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SOMATIC EMBRYOGENESIS IN STRAWBERRY (*FRAGARIA ANANASSA*) VAR. CAMAROSA

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ABSTRACT
Micropropagation is an effectively used tissue culture technique for mass multiplication of plants. The plant propagation may be achieved either through organogenesis or somatic embryogenesis. Somatic embryogenesis is a better alternative because of its high propagation rates, the presence of both shoot and root meristems in same propagation unit. Indirect somatic embryogenesis was obtained from leaf disc (5 × 5 mm) explants obtained from in vitro raised shoots of Strawberry (*Fragaria ananassa*) var. Camarosa on MS medium supplemented with different concentrations and combinations of 2,4-D and BAP. 2mg/lit 2, 4-D and 0.5 mg/lit BAP showed early callusing and induction of somatic embryos with 100% response. Embryo development was better on hormone free medium (MS basal) as compared to 0.1 and 0.2 mg/lit 2, 4-D. The somatic embryos thus developed were germinated on MS basal medium supplemented with 0.5mg/lit GA_3 with 8 % conversion frequency.

KEYWORDS: 2,4-D, BAP, *Fragaria ananassa*, Somatic embryogenesis.

INTRODUCTION

The process of formation of an embryo is called embryogenesis. Embryogenesis starts from a single embryogenic cell, called a zygote (the product of the fusion of an egg and a sperm during fertilization), or an undifferentiated callus tissue. Embryos developing from zygotes are called zygotic embryos, while those derived from somatic cells are called somatic embryos. During the embryonic development, the polar axis of the plant is established, domains that set up the organization of the plant body are defined, and the primary tissue and organ systems are delineated. Somatic embryogenesis is another important way to regenerate new plants in plant tissue culture. Generally, somatic embryo develops from single cell. This cell undergoes cell divisions to form a group of meristematic cells. The constituent cells of this group continue to divide to give rise to somatic embryos through the stages similar to the zygotic embryogenesis *viz.* globular, heart shaped, torpedo shaped and cotyledonary stage. Somatic embryos may be primary (regenerate from explant / callus) or secondary (regenerate from the tissues of other somatic embryos or a part of generating somatic embryo.)

In several species of Citrus and Mangifera the development of adventive embryos from nucellar cells is a normal feature. However, the nucellar embryos attain maturity only if they are pushed into the embryo sac at an early stage of development, or else they fail to mature. These in vivo observations would suggest that for their growth and development embryos require a special physical and chemical environment available only inside the 'magic bath' of the embryo sac.

Strawberries (*Fragaria ananassa* Duch) are one of the most important fruits. In 2010, the worldwide production of strawberries has been reported to be around 4.3 million tonnes. Such a huge production is dependent upon efficient nursery production of certified transplants. This in turn requires enough *in vitro* disease indexed plants from nuclear stocks for further propagation in the nursery fields. Micropropagation is the tool for the multiplication of disease-free strawberry plants. Potentially micro propagation of strawberry plants can be carried out via somatic embryogenesis. Somatic embryogenesis is the better alternative because of its high propagation rates, the presence of both root and shoot meristems in the same propagation unit. Applications of somatic embryogenesis are clonal or mass propagation, elimination of viruses, source tissue for genetic transformation, development of synthetic seeds. Although the somatic embryogenesis was described long ago in strawberry by 1984 using cotyledon, where few embryos were produced. However, until date, the frequencies of strawberry improvement and regeneration are significantly lower than the expected. Therefore, the present investigation was undertaken for production of somatic embryo from leaf and further regeneration in order to improve the plantlet regeneration via somatic embryogenesis.

MATERIALS & METHODS

Preparation of Culture Medium

The media formulated in the lab according to the composition of Murashige and Skoog referred as MS medium selected as the optimal culture medium. Stock solutions of generally 40 X macroelements, 1000 X for microelements, 100 X for the organic constituents were prepared. These stock solutions were stored in a freeze at 4^oC and were mixed in desired proportions only before use. None of the stock solutions was stored for more than 15 days. The reagents used were of Analytical Reagent

Grade. Each salt was dissolved separately one after one to avoid precipitation.

Sterilization of the Nutrient Media

Following preparation of the medium and prior to addition of agar, the pH adjusted to 5.8. The medium poured into culture jars of size 350 ml where each jar contained 20ml of the medium and capped with polypropylene caps. The culture jars were autoclaved at 121^{o}C and 15 psi pressure for 20 minutes.

Collection of plant material

In vitro raised plants collected from VSBT Plant Biotechnology Laboratory. Plants were of 40-45 days old. Only healthy plants having uniform leaf size selected for somatic embryogenesis. Plants taken aseptically in Laminar and the leaves dissected into 5 × 5 mm leaf disc.

Medium optimization for somatic embryogenesis

Callus induction and Subculturing

Leaf discs (5 ×5 mm) cultured on the surface of media solidified with agar under sterile conditions in a 350 mL glass jar containing 40 mL medium. This medium was supplemented with different concentrations and combinations of 2, 4-D and BAP. All possible combinations of five concentrations (1.0, 2.0, 3.0, 4.0, 5.0 mg/lit) of 2, 4-D and three concentrations of BAP (0.5, 1.0, 2.0) were prepared. Media solidified with 8.0 gm/lit agar and the pH adjusted to 5.7 before autoclaving (121 0C, 15 min). 4 discs per bottle and 5 bottles of each combination were inoculated. Cultures were incubated at 25±2 0C in dark for two weeks and then transferred under light (16/8 h photoperiod at 2000 lux white light) at 21 ˚C for three weeks.

After this time cultures were subcultured on MS medium containing 2.0 mg/lit 2, 4-D and 0.5 mg/lit BAP (100 % response) and grown for three weeks (same light and temperature condition).

Embryo development and maturation

After six weeks of incubation 4 embryogenic callus per bottle and 40 bottles of three different MS medium containing 0.0, 0.1, 0.2 mg/lit 2, 4-D were transferred and incubated under photoperiod of 16/8 h at 2000 lux white light at 25 ±2^{o}C temperature for three weeks.

Embryo germination

Callus containing cotyledonary stage embryo were transferred to MS media containing three different concentrations of GA_3 (0.5, 1.0, 1.5 mg/lit) and incubated under photoperiod of 16/8 hrs at 2000 lux white light at 25±2^{o}C temperature for three weeks.

Hardening

Medium for hardening, soilrite sterilized by autoclaving (15 psi for 20 min). Protray then cleaned under tap water and tunnel was prepared with the help of bamboo sticks and polythene paper around tray. Sterile soilrite filled in protray. Plantlets generated *in vitro* taken in petri plate and first washed with filter water to remove the medium adhering to the roots. They treated with fungicide solution such as bavistin (0.1 % v/v) for 10 min. Then single plantlet planted in each pot of protray. Filter water sprayed using sprayer over plants and in the overhead space inside the polytunnel thrice a day to maintain desired humidity in the atmosphere around the plantlets.

RESULTS & DISCUSSION

Induction of embryogenic callus

The cultured leaf discs showed curling after 3 days of inoculation. The callus induction observed after nine days of incubation at the cut edges of explants. The best results were obtained on MS basal medium supplemented with 2.0 mg/lit 2, 4-D in combination with 0.5 mg/lit BAP with 100 % response of the explants within 20 days of incubation. The callus developed was white or greenish, granular and slightly hard. The callus multiplied by repeated subculturing on the same medium after every 30 days.

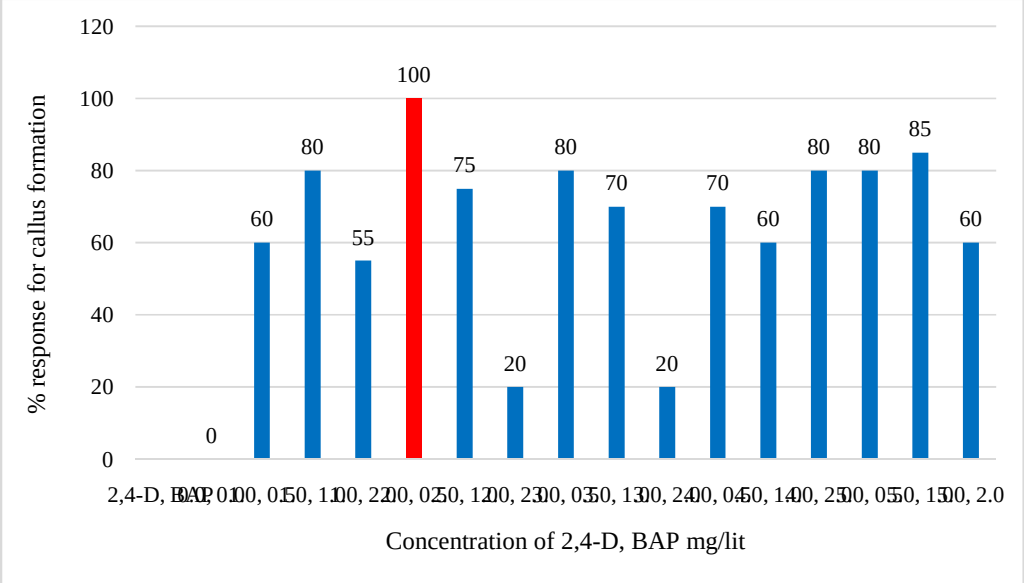

FIGURE 1: Effect of plant growth regulators on embryogenic callus formation in leaf discs of *Fragria ananassa* var. Camarosa

Manosh Kumar Biswas *et al.* (2007) have observed callus induction within 14–21 days from leaf discs of Strawberry on MS basal medium supplemented with only NAA. Omar *et al.* (2013) observed best callus induction in strawberry var. Sweet Charlie in the range of 3-4mg/lit 2, 4-D in combination with 0.5-1.0mg/lit BAP and 5% sucrose whereas the best results for callus culture in strawberry var. Chandler were observed on medium supplemented with 1.0-2.0mg/lit 2, 4-D in combination with 2mg/lit BAP. This suggests that the combination of auxin and cytokinine is essential for callus induction in strawberry. In the present studies, also the combination of auxin and cytokinine found superior for callus induction.

Giovanni Garro-Monge *et al.* (2008) observed highest embryogenic callus frequency when leaf bases were cultured on MS basal medium supplemented with 0.25 mg/lit 2,4-D and 1mg/lit kinetin. He also observed when high concentrations of 2,4-D and low kinetin concentrations were used embryogenic callus induction from apical meristems and leaf bases decreased in *Aloe barbadensis*. K. S. Nagesh *et al.* (2010) and Husaini *et al.* (2011) observed in *Curculigo orchioides* Gaertn on the medium supplemented with 0.5-4.0 mg/lit BAP alone and in combination with 2, 4-D or NAA at 0.1-0.5 mg/lit they produced smooth, round structures within 4 weeks. High frequencies (23±0.8) occurred on MS medium containing 1 mg/lit BAP.

PLATE 1: Effect of plant growth regulators on embryogenic callus formation in leaf discs of *Fragria ananassa* var. Camarosa; (a, b & c) 100 % response of the explants on MS basal medium supplemented with 2.0 mg/lit 2, 4-D in combination with 0.5 mg/lit BAP.

Embryo Development

For development of somatic embryos from the embryogenic callus, MS basal medium found to be superior over the MS basal medium supplemented with low concentrations of auxin *i.e.* 2, 4 –D. On MS basal medium 8 % response was obtained whereas on MS basal medium supplemented with 0.1 and 0.2 mg/lit 2,4-D the callus turned brown after 1 month of incubation. Gona Karimi Kordestani *et al.* (2008) have observer globular stage embryos developed into cotyledonary ones within 1–2 weeks after transfer to hormone-free media containing different concentrations of sucrose. The percentages of globular stage embryos developing into cotyledonary ones at different concentrations of sucrose in culture media. Increasing sucrose concentrations enhanced the development of globular stage embryos into cotyledonary ones. Gaj M.D. (2011) subcultured somatic embryos onto auxin-free medium and resulted in their conversion into plantlets with an average frequency of 80 % in the culture of Arabidopsis thaliana.

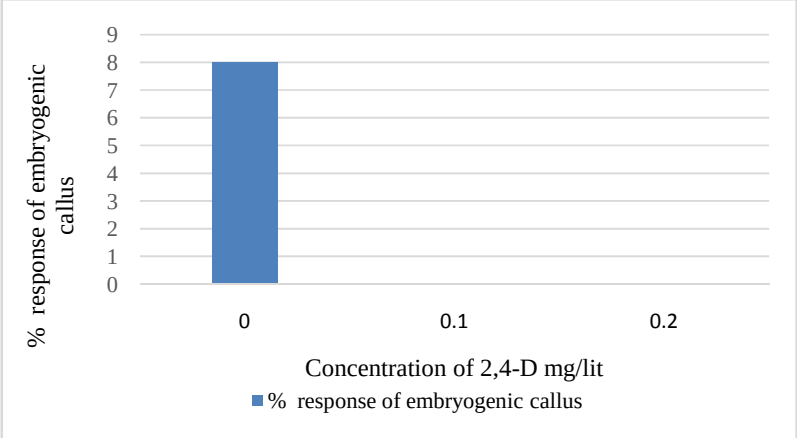

FIGURE 2: Effect of 2, 4-D on embryo development on embryogenic callus produced from leaf disc of *Fragria ananassa* var. Camarosa

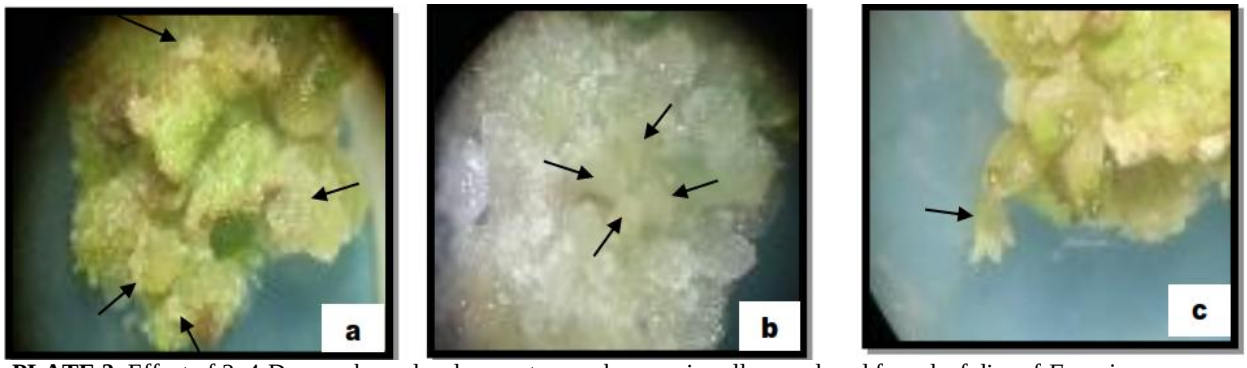

PLATE 2: Effect of 2, 4-D on embryo development on embryogenic callus produced from leaf disc of *Fragria ananassa* var. Camarosa: a) The Globular stage b) The Heart stage c) The torpedo-shaped and cotyledonary stage.

Embryo Germination

The cotyledonary embryos germinated after one week of subculturing and the maximum germination 6/callus was obtained on MS medium containing 0.5 mg/lit GA_3 followed by germination of 4 somatic embryos on MS basal medium supplemented with 1.0 mg/l GA_3. Whereas only 3 embryos germinated on MS basal medium containing 1.5 mg/lit GA_3 within 3 weeks of transfer to embryo germination medium. Manosh Kumar Biswas *et al.* (2007) observed the cotyledonary stage embryos which then he isolated and cultured it on MS medium supplemented with 0.1 mg/l GA_3 and 0.1 mg/l IBA. After four weeks of culture, the cotyledonary embryos had developed into plantlets. Husaini *et al.* (2007) and Husaini *et al*. (2008) showed similar findings by using TDZ.

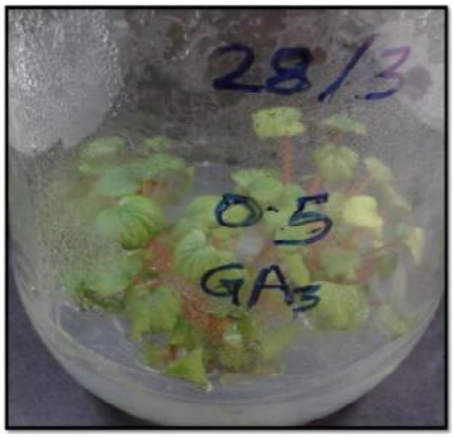

PLATE 3: Effect of GA_3 on germination of somatic embryo after 4 weeks of culture: 6 embryos per callus were germinated on MS basal medium supplemented with 0.5 mg/lit GA_3.

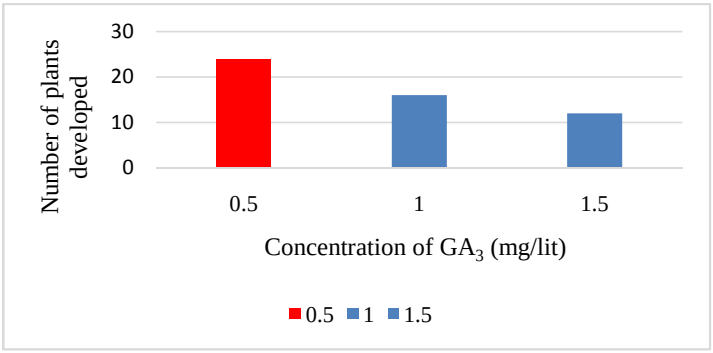

FIGURE 3: Effect of GA_3 on germination of somatic embryo after 4 weeks of culture

Primary hardening and acclimatization

Plantlets developed from somatic embryos planted in pots filled with soilrite hardened after 25 days. After 10 days, the plantlets were green and healthy. The length of plantlets as well as size of leaves was increased (temp. 32˚C and humidity 90%). Efficiency of survival of plantlets were maximum (100 %) in soilrite. Manosh Kumar Biswas *et al.* (2007) were transferred plantlets developed from somatic embryos into plastic bags containing sterile sand and garden soil (1:1) and maintained in a growth chamber for two weeks followed

by transferred to greenhouse. One month after transfer, 90–95 % plantlets survived and grew normally.

CONCLUSION

It was observed that, 2.0 mg/lit 2, 4-D and 0.5 mg/lit BAP can increase the efficiency of callus formation as compared to less or more concentration of 2, 4-D and BAP. For the development of somatic embryo MS basal medium is better as compared to reduced concentration of 2,4-D. High germination percentage of somatic embryos can be achieved on the MS media supplemented with 0.5 mg/lit GA_3. Soilrite is the best medium for the primary hardening of plantlets developed from somatic embryos in strawberry var. Camarosa.

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