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# *IN VITRO* PROPAGATION OF EMBRYONIC EXPLANTS OF SATKARA (*Citrus macroptera* Montrouz)

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**ABSTRACT**

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*Citrus macropter* is a wild or semi wild *Citrus* species grow well in hill-forest. The present investigation was conducted to study the effects of auxin and cytokinin on callus induction from embryonic explants of *Citrus macroptera* Mont. The embryonic explants produced semi-hard greenish and creamy-white callus and adventitious buds within 4-5 week of culture. The highest percentage of callus is produced in media containing 2.0 $mgl^{-1}$ 2,4-D+1.5 $mgl^{-1}$ KIN were used. The rate of adventitious shoot regeneration from callus was also influenced different concentration of BA in MS medium. The highest shoot regeneration rate was observed in embryogenic callus on MS media supplemented with 1.5 $mgl^{-1}$ BA. Best rooting achieved within 28 days after the isolated adventitious shoots were transferred to a rooting media containing 1.0 $mgl^{-1}$ IBA. *In vitro* cultured plantlets transfer to pot soil and successfully acclimatized with natural condition.

***Key words:*** *in vitro propagation, auxin, cytokinin, callus, Citrus macroptera Mont*

## INTRODUCTION

*Citrus macroptera* is locally known as Satkara in the Sylhet Division of northeastern Bangladesh and Meghalaya, Assam and Mizoram in India. Sat meaning seven or its multiples and kara meaning segments as most of the fruits possess 14 segments. This species is found wild as well as in semi-domesticated form in the forest. South-east Asia, Australia, the intervening islands between Asia and Australia and central Africa are recognized as important centers of origin of Citrus and related genera (Tanaka 1958; Swingle and Reece, 1967). It is not consumed fresh like mandarin, Shaddak orange and lemon. This species inspite of producing very hard and large fruits, which are very aromatic and too acidic in taste for eating raw as a table fruit, has shown some promise. In Bangladesh, both green and mature fruits are used for flavoring beef, mutton, fish curries, dal and vegetables. The fruit is also used in the preparation of pickles, available in many super shop of Bangladesh. Fresh fruits, bottled juice and dried fruit peel were being used by local people for edible purposes and sold on small village shops in Sairang near west Aizawl in Mizoram. Juice mixed with sugar is used as a squash and relished by local people as it gives soothing effect to the stomach. This fruits are used by local tribes of Assam for medicinal purpose (Ghosh 1990). The sweet flavor of fruits due to essential oil of flavedo parts may be used in flavoring purposes in perfume industries and as an ingredient in preparing traditional soap and cleanser. Though this fruits are not commercially distributed other region of Bangladesh but every year high amount fruits are taken by immigrant local people of Sylhet in UK, USA and Middle East countries. Unfortunately the production of this popular fruit is not satisfactory in Bangladesh. Main Reason is that, Dwaki and Jaintia hills of Meghalaya bordering Bangladesh had a lesser occurrence of *C. macroptera* as earlier reported by Singh *et al.* (2001). This species is under threaten condition in bordering area of Bangladesh-India, Jaintia hills of Meghalaya and Assam of northeastern India reported by Malik *et al.* (2006). *Citrus macroptera* are affected by many disease which are causes the lesser fruit production and survivability of the plant. This initiative was taken to produce quality plantlets for crop improvement.

## MATERIALS AND METHODS

This research work was conducted at Plant Genetic Engineering Laboratory of Genetic Engineering and Biotechnology Department, Shahjalal University of Science & Technology, Sylhet, Bangladesh. Mature embryos of *C. macroptera* were used as explants in the experiment. Ripen *C. macroptera* was collected from Bandar bazar of Sylhet city, Bangladesh. Seeds separated and dried in the sun for the study.

### *Preparation of explants*

At first seed coats were removed manually to obtain embryos. Then embryos were exposed to sterilizing agents for varying durations, either before or after removing the embryo sec to identify the ideal surface sterilization conditions. Embryos were treated with 70% ethanol for 2-3 minutes, followed by few drop of tween-20 for 5 minutes, then with 0.1% Mercuric Chloride solution for 5 minutes then washed with sterilized distilled water for several times. The embryos were then placed on the sterilized petriplate having sterile filter papers with the help of forceps to remove excess water. Embryo sac removed carefully with sterile forceps before inoculation. Embryos were then placed individually in culture tubes. The culture tubes were then incubated 27±2°C temperature under florescent light of 16 hours photoperiods.

### *Callus induction*

Callus induction was initiated in 25 x 150 mm culture tubes containing 10 ml of MS (Murashige and Skoog, 1962) medium containing 3% sucrose and solidified with 0.7% agar having different concentrations of callus inducing growth regulators in different combination. Ten explants were used for each treatment and thirty test tubes were inoculated in each time. Visual observations were taken every three days and the effect of different treatments was quantified on the basis of percentage of explants showing response for callus induction.

### *Shoot regeneration*

Shoot regeneration was performed in 25 x 150 mm culture tubes containing 10 ml of MS medium containing 3% sucrose and solidified with 0.7% agar, having different concentrations of shoot regenerating hormones. In the case of shoot regeneration from callus, a healthy green portion of callus was taken and cut into pieces, and these pieces were then placed on a shoot regeneration medium. Visual observations were taken every three days and the effect of different treatments was quantified on the basis of percentage of calli showing response for shoot regeneration.

### *Rooting of regenerated shoot*

Rooting was performed in 25 x 150 mm culture tubes containing 10 ml of MS medium containing 3% sucrose and solidified with 0.7% agar, having different concentrations of root regenerating hormone IBA individually. Visual observations were taken every three days and the effect on different shoots was quantified on the basis of percentage of shoots showing response for rooting.

### *Data collection and analysis*

Weekly visual observation of culture was made and frequency of culture showing callus, shoot and root formation was recorded. Completely randomized design has been used in each experiment. The data pertaining to shooting and rooting per culture were analyzed using Microsoft Office Excel 2007.

### *Transfer to the soil*

Rooted plantlets transferred to plastic pots filled with 2:1 sterilized garden soil and compost. The potted plants (one plantlet per pot) were then put into large polythene bags (25 cm × 15 cm) to maintain high humidity. Open portion of the large bag was made airtight and kept them in growth chamber under artificial illumination. Within 7-9 days the covering bags were finally removed. Potted plants were acclimated with natural environment.

## RESULTS

### *Effect of cytokinin and auxin on callus induction*

Differential morphogenic responses of mature embryos of *C. macroptera* in MS medium supplemented with different concentrations and combination of cytokinin and auxin were observed and the results of the experiment are shown in the Table 1.

Highest callus forming 87.5% explants were recorded in the media having 1.5 $mgl^{-1}$KIN with 2 $mgl^{-1}$ 2,4-D (Fig1.A and Table 1). The similar effects (62.5%) are found in 1.5 $mgl^{-1}$BA (Fig1.D and Table 1) and 1.5 $mgl^{-1}$KIN (Fig1.C and Table 1) combination with 4$mgl^{-1}$NAA and 3$mgl^{-1}$NAA respectively. It also reveals that the concentrations of NAA had significant effects on callus induction combination with BA and KIN.

On the other hand, the effects in callus induction of BA and KIN with different concentration of 2,4-D was recorded. Maximum (87.5%) callus induction capacity observed in 1.5 $mgl^{-1}$KIN combination with 2 $mgl^{-1}$ 2,4-D (Fig1.A and Table 1 ). Callus induction capacity was observed 62.5% in case of 1.5 $mgl^{-1}$BA combination with 3 $mgl^{-1}$ 2,4-D (Fig1.B and Table 1 ). If the 2,4-D concentration increased 3 $mgl^{-1}$ to 4 $mgl^{-1}$ with BA and KIN then callus induction decline. On the other hand, when NAA concentration increased 3 $mgl^{-1}$ to 4 $mgl^{-1}$ with KIN callus induction rate decrease but in case of 1.5 $mgl^{-1}$BA rate of callus production increase.

Table 1. Effect of different concentration and combination of auxin and cytokinin in callus induction from embryonic explants of *C. macroptera* after 3 weeks

| Growth regulators concentration ($mgl^{-1}$) | Frequency of callus induction (%) | Degree of callus formation | Callus with buds | Direct germination |
|---|---|---|---|---|
| **NAA + BA** | | | | |
| 1 + 1 | | - | - | Yes |
| 2 + 1 | | - | Yes | - |
| 3 + 1 | 37.5 | ++ | - | - |
| 4 + 1 | 25 | + | - | - |
| 1 + 1.5 | | - | - | Yes |
| 2 + 1.5 | | - | Yes | - |
| 3 + 1.5 | 50 | ++ | - | - |
| 4 + 1.5 | 62.5 | +++ | - | - |
| **NAA + KIN** | | | | |
| 1 + 1.5 | | - | - | Yes |
| 2 + 1.5 | 37.5 | ++ | - | - |
| 3 + 1.5 | 62.5 | +++ | - | - |
| 4 + 1.5 | 50 | ++ | - | - |
| **2, 4-D + BA** | | | | |
| 1 + 1 | | - | - | Yes |
| 2 + 1 | | - | - | - |
| 3 + 1 | | - | - | - |
| 4 + 1 | 12.5 | + | - | - |
| 1 + 1.5 | | - | - | Yes |
| 2 + 1.5 | 37.5 | ++ | - | |
| 3 + 1.5 | 62.5 | +++ | - | - |
| 4 + 1.5 | 25 | + | - | - |
| **2, 4-D + KIN** | | | | |
| 1 + 1.5 | 37.5 | ++ | - | - |
| 2 + 1.5 | 87.5 | +++ | - | - |
| 3 + 1.5 | 50 | ++ | - | - |
| 4 + 1.5 | 25 | + | - | - |

**Rate of Response:** - none, + low, ++ moderate, +++ high

***Regeneration of plated calli***

When the mature embryos were cultured on callus inducing medium, different types of callus were formed within 4–5 weeks of culture. The produced calli of convenient size were transferred on MS medium supplemented with different concentrations of BA.

Table 2. Effect of different concentrations of BA on multiple shoot formation from plated callus after 4 weeks

| Different concentrations of BA ($mgl^{-1}$) | Shoot/callus (M±SE) | Shoot length(mm) (M±SE) |
|---|---|---|
| 0.5 | 1.20±0.39 | 1.50±0.44 |
| 1.0 | 1.60±0.99 | 1.75±0.62 |
| 1.5 | 2.80±0.39 | 2.31±0.66 |
| 2.0 | 1.40±0.78 | 2.88±1.09 |
| 2.5 | 1.20±0.39 | 1.67±0.41 |

M=Mean, SE=Standard Error

It is evident from Table 2 that on MS media fortified with 1.5 $mgl^{-1}$BA produce the highest number of regenerated shoot which was 2.80±0.39 (Fig1.F and Table 2) and the maximum frequency of shoot regeneration was observed 40%. The highest shoot length was observed at the concentration of BA (2 $mgl^{-1}$) which was 2.88±1.09 mm (Table 2). Shoot regeneration frequency decrease with the decrease (1 $mgl^{-1}$) or increase (2 $mgl^{-1}$) in BA level.

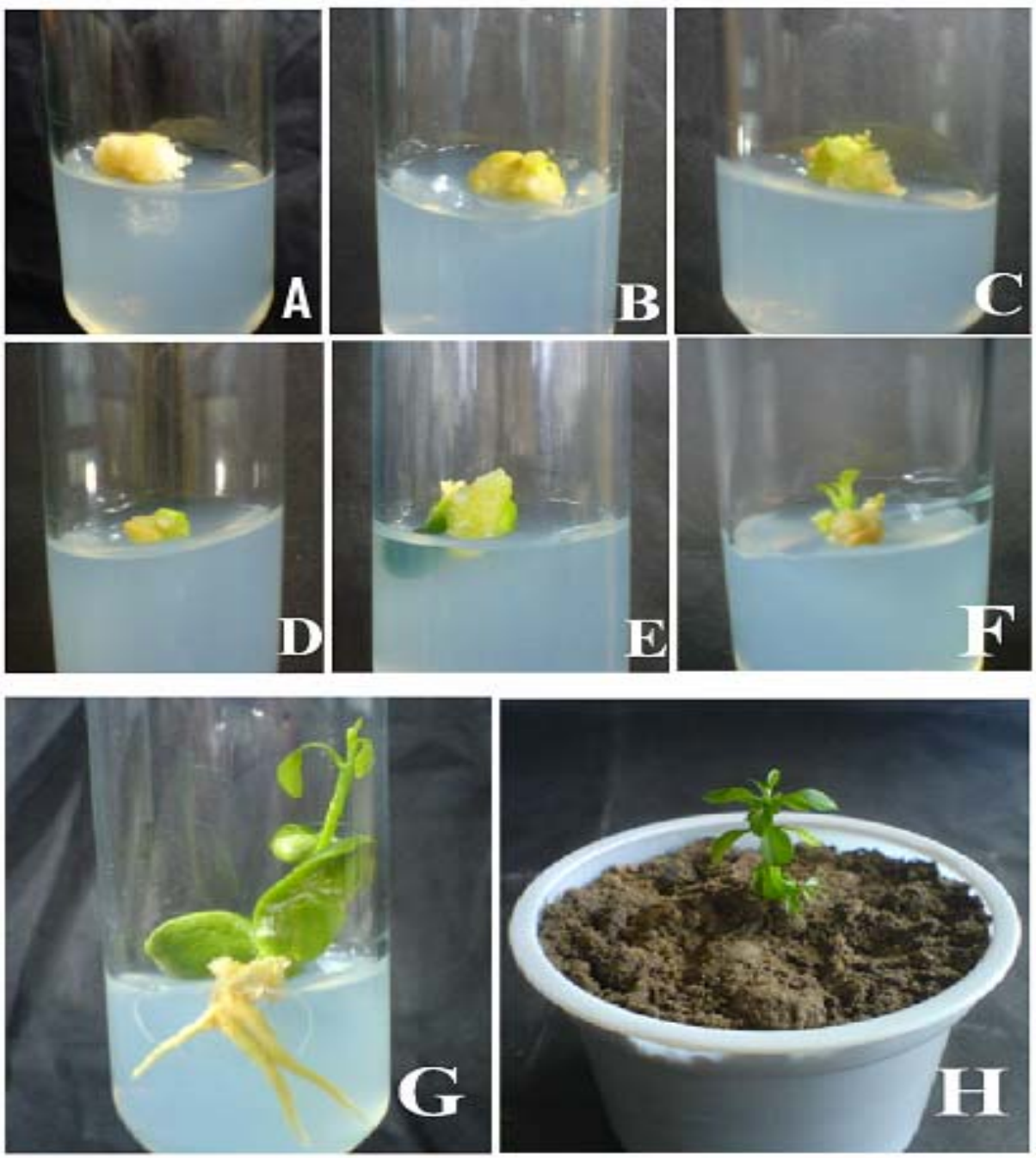


**Figure 1.** Effect of auxin and cytokinin in callus induction. (A) Induction of callus in MS medium + KIN(1.5 $mgl^{-1}$) + 2,4-D(2 $mgl^{-1}$); (B) Induction of callus in MS medium + BA(1.5 $mgl^{-1}$) + 2,4-D(3 $mgl^{-1}$); (C) Induction of callus in MS medium + KIN(1.5 $mgl^{-1}$) + NAA(3 $mgl^{-1}$); (D) Induction of callus in MS medium + BA(1.5 $mgl^{-1}$) + NAA(4 $mgl^{-1}$); (E) Induction of callus in MS medium + BA(1 $mgl^{-1}$) + NAA(3 $mgl^{-1}$); (F) Proliferation of shoot in MS media + BA (1.5 $mgl^{-1}$); (G) Proliferation of root in MS media + IBA (1.0 $mgl^{-1}$); (H) Regenerated plantlet under natural condition in the plastic pot

### *Rooting of in vitro grown shoot*

Individual shoot from *in vitro* grown shoot clumps were excised and after trimming they were transferred to rooting media. Rooting media composed of MS basal salt having 0.6% agar, 3% sucrose and fortified with different concentrations of IBA *viz*; 0.5, 1 and 2 $mgl^{-1}$. Some of this media composition showed good performance in root induction. The highest number of root per explants was observed 3.60±0.78 after 28 days of inoculation in MS media having 1 $mgl^{-1}$ IBA (Fig1.G and Table 3). The lowest number of roots per explants was observed 1.40±0.44 after 28 days of inoculation in the media having 2.5 $mgl^{-1}$ IBA. Data were recorded as mean number of roots per explants and mean length of the longest root per explants in centimeter after 28 days of inoculation and are tabulated in table 3.

Table 3. Effect of different concentrations of IBA on root formation after 4 weeks

| Different concentrations of IBA ($mgl^{-1}$) | Root/explants (M±SE) | Root length (cm) (M±SE) |
|---|---|---|
| 0.5 | 2.40±1.00 | 1.83±0.73 |
| 1.0 | 3.60±0.78 | 2.06±0.82 |
| 1.5 | 3.00±0.62 | 2.13±0.18 |
| 2.0 | 1.60±1.00 | 2.25±0.62 |
| 2.5 | 1.40±0.44 | 1.14±0.28 |

M=Mean, SE=Standard Error

***Performance of regenerated plantlets under in vivo condition***

It was found that more than 85% of plantlets survived during initial establishment. In general, plantlets with active growth of primary roots (1-2 cm) showed greater survival and faster initial growth as compared to the plantlets having longer and branched root system (3-5 cm) at the time of transplantation.

## DISCUSSION

*Citrus* seeds have a very short life because they are injured by drying during storage and thus lose their viability (Johnston 1968). Accordingly we mainly used freshly isolated seeds from fruits. Pierik (1987) reported that for callus induction many factors such as genotype, composition of the nutrient medium and physical growth factors such as light, temperature, humidity, and endogenous supply of growth regulators are important. Several reports have been published about the effects of plant growth regulators on callus culture in different *Citrus* species such as *C. jambhiri* Lush, *C. indoor*, *C. sinensis* Osbec (Uchimiya *et al.* 1983), *C. reticulate*, *C. aurantium*, *C. paradise*, *C. aurantifolia* and *C. reticulate* (Altaf *et al.* 2009). In the present study it was observed that 2,4-D and KIN was the best combination for callus induction in *C. macroptera* (Fig 4A). Altaf *et al.* (2009) reported that Rough lemon (*Citrus reticulata*) and Gada dehi (*Citru saurantium*), both responded in the same way with different combinations of both KIN and 2,4-D had best callusing response in embryonic explants. In the study, the number of direct shoot regeneration was observed 1.20±0.39, 1.60±0.99, 2.80±0.39 and 1.40±0.78 and 1.20±0.39 from the cultured callus using $0.5mgl^{-1}$, $1.0mgl^{-1}$, $1.5mgl^{-1}$, $2.0mgl^{-1}$ and 2.5 $mgl^{-1}$ concentration of BA respectively. Maximum (40%) shoot regeneration observed in 1.5 $mgl^{-1}$ BA concentration. For rooting, the developed shoots cut off and segments were cultured on MS medium supplemented with different concentration of IBA (0.5 $mgl^{-1}$, 1.0 $mgl^{-1}$, 1.5 $mgl^{-1}$, 2.0 $mgl^{-1}$ and 2.5 $mgl^{-1}$). We obtained 80% rooting results in the media containing 1 $mgl^{-1}$ IBA and the highest number of root was observed 3.60±0.78. Low rooting efficiency has been previously reported as major problem for *in vitro* production of *Citrus* plants (Duran-Vila *et al.* 1989). Belarmino and Posas (1999) observed that eight-week-old shoots efficiently produced roots in MS containing 0.5 to 1.0 mg/1 IBA compared to four-week-old shoots. The rooted plantlets also showed healthy and steady growth.

## CONCLUSION

*In vitro* propagation of Satkara (*Citrus macroptera* Mont.) were conducted with a view to develop an ideal protocol for large scale production of as well as detrermination of the most suitable medium compositions for the best response, standardization of growth regulators for maximum callus induction, shoot proliferation and root induction *in vitro* from embryonic explants. BA and KIN used as cytokinin on the other hand 2,4-D and NAA used as auxin. Our results show that up to 87.5% of embryonic explants can produce calli on MS with 2.0 $mgl^{-1}$ 2,4-D combination with 1.5 $mgl^{-1}$ KIN which can produce maximum (40%) shoots on MS with BA 1.5 $mgl^{-1}$. Rooting can be induced in up to 80% of these shoots by using MS medium supplemented with 1.0 $mgl^{-1}$ IBA. Data generated in this study could be practically useful for efficient in vitro propagation of this plant.

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