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Micropropagation of strawberry by multiple shoots regeneration tissue cultures

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An experiment was carried out to examine the effects of different combinations of plant growth regulators *in vitro* micropropagation of strawberry. Strawberry of the Rosaceae family, is a perennial, stoloniferous herb and popular in genetic engineering. The present study showed the procedure for propagation of *Fragaria* using nodal segments from *in vitro* germinated plants. The best concentration of BAP for bud induction was 0.5 mg/l plus KIN 0.2 mg/l. Histochemical analysis showed that only a direct induction of more teratological protuberances that arise around the cut end of the explants. The maximum number of roots per explant was obtained in elongation medium in MS combined with BAP 0.1 mg/l with auxin IBA 0.2 mg/l. In this medium, all of them the seedlings had safe and excellent rooting. The rooted plants were transferred to the greenhouse condition where they normally grew, matured and flowered with a survival rate of 100%. It is concluded that the present protocol can be efficiently used for mass propagation of strawberry.

Key Words: Micropropagation, *Fragaria*, Nodal segments, Rosaceae
Abbreviations: BAP—6-benzylaminopurine; IBA— indole-3-butyric acid; KIN: kinetin; AC: activated charcoal.

Introduction

Strawberry is a member of the genus Fragaria in the Rosaceae family is a perennial, stoloniferous herb and popular fruit. Strawberry is a major berry crop around the world.It has been commercially cultivated in many counties in the world (Biswas *et al.*, 2007). One of the important goals of the agricultural policy is to increase the acreage of strawberry to meet the demand of local fresh market, processing and export. The strawberry fruits are rich of vitamin C, B1, B2, protein, calcium, potassium, copper and iron, most of the nutritious elements essential for human being (Nehra *et al.*, 1994). They are important in transformation genes and genetic engineering that genetic engineering of

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strawberry has already been reported by Jame *et al*. (1989), Nehra *et al*. (1990). Biotechnological approaches, especially genetic engineering, are an alternative efficient strategy to implement strawberry improvement.

Importing mother plants is expensive. Healthy stocks used for propagation through conventional methods are not available. The *in vitro* culture of nodal cutting has been successful in the micropropagation of strawberry plants (Karhu and Hakala, 2002). It was have an opinion that several of plants can be produced within a year from a few mother plants by tissue culture technique (Boxus, 1983; Nehra, *et al* 1994). In this technique is useful if of the introduction of new cultivars. Propagation of strawberry is achieved either by runners or by in vitro micropropagation. The division of branches and runners of strawberry are not always suitable for this type of cultivation because of their emotional sensitivity of pathology agents (Dijkstra, 1993; Swartz *et al*., 1981; Nehra, *et al*., 1994). Micropropagation of strawberry from runners for beginning has been reported and may be refer to efficiently generate a large number of disease free plants (Boxus, 1974). In addition, the storage of tissue cultured propagules requires less space than traditional runner plant and the in vitro storage can be initiated at any time during the production cycle (Swartz *et al*., 1981). Micropropagated strawberry plant has been introduced to prevent most of the plant and soil transmissible diseases.

This is the first time to report on *in vitro* propagation of strawberry in iran. In the present examination of a simple protocol has been developed to propagate strawberry through tissue culture methods from nodal segments.

Material and methods

The experiment was carried out at the Tissue Culture Lab of the Agricultural Biotechnology Research Institute of Iran in 2011. Seeds of strawberry native cultivar were obtained from the Collection of Agricultural Research Center.

Plant material

Seeds were washed with tap water for 5 - 10 minutes to remove surface contamination and then sterilized by immersing in 70% ethanol for 1 minute with vigorous shaking followed by 20 minutes in 4% sodium hypochlorite containing one drop of Tween 20. The seeds were then rinsed three times with sterile distilled water in laminar flow cabinet to remove minor amounts of disinfection liquid.

Culture media and conditions

For germinating, the seeds were cultured in mugs on 30 ml of standard Murashige and Skoog medium (1962) containing 3 % (m/v) sucrose and 0.6% (m/v) agar. Cultures were incubated in a growth chamber at temperature of 24 °C, a 16h photoperiod provided with a light intensity of 2000 lux provided by white fluorescent lamps.

Shoot multiplication

Nodal segments (0.5-1 cm length) were cultured on MS medium supplemented with BAP with four concentrations (0, 0.1, 0.5, 1 and 1.5 mg/l) combined with three concentrations KIN (0, 0.2 and 0.5 mg/l) or IBA (0, 0.2 and 0.5 mg/l). Sterilization performed by autoclaving at 121ºC for 20 minutes. PH was adjusted at 5.8 before adding 0.6% (w/v) agar and 0.4% (w/v) activated charcoal. Five explants (nodal segments) were aseptically cultured in a mug containing 50 ml of the induction medium. The mugs were covered and sealed with household plastics foil for a period of 5 weeks and then transferred to the same conditions as mentioned above.

Bud elongation

After 3 weeks, the regeneration of explants trancfered to elongation medium, the elongation medium was supplemented with BAP 0.1 mg/l and IBA 0.2 mg/l. After 5 weeks, the rooted plantlets were acclimatized and out-planted in pots contained sterilized peat mass and vermiculite (3:1 ratio). The pots were covered with clear beaker having a few holes on it and were frequently watered to keep high humidity in a phytotron for 10 days. Hardened plantlets were out-planted in a greenhouse set at a day temperature 21ºC, a night temperature 19ºC, relative humidity 85% a 16 h photoperiod provided with a light intensity of 3000 lux provided by white fluorescent lamps.Immediately after planting, the plantlets were irrigated and adequate soil moisture was maintained through daily watering.

Experimental design and statistical analysis

The experiment was carried out in factorial experiment in completely randomized design. Data were statistically analyzed using the SAS software (version 8). When the ANOVA indicated significant treatment effects (5 or 1%) based on the F-test, the Duncan's Multiple Range Test ($P \leq 0.05$) was used as a

method to determine which treatments were statistically different from other treatments.

Histological studies

The histological staining was carried out to establish the ontogeny of explants containing shoot buds. Explants at different stages of their development (3, 8 and 12 days of regeneration) were fixed in FAA (formalin, acetic acid, absolute alcohol: 10, 5, 85; v/v) for 24 hours. After dehydrating in ethanol (70, 95 and 100%) and xylene, textures were fixed in paraffin, sectioned with a microtome at 30 µm and then stained with metylen blue. The sections were prepared in a lam for observation under a light microscope.

Rooting and acclimatization

Most of shoots in the elongation medium that had 0.1mg/l BAP with follow 0.2 mg/l IBA were rooted as well. In general, 4 weeks-old rooted plantlets were acclimatized and out-planted in pots contained sterilized peat mass and vermiculite (3:1 ratio). The pots were covered with clear beaker having a few holes on it and were frequently watered to keep high humidity in a phytotron during 25 days. Hardened plantlets were out-planted in a greenhouse set at a day temperature 21ºC, a night temperature 19ºC, relative humidity 85% and a day length of 12 h. Immediately after planting, the plantlets were irrigated and adequate soil moisture was maintained through daily watering. The proliferated plants showed 100% survival rate during hardening and acclimatization.

Results

To optimize the media for regeneration of *Strawberry* in a preliminary experiment, nodal explants from grown plants of *Fragaria sarian* L. were inoculated on MS medium with different concentrations of growth regulators of BAP with four concentrations (0, 0.5, 1 and 1.5 mg/l) alone or with three concentrations KIN (0, 0.2 and 0.5 mg/l) or IBA (0, 0.2 and 0.5 mg/l). The multiplication efficiency of nodal segments from plants was significantly when estimated four to four weeks. The results showed that about 3-5 multiple shoot buds developed from explants derived from 4-5 old week seedlings germinated *in vitro* on the shoot bud induction media (Fig. 1). Different concentrations and combinations of growth regulators showed different responses in terms of number of buds, the length of stem and the percentage of rooting and regeneration (Table 1; Table 2).

Table 1. Effect of growth regulators BAP, KIN on the Number of bud, Stem length, the percentage of rooting and regeneration of explants

Growth regulators (mg/l)		regeneration (%)	Stem length	Number of bud	Rooting (%)
KIN	BAP				
0	0	7 g[1]	7 e	0.9 ef	5 h
0	0.2	14 fg	1.6 d	1.1 ef	12 gh
0	0.5	12 g	1.5 d	0.8 f	12 gh
0.5	0	21 ef	1.2 cd	1.6 de	15 fg
o.5	0.2	100 a	5.3 a	1.5 a	100 a
0.5	0.5	27 e	2.5 c	1.2 cd	19 efg
1	0	37 cd	2.4 c	1.9 cd	26 de
1	0.2	69 b	3.9 b	1.4 b	77 b
1	0.5	39 c	2.5 c	1.2 cd	32 cd
1.5	0	36 cd	2.5 c	2.4 c	35 c
1.5	0.2	22 ef	1.5 d	1.9 cd	22 ef
1.5	0.5	29 de	2.3 c	2.2 cd	18 efg

[1]The data with similar letters are not significantly different

Table 2. Effect of growth regulators BAP, IBA on the Number of bud, Stem length, the percentage of rooting and regeneration of explants

Growth regulators (mg/l)		regeneration (%)	Stem length	Number of bud	Rooting (%)
BAP	IBA				
0	0	11 de[1]	1bc	0.9 cd	7 a
0	0.2	7 e	0.9 c	0.6 d	8 de
0	0.5	12 cde	1.5 bc	0.9 cd	9 cde
0.1	0	19 bcde	2.6 ab	3 a	16 bcde
o.1	0.2	39 a	3.5 a	3 a	46 a
0.1	0.5	17 bcde	2.3 abc	1.6 bcd	21 bc
0.5	0	25 abcd	2.6 abc	2.5 ab	25 b
0.5	0.2	31 ab	1.5 bc	2.1 ab	23 b
0.5	0.5	31 ab	1.6 bc	2.4 ab	26 b
1	0	25 abcd	1.9 abc	2.1 ab	20 bcd
1	0.2	26 abc	1.7 bc	1.9 abc	23 b
1	0.5	29 ab	2.1 abc	2.3 ab	27 b

[1]The data with similar letters are not significantly different

Fig. 1. Multiple shoots proliferation strawberry in regeneration media

Fig. 2. Nodal segments of Strawberry resulted from aseptic seedlings as explants on medium MS in combination with growth regulators.

After five weeks of culture the maximum number of microshoots per explant was obtained in MS medium supplemented with BAP with KIN, where 90% explants showed shoot prolferation and shoot developed. The best concentration of BAP for bud induction was 0.5 mg/l plus KIN 0.2 mg/l (Table1).The maximum number of roots per explant was obtained in elongation medium in MS combined with BAP 0.1 mg/l with auxin IBA 0.2 mg/l. In this medium all of them the seedling had safe and excellent rooting. When BAP concentration was increased in medium the rate of shoot multiplication recorded. When BAP was supplemented with KIN the rate microshoots per explant was increased, but when BAP was supplemented with IBA the rate microshoots per explant was recorded and microshoots had elongation. Subcultures were done every 25 days interval. Nodal segments from the proliferated shoots were subcultured again for further multiple shoot induction (Fig. 2). Regenerated multiple shoots were cut and individual shoots were placed in MS medium containing different concentrations of BAPand IBA for elongation induction.

As the part of our results, we showed that media containing BAP in combinations with other IBA had the least effectiveness on regeneration parameters especially the number of bud per explants (Table 2). All of them the explant were rooting in the same medium in elongation medium (Fig. 3).The proliferated plants showed 100% survival during hardening and acclimatization (Fig. 4). There were no observable variations between the parent plants and *in vitro* propagated plants. The transplanted plantlets established well in a glasshouse (Fig. 6).

Histochemical staining of the regenerated showed initial meristems and primordial formation in regions of bud formation after 12 days of culturing (Fig. 7).

Fig. 3. Seedling of strawberry with expanded buds and extended roots after 30 days of regenerating

Fig. 4. Hardened plantlets of Strawberry after transferring from *in vitro* condition

Fig. 5. Tissue culture plant bearing fruit after transferring from *in vitro* condition.

Fig. 6. Strawbeery whole plants out-planted in a greenhouse

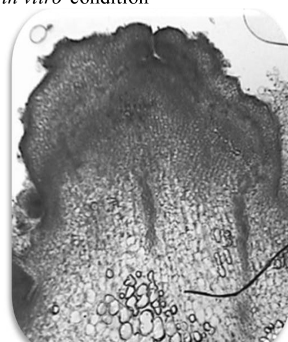

Fig. 7. Section of the embryo originated from bud induction after 12 days of culture. The development of the mature meristem was observed 30 µm

Discussion

There are some diverse reports regarding to regeneration of strawberry as well as the key role of plant growth regulators for regeneration (Adel and Sawy, 2007; Biswas *et al*. 2007; Karhu and Hakala, 2002; Meyer *et al*., 2003; Harker *et al*., 2000; Boxus, 1974; Popov and Trushechkin, 1975; Abramenko, 1983).

The best treatment of cytokinins producing the highest number of buds per explant, the length of the stem and the percentage of rooting and regeneration was BAP 0.5 mg/L with 0.2mg/L KIN. The importance of BAP for regeneration of Strawberry has been emphasized by (Adel and Sawy2007; Biswas *et al*. 2007; Sakila, *et al*. 2007; Harker *et al*., 2000) who showed BAP for regeneration of strawberry is important. Hu and Wang reported that high

concentration of cytokinin reduced the number of micropropagated shoots(Hu and Wang, 1983). Similar results have already been reported in *Fragaria indica* andr (Bhatt and Dhar, 2000).

This was in accordance with other studies in which regenerated strawberry have been obtained that MS medium supplemented with 0.5 mg/L BAP with IBA 1 mg/L was effective for shoot multiplication in nodal segment of strawberry (Adel and Sawy, 2007). In our study, the concentration of BAP lesser than 0.5 mg/L with cytokinin KIN was less effective for regeneration of strawberry. The others researcher that showed the concentration of BAP 1 mg/L with cytokinin KIN 0.5 mg/L was effective in regeneration of strawberry (Biswas *et al*., 2007). The others in their reported showed the best medium for regeneration of strawberry was the medium MS plus of BAP 1.5 mg/L and cytokinin KIN 0.5 mg/L, and the plantlets was produced root in medium MS plus of IBA 1 mg/L (Sakila *et al*., 2007).

The axillary shoots further produced multiple shoot buds when cultured in bud induction medium. The *in vitro* established plantlets were hardened in a phytotron with a survival rate of 90-95% and were then transplanted in glasshouse Overall findings of the present study are significant in obtaining the maximum regeneration with minimum concentrations of growth regulator. In conclusion, we have developed a promising method for an efficient regeneration from nodal explants of *Fragaria* using BAP and NAA. The survival rate was 100%. The regenated plants started to produce fruits in 2 to 3 month after transplanting (Fig. 5)

A summary of the optimized protocol of strawberry mass propagation is shown in (Fig. 6). In conclusion we have developed the protocol of efficient and effective shoot multiplication and high survival rate of the shoot at initiation stage for commercial micropropagation in strawberry. Favorable morphogenic response observed in apex explants may be due to the fact that they were obtained from young tissues, which have a higher capacity to respond to *in vitro* culture than older explants.

Our experiment indicated the explants include shoots bud of different process of their development. After three days of culturing only parenchyma cells were observed, whereas apical meristem in maturity situation was seen after 12 days (Fig. 7).

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