roats and the microtubers will increase up to the end of the inductive period.

According to Yu *et al.* (2000) concentration of total sugar should be constantly reduced in consequent with the growth of microtubers. During the sixth week, the growth of microtubers will gradually stop. Studying the charges of sugar concentration shows that sucrose concentration during the first two weeks of microtubers growth will drop highly from 80 to 6 g L^{-1}. Mainly part of sucrose changes in to glucose and fructose. The total concentration of sugar during the second week will decrease to less than 20 g L^{-1}, to the end of sixth week. Sucrose hydrolyze will produce and even amount of fructose and glucose. Thus, glucose concentration is always lower than the fructose concentration that indicating the better absorption of glucose than fructose by the explants.

The reported suitable limitation for sucrose in inducing culture of microtuber is between 60 to 80 g L^{-1} (20, 21). The higher or lower than the declared concentrations of sucrose would be late microtuberization, decrease number microtubers and shortening of them (Seabook and Coleman, 1993). It is likely that sucrose has a two type role in microtubers growth a part as a carbon source which in absorbed easily by microplants and changes into starch in growing microtubers and in the concentration of 80 g L^{-1} provides suitable osmolarite for the microtubers growth (Yu *et al.*, 2000). When the concentration of sugar is low (40 g L^{-1} instead of 80 g L^{-1}), or when a mixture of glucose and fructose is replaced for sucrose, the growth speed of microtubers decreases. Sucrose as a carbon source for the microtubers is prior to the products obtained from its hydrolyze and the available amount of sucrose is an important factor in indicating the size of microtubers. Fujino *et al.* (1995) indicated that adding 8% sucrose to the culture medium would be stopped the lengthening of shoots and enlargement the subapical region of them. The swellening of this region begins at first with lateral enlargement and after, priclinal division. Then, divided cells get enlarged from their sides. This appeared that between the microtuber growth and the sugars levels is relation.

IAA controls strongly the lengthening of stolons and cause the formation of small tubers which are attached to the stem. Exogenetic cytokinin don't influence the formation and lengthening of the stolons. The endogenic concentration of GA_1 during the lengthening of the stolons is up and during the enlargement of stolons

tips in inducing culture, reduces, while the level of GA_1 in non-inducing culture is up. GA_1 concentration with the sucrose concentration present in the culture, have negative relation. It seems that GA_1, is the active GA that is produced during the formation of tubers. The result showed that GA is one of the main controlling factors of tubers reproduction and ABA against with GA, stimulate tubers reproduction and sucrose through influencing the level of GA. Controls the production and formation of the tubers (Yu *et al*., 2000).

Gopal *et al.* (1998) believes that a photoperiod (10 h) with low intensity light when followed with suitable temperature of (20°C at day and 18°C at night) in many genotypes would be increased the microtubererization and co-occurrence of these factors with the higher levels of BAP could be increased the yield of microtubererization and bigger tubers.

The size of microtubers in *in vitro* growth container were influenced by environmental condition (for example photoperiod, gases like ethylene and CO_2 and environmental compounds) and culture medium (Takayama *et al*., 1994). One of the important factors in culture medium is carbon source which its type and concentration have significant influence on the growth of tubers. Sucrose are introduced as suitable sources of carbon.

In inducing phase of microtubers, the buds which were constantly at the bottom of reproducing solution, are not able to produce microtubers. The growth of microtubers is irregular, which can be seen in the size of microtubers. The irregular growth of buds and their weight increase make it possible to put them in 3 groups.

- Fast growth tubers (usually are produced from the growth of lateral buds of green shoots).
- Medium growth tubers (usually are produced from the lateral buds of white stolons).
- Slow growth tubers (which are usually produced out of the change in growth pattern of last Meristem and the lower part of stolons.

Then differences in a way can indicate the difference rate of exogenetic ABA:GA_3 in different parts of their plant (3). The dormancy of microtubers is one of the properties of potato cultivar and has positive relation with the ABA level of tissues and the sleeping period of micro tubers.

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