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Establishment of a Standard Protocol for *In Vitro* Meristem Culture and Plant Regeneration of *Citrus aurantifollia*

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Abstract: *The present investigation was conducted to establish a standard method for meristem culture and plant regeneration with root induction in citrus (Citrus aurantifollia) followed by aclimatization in the natural condition. Dissected apical meristem (20-25 days old field) was cultured onto filter paper bridge in liquid and semi solid MS media supplemented with different concentrations and combinations of auxin (NAA, IBA), cytokinin (BAP) and GA3. Among various combinations, 1.0mg/l BAP + 0.20 mg/l GA3 was found to be the best medium formulation for the primary establishment of isolated meristem. However, efficiency of meristem development was enhanced by addition of 0.50 mg/l GA3 with 1.5 mg/l BAP + 0.5 mg/l NAA. The developing meristems were rescued aseptically and cultured onto solidified MS media containing different concentrations and combinations of auxin, cytokinin and GA3 for shoots and roots multiplication. Among all treatments, semi solid MS medium having 1.5 mg/l BAP + 0.5 mg/l GA3 established as best medium formulation for proper shoot regeneration and elongation. Sub-culturing the in vitro grown shoots with different concentrations of auxin for root induction, best result (100%) was found for MS medium having 0.5mg/l NAA. Finally, well rooted plantlets were gradually acclimatized and successfully established in the field condition.*

Keywords: *Auxin, Acclimatization,Citrus aurantifollia, Meristem*

I. Introduction

Plant tissue culture is an essential component of plant biotechnology offering novel approaches to plant production, propagation and preservation. It is broadly defined as the growth, differentiation and maintenance of cells, tissues, organs or whole plants on artificial nutrient medium under in vitro condition. It is an important tool in both basic and applied biological sciences as well as in commercial applications [1]. The most popular application of plant tissue culture is micro propagation which is an alternative to vegetative propagation of plants. It is now being used for large-scale multiplication of many plant species of diverse groups [2].

1.1 Background

During the last three decades major advances have been made in this fascinating field of research and from being a scientific art, it has become a rapidly expanding technology [3] & [4]. Citrus species are infected with systemic diseases caused by fungi, viruses, bacteria, mycoplasma etc. Attack of pathogens does not always lead to the death of the plant but very often the infection caused by pathogens considerably reduces the yield and quality of the plant. While pathogens are nearly always transferred in plants through vegetative propagation, viral diseases occur in virtually all seed propagated as well as vegetatively propagated crop species [5]. Eradication of pathogens is highly desirable to optimize the yields and also to facilitate the movement of materials across the international boundaries. While *Citrus aurantifollia* infected with bacteria and fungi may respond to treatments with bactericidal and fungicidal compounds, there is no commercially available treatment to cure virus-infected plants. The therapeutic chemicals capable of eradicating virus from infected plants are not available through the use of virazole and vidarabine (antimetabolites) in the culture medium have resulted in the production of virus-free lily and apple plants [6]. To produce disease-free *Citrus aurantifollia*, a healthy nucleus stock could be developed by selecting out one or more healthy plants and then multiplying them vegetatively. Apical meristems in the infected plants are generally either free or carry a very low concentration of the viruses [7]. There are many explanations for this, such as viruses move readily in plant body through the vascular system which in meristems is absent and a high metabolite activity in the actively dividing meristematic cells does not allow virus replication and a high endogenous auxin level in shoot apices may inhibit virus multiplication.

A Knowledge of the gradient of virus distribution in shoot tips encouraged to obtain virus-free plants from infected individuals of Dahlia through shoot-tip cuttings [8]. Morel and Martin (1952) applied tissue culture techniques for elimination of viral infection in Dahlia [9]. Since then meristem culture has now become a popular horticultural practice towards production of pathogen-free plants stock.

1.2 Objective of the study

Citrus aurantifollia is highly cross pollinated crop like other Citrus species that is why it shows genetic variability in different varieties from major to minor. Limes are susceptible to tristeza virus, but the more serious threat is bacterial canker (*Xanthomonas campestris*; citri), which shortens the life of trees by griddling the trunk. But in vitro regeneration is highly effective biotechnological approach to develop true to type plants as well as diseases or insects resistant plants. The present study was undertaken to determine the culture conditions for meristem culture and establish the in vitro plant regeneration techniques from in vitro grown seedling of *Citrus aurantifollia* for production of virus free plant.

II. Materials and Methods

2.1 Plant materials

In the present research work, shoot apical meristem from 20-25 days old field grown plants plants of Lime (*Citrus aurantifollia*) was used as plant materials.

2.2 Other materials

All chemical compounds including macro and micro nutrients, sugar, agar, $HgCl_2$, 70% ethyl alcohol etc. were used in the present study, grade product of Sigma Chemical Co., St. Louis, MO, U.S.A. The vitamins, amino acids and growth regulators were also the products of Sigma, UNI- Chemical Company, China and S.R.L, India.

2.3 Preparation of culture media

The most convenient way to prepare MS medium using commercially available prepacked MS salt formulation supplied by companies such as the Sigma Chemical Company or North Carolina Biological Supply Company. But in this research work MS medium was prepared using all of the individual chemical compounds recommended by [10]. In this way, the first step for the preparation of culture media (MS) was the preparation of the stock solutions. As different media constituents were required in various concentrations, individual stock solutions (I-VIII) were prepared for ready use during the preparation of culture media

2.4 Preparation of stock solution for growth regulators

In addition to the MS-nutrients, it is generally necessary to add one or more growth regulators such as auxins, cytokinin or gibberellic acid to the media to support desired morphological response of tissue and organs [11]. Three types of stock solutions such as main stock(1mg/ml), moderate stock-1(0.1mg/ml) and moderate stock-2(0.01mg/ml) were prepared for each of the growth regulators.

2.5 Preparation of explants and inoculation of apical meristems

Explants of lime were collected from open field. The plant materials were taken in a conical flask and thoroughly washed under running tap water for 1-2 hours to reduce the level of surface microorganisms. Then the plant materials were sterilized by different reagents in aseptic condition of laminar airflow cabinet. The shoot tips (1-2 cm) were excised with the help of sharp blade and collected in a petridish. The exposed meristem tips that appeared as a shiny dome (0.2-0.3 mm) were gently isolated with sharp blade. The culture tubes were deplugged and a singly isolated meristem tip was carefully placed on the M-shaped filter paper bridge of the culture tubes containing MS liquid medium with hormonal supplements for the establishment of primary meristem culture and kept in the incubation chamber.

2.6 Sub-culture for meristem

Two to three weeks after primary culture in liquid medium, the explants those showed morphogenesis response were removed aseptically from the culture tubes and transferred carefully into semi solid MS medium supplemented with different growth regulators for shoot multiplication directly. Then they were transferred into test tubes containing the same or different growth regulator supplemented in semisolid MS media for root induction.

2.7 Sub-culture for shoot multiplication and rooting

Successful shoots formation became evident when the explants grew into a small, leafy structure with several auxiliary branches; they were rescued aseptically from the culture and again cultured to freshly prepared medium containing different combinations of hormonal supplements for multiplication of shoots. When these shoots grew about 4-5 cm in length, they were separated aseptically from the culture vessels and the separated individuals were transferred to freshly prepare rooting media containing different combinations of hormonal supplements.

2.8 Acclimatization and transplantation of regenerated plantlets

After developing sufficient roots system the regenerated plantlets were considered ready to transfer in soil and brought out of the controlled environment of plant growth room. The roots of the plantlets were gently washed under running tap water to remove agar attached to the root zone.

2.9 Methods of scoring growth response for shoot induction

Among the regenerated explants which showed advantageous shoots proliferations were recorded after required days of culture. Percentage of explants induced to develop auxiliary or advantageous shoots were calculated using following formula (Equation 1):

$$\textbf{\% of explants induced shoots} = \frac{\text{no. of explants induced shoot}}{\text{total number of explants cultured}} \times 100$$

Equation no. - 1

2.10 For development of adventitious shoot bud

Mean number of adventitious shoot buds per culture was calculated using the following formula (Equation 2):

$$\text{Mean (M)} = \frac{\left[\sum_{1-i}^{n} Xi\right]}{n}$$

Where, X = the individual reading recorded on each plant.
n = Number of observations.
i = 1, 2, 3----------n
Standard error (SE) was calculated using the following formula.

$$\text{Mean (M)} = \frac{\sum xi}{N}$$

$$\text{Standard error (SE)} = \frac{S}{\sqrt{N}}$$

$$\text{Where, } S = \sqrt{\frac{\sum X_1^2 - \frac{\left(\sum X_1\right)^2}{N}}{N-1}}$$

M = Average weight of callus,
∑ = Summation,
Xi = Total weight of callus,
N = Number of observation
∑ = Summation**.**

Equation no. - 2

2.11 Methods of scoring for the Percentage of explants induced roots

Among the explants, which showed advantageous root proliferations were recorded after required days of culture. Percentage of explants induced to develop advantageous roots were calculated using following formula (Equation 3):

$$\text{\% of explants induced roots} = \frac{\text{no. of explants induced roots}}{\text{total number of explants cultured}} \times 100$$

Equation no. - 3

III. Results

3.1 Primary establishment of isolated apical meristem

In liquid MS_0 medium maximum 60% meristems were induced to develop shoots, whereas 50% response was observed in semi solid medium. The meristems initiate their initial growth by increasing in size and gradually changed to light green colour within 12-20 days of inoculation.

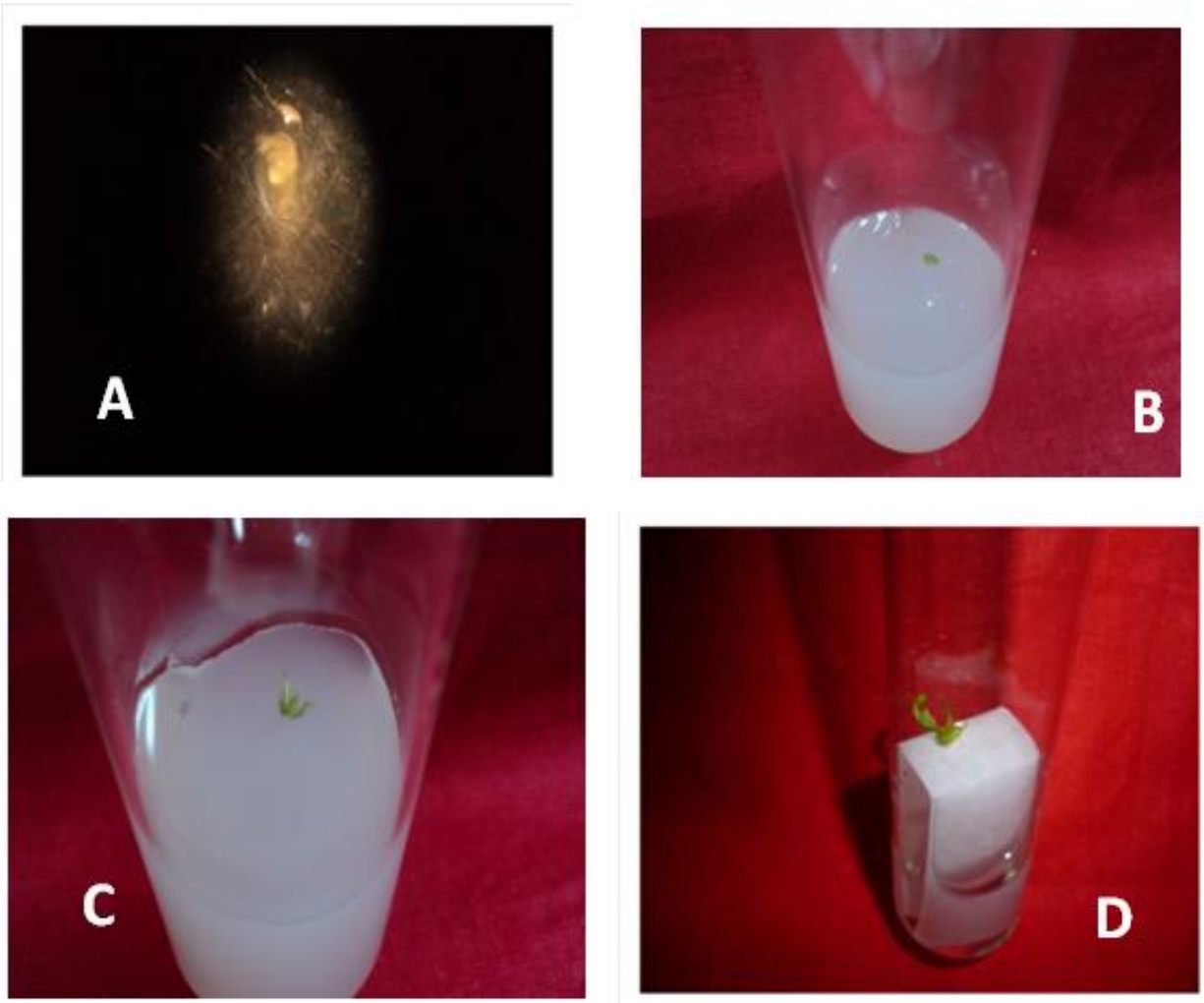


Figure 1: A: Isolated Meristem, B: Establishment of meristem in MS medium, C: Meristem establishment in MS solid medium supplemented with 1.0 mg/l BAP, D: Meristem establishment in MS liquid medium supplemented with 1mg/l BAP + 0.1mg/l GA_3

3.2 Effect of BAP

Results of this study have been presented in Table-1. It has been observed that the cultured meristems started their initial growth by increasing in size and gradually changed to light green in colour within10-19 days of inoculation. The cultures exhibited highest 80% response for fresh growth when they were cultured on MS liquid and semi solid media supplemented with 1.5 mg/l BAP. Second highest response (70%) was obtained when they were cultured on MS liquid medium containing 1.0 mg/l BAP. This experiment indicated that different concentrations of BAP played a major role in establishing the isolated meristem culture of Citrus. Experimental results revealed that 1.5 mg/l BAP was appeared as the optimum dose for primary establishment of isolated meristems and the performance declined when the BAP concentration was decreased or increased from the optimum dose.

3.3 Effect of BAP with GA_3

Depending on the supplemented BAP and GA_3 the meristems resumed new growth within 12-16 days of culture. When the excised meristems were cultured in MS liquid medium supplemented with 1.00 mg/l BAP + 0.20 mg/l GA_3 showed highest 80% of response. Media having 1.0 mg/l BAP + 0.10 mg/l GA_3 gave the lowest percentage (50%) of response within 12-16 days of inoculation. The remaining treatments of BAP with GA_3 also gave satisfactory result in comparison to other treatments for primary establishment of apical meristem.

Table-1: Effect of different concentrations and combinations of growth regulators in MS liquid and semi solid media on primary response of meristem isolated from field grown plant shoots. Each treatment consisted of 10 explants and data were recorded after 21 days of culture.

Hormonal supplements(mg/l)	**Response of meristem culture**			
	Liquid medium		**Semi solid medium**	
	Days to responded	% of explants responded	Days to responded	% of explants responded
MS_0	12-16	60	12-16	50
BAP				
0.50	11-18	60	12-19	50
1.00	10-15	70	11-14	60
1.50	**12-16**	**75**	**12-15**	**80**
2.0	11-17	70	12-16	75
BAP + GA_3				
1.0+0.1	12-16	50	11-16	50
1.0+0.2	**11-16**	**90**	**12-15**	**80**
1.5+0.20	12-17	80	12-17	60

3.4 Shoot regeneration and elongation from established meristem

To evaluate the best performance for shoot regeneration and elongation, the primary established meristems were cultured on semi solid MS medium supplemented with different concentrations and combinations of growth regulators. Data were recorded on percentage of explants responded and number of shoots per culture and presented in (Table-2 and Fig. 2). Different treatments for shoot regeneration and elongation of established meristem are stated below:

3.5 Effect of BAP

Results of this study have been presented in Table -2. The cultures exhibited highest 80% response when they were cultured on MS semi solid media supplemented with 1.5mg/l BAP followed by 60% was obtained when they were cultured on MS medium containing 1.0 mg/l BAP. On the other hand, the lowest percentage of culture response was found 50% on MS medium containing 2.0 mg/l BAP. The highest number of shoots per culture was 5 when they were cultured on MS medium containing 1.5 mg/l BAP followed and the lowest number of shoots per culture was 2 on MS medium containing 2.0 mg/l BAP.

3.6 Effect of BAP with GA_3

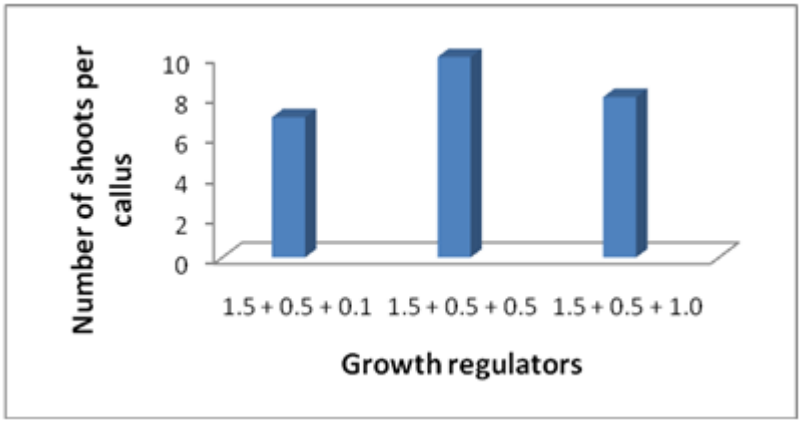
Depending on the supplemented BAP and GA_3, when the established meristems were cultured on MS medium supplemented with 1.5 mg/l BAP + 0.50 mg/l GA_3 showed highest 90% of response followed by 80%on MS medium supplemented with 1.5 mg/l BAP + 0.1 mg/l GA_3. Media having 1.5 mg/l BAP + 1.0 mg/l GA_3 gave the lowest percentage (60%) of response. The highest number of shoots per culture was 7on MS medium supplemented with 1.5 mg/l BAP + 0.50 mg/l GA_3. On the other hand, the lowest number of shoots per culture was 4 on MS medium supplemented with 1.5 mg/l BAP + 0.1 mg/l GA_3.

3.7 Effect of BAP and GA_3 with NAA

When the excised meristems were cultured in MS medium supplemented with 1.5 mg/l BAP + 0.5 mg/l GA_3 + 0.5 mg/l NAA showed highest 100% of response followed by 90% on MS medium supplemented with 1.5 mg/l BAP + 0.5mg/l GA_3 + 0.1 mg/l NAA.MS medium having 1.5 mg/l BAP + 0.5 mg/l GA_3+1.0 mg/l NAA gave the lowest percentage (80%) of response. The highest number of shoots per culture was 10 on MS medium supplemented with 1.5 mg/l BAP + 0.5 mg/l GA_3 + 0.5mg/l NAA. On the other hand, the lowest number of shoots per culture was 7 on MS medium supplemented with 1.5 mg/l BAP + 0.5 mg/l GA_3 + 0.1 mg/l NAA. The remaining treatments of BAP and GA_3 with NAA gave satisfactory result in comparison to other treatments for shoot regeneration as well as elongation.

Table-2: Effect of different concentrations and combinations of auxin and cytokinin for shoot regeneration and elongation from primary established meristems.

Growth regulators (mg/l)	Percentage of culture responded	Number of shoots per culture
BAP		
0.5	55	1
1.0	60	2
1.5	**90**	**5**
2.0	50	4
BAP + GA_3		
1.5 + 0.1	80	4
1.5 + 0.5	**90**	**7**
1.5 + 1.0	60	6
BAP + GA_3 + NAA		
1.5 + 0.5 + 0.1	90	7
1.5 + 0.5 + 0.5	**100**	**10**
1.5 + 0.5 + 1.0	80	8



Graph 1: Response of meristem culture in liquid medium and semi solid medium with hormonal supplements.

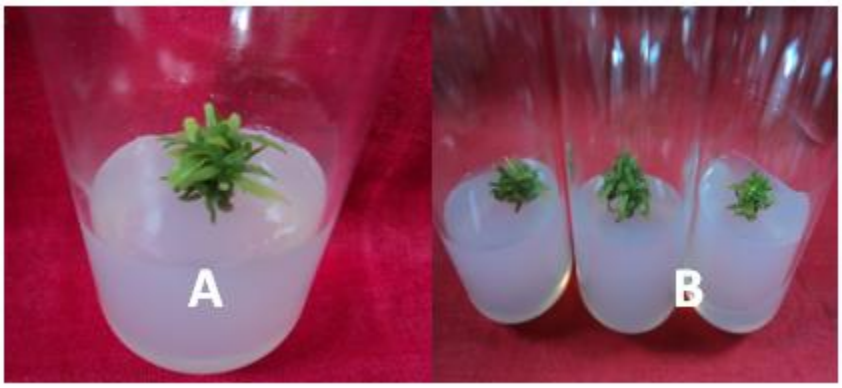


Figure 2: A: Shoot initiation in MS medium supplemented with 1.5mg/l BAP + 0.5mg/l GA_3 + 0.5mg/l NAA, B: Shoot elongation in MS medium supplemented with 1.5mg/l BAP + 0.5mg/l GA_3 + 0.5mg/l NAA

3.8 Root induction from in vitro grown shoots

For root formation, in vitro grown shoots (about 3.4 cm height) were cultured individually on MS medium supplemented with different concentrations of IBA. The summarized results are presented in (Table -3 ,Table-4 and Fig. 3).

Figure 3: A: Root induction from in vitro grown shoot in ½ MS with 0.5mg/l IBA, B: Acclimatized plantlets in pot soil after 7 days and C: Establishment of plant in field after 45 days.

3.9 Effect of IBA on root induction

Among the different concentrations of IBA, 0.5 mg/l was found to be best for root induction and cent percent of root induction was achieved in the cultures. In this treatment, the highest mean number of roots per shoot was10 after 28 days of sub culture and the root induction frequency (96%) were recorded in MS medium having 0.5 mg/l IBA. Further increase or decrease in the concentration of IBA, rooting performance was decreased.

3.10 Effect of NAA on root induction

Among the different concentrations of NAA treated, highest number of roots (10),was observed nature of roots was long and thin and root induction frequency (90%) were recorded in MS medium having 0.5 mg/l NAA. On the other hand, the lowest number of roots (3), and root induction frequency (60%) were recorded in MS medium having 0.1mg/l NAA.

Table-3: Effect of different concentrations of IBA on root induction from in vitro grown shoots.

	In vitro development shoot with ½ MS	Used growth regulator mg/l	% of explants produce root	Average no. of roots after 14 and 21 days
Root induction	IBA			
	0.10	55	4.00	9.25
	0.50	**90**	**8.10**	**18.20**
	1.00	70	5.50	11.75

Table-4: Effect of different concentrations of NAA on root induction from in vitro grown shoots.

Growth regulators (mg/l)	% of culture responded (M)	No. of roots per shoot	Nature of roots
NAA			
0.1	60	3	Long, thin
0.5	**90**	**10**	**Long, thin**
1.0	70	5	Long, thin

3.11 Acclimatization and transfer of plantlets to soil

When the plantlets were 6-8 cm long and well rooted, they were ready for hardening and transplantation into the soil. The caps of the culture vessels containing the plantlets were removed and the plantlets were kept in growth room for 2 days. Then the cultures were transferred gradually from growth chamber to open room and kept there for 3 days. Plantlets were subsequently transferred to larger pots and gradually acclimatized to outdoor conditions where survival rate of the transferred plantlets to the nature was 100%.

IV. Discussion

In the present investigation attempts were made to standardize a reproducible protocol for in vitro clonal propagation of *Citrus aurantifollia*. However, meristem culture is one of the important methods to produce virus free stock plants [12]. Many important medicinal plants and horticultural crops (e.g. bitter gourd, potato, tomato, orchids, lady's finger and strawberries) are routinely free of viral contamination by using this procedure [13],[14] & [15]. Smith and Murashige (1970) accomplished the first true meristem culture of an isolated angiosperm meristem into a complete plant [16].

There have been many reports on using Calcium hypochlorite [17] or Sodium hypoclorite [18] for surface sterilization of explants. $HgCl_2$ solution (0.5%) was used as surface sterilants in this investigation but

non-judicious application of $HgCl_2$ solution in uncertain duration may lead to microbial contamination of the culture as well as tissue killing.

Studies on the regeneration systems of citrus species reported so far had shown the critical effect of cytokinin concentration and types of cytokinin-auxin ratios in regeneration from various types of explants [19],[20] & [21]. Use of shoot tips as the explants was found to be an effective method for in vitro clonal multiplication of *Citrus aurantifollia* [22]. We studied the morphogenetic response of meristem of *Citrus aurantifollia* cultured on MS medium supplemented with different concentrations of BAP alone and in combination with GA_3. After 12-16 days meristematic positive response was found. Best response was found in the medium supplemented with 1.5 mg/l BAP and 1.00 mg/l BAP + 0.20 mg/l GA_3. Then the explants were transferred to shooting medium. After four weeks, shoot buds developed from meristem (fig. 1B). The maximum number of shoots were produced in medium supplemented with 1.5 mg/l BAP, 1.5mg/l BAP with 0.50 mg/l GA_3, 1.5 mg/l BAP with 0.50 mg/l GA_3 and 0.5 mg/l NAA (Table-3). Among these MS medium, supplemented with 1.5 mg/l BAP + 0.50 mg/l GA_3 + 0.5 mg/l NAA showed highest 100% of response followed by 90% on MS medium supplemented with 1.5 mg/l BAP + 0.5 mg/l GA_3 + 0.1 mg/l NAA. MS medium having 1.5 mg/l BAP + 0.50 mg/l GA_3 + 1.0 mg/l NAA gave the lowest percentage (80%) of response. However, Altaf et al. (2008) showed that 2 mg/l BAP with 0.50 mg/l NAA induced multiple shoots from *Citrus jambhiri* [23]. The result of the present investigation supports with the mentioned results. Addition of 0.50 mg/l NAA to the medium containing relatively high concentration of BAP did not improve the rate of shoot proliferation in sour pummelo. This result is in agreement with that of Omura and Hidaka,1992 [24], who found that the addition of 0.50 mg/l NAA is not as critical as that of different concentration of BAP for shoot tip culture of Satsuma mandarin. The results of the present investigation differ with the above results. In addition, Paudal and Haq (2000) found that addition of low concentration (0.10 mg/l) NAA to medium containing relatively high concentration of BAP (2.5 mg/l) did not improve the rate of shoot proliferation in pummelo [20]. One reason for shoot proliferation without addition of any auxin to the medium may be due to the ability of tissues to synthesize the required amount of auxin endogenously [16]. Different concentration of IBA and NAA such as; 0.1 mg/l, 0.5 mg/l and 1.0 mg/l were used in MS medium for rooting excised shoots. The highest number of roots was produced with 0.5 mg/l NAA (Table-4). A study with *citrus grandis* showed similar results with maximal rooting at 0.5 mg/l NAA and a decrease in the frequency of rooting below this NAA concentration [20]. However, in the study by Parthasarathy and Nagarju (1996) [25], MS medium supplemented with 0.05 mg/l NAA was found to be the best for rooting in many Citrus species, excepting Musambi for which the best concentration was 0.2 mg/l NAA.

Among the auxins, IBA were less effective for rooting of *Citrus aurantifollia* than NAA [25]. Root length decreased with higher concentration of NAA and the longest root was found in the 0.5 mg/l NAA treatment (Table-4). Similarly, various rooting responses of Mandarin varieties to different types of auxin were reported by Omura and Hidaka [24]. After four weeks of culture in rooting medium, the elongated plantlets were decapitated and the effect on the growth of axillary shoots was studied. The decapitated plantlets showed the development of two to five axillary shoots within two weeks of culture (Fig. 3). Induction of axillary shoots by decapitation of in vitro generated plantlets has been reported in many plant species [26] & [27]. However, so far there have been no reports of micropropagation of Citrus species from axillary shoot tip induced by decapitation The regenerated plantlets showed 70% survival when transplanted. The success rate was recorded by emergence of two or three new leaves. Morphologically, these plants were slightly yellowish when compared to the parent plant.

References

[1]. Bhojwani, S. S., Plant Tissue Culture: Applications and Limitations, VIII (Elsevier. Sci. Publ., Amsterdam, The Netherlands, 1990) 1-46.

[2]. Debergh, P. C. and Zimmerman, R. H., Micropropagation: Technology and Application (Kluwer Academic Publ. Dordrecht. The Netherlands, 1990 **)** 485.

[3]. Haminersehlag, F. A. and Litz, R. E., Biotechnology of Perennial Fruit Crops, xxi (CAB Intl., London, 1992) 550.

[4]. Bapat and Rao, Regeneration of plantlet from immature leaflet derived callus culture of *Aegle marmelos* (L.) Corr, Plant Tissue Cult**,** 8, 1993, 77-82.

[5]. Kartha, K. K., Elimination of virus. In: Vasil IK (eds) Cell culture and somatic cell genetics of plants, Laboratory procedure and their applications, V-I (Academic Press, Inc.,Orlando, 1984) 577-585.

[6]. Pierik, R. L. M., In vitro culture of higher plants, (Dordrecht, The Netherlands: Martinus Nijhoff Publishers, 1989) 344.

[7]. Razdan, M. K., An Introduction to Plant Tissue Culture, VII (Oxford and IBH Publ. Co. Pvt. Ltd. New Delhi, India 1993) 398.

[8]. Holmes, F. O., Elimination of spotted wilt from a stock of Dahlia, Phytopathology, 38, 1948, 314.

[9]. Morel, G., Martin, C., Gu´eris on de dahlias atteints d'une maladie `a virus, Comptes Rendus de l'Academie des Sciences, Paris 235, 1952, 1324–1325.

[10]. Murashige, T. and Skoog, F., A revised medium for rapid growth and bio assays with tobacco tissue cultures, Physiol. Plant., 15, 1962, 473-497.

[11]. Bhojwani, S. S. and Razdan, M. K., Plant Tissue Culture: Theory and practice, (Elsevier Science Publishers, Amsterdam**,** 1983) 1-502.

[12]. Uddin, M. N., Bari, M. A., and Rahman, M., Meristem culture in eggplant (*Solanum melongena* L.*)* for production of disease free cultivars in Bangladesh, J. Bio-sci., 12, 2004, 1-7.
[13]. Huda, A. K. M. N. and Sikdar, B., In vitro plant production through apical meristem culture of bitter gourd (*Momordica charantia L.*), Plant Tissue Cult. & Biotech., 16(1), 2006, 31-36.
[14]. Prakash, J. and Pierik, R. L. M., Plant biotechnology: Commercial prospects and problems: (Eds), (International Science Publishers, New York, 1993) ISBN 1-881570-31-2, hardback, 289.
[15]. Ali, A. M., Meristem culture for virus-free plant production *Abelomschus esculentus* (L.) Mocnch, M.Sc. Thesis, Breeding Laboratory, Botany Dept. Rajshahi University, 1998.
[16]. Smith, R. H. and Murashige, T. A., In vitro development of the isolated shoot apical meristem of angiosperms, Ameri. J. Bot., **57**, 1970, 561-568.
[17]. Asaduzzaman, M., In vitro Regeneration and GUS Gene Transformation in Rice (*Oryza sativa* L.), Ph. D. Thesis, Rajshahi University, Bangladesh, 2005.
[18]. Jones, J. B., Determining marketes and market potential. In: Tissue Culture as a Plant Production system for Horticultural Crops, R. H. Zimmerman, E. A. Hammerschalag and L. R. H. Grieshbach (eds.), (Dordreeht. Martinus Nijhoff pyblishers, 1986.) 175-182.
[19]. Duran-Villa, N., Ortega, V., Navarro, L., Morphogenesis and tissue culture of three Citrus species, Plant Cell Tissue Organ Cult., 16, 1989, 123–133.
[20]. Paudyal, K. P. and Haq, N., In vitro propagatipon of Pummelo (*Citrus grandis* L. Qsbeck), In Vitro Cell. Dev. Biol. Plant 36, 2000, 511–516.
[21]. Silva, R..P., Almeida, W. A. B., Souza, E. S., Filho, F. A. A. M., In vitro organogenesis from adult tissue of 'Bahia' sweet orange (*Citrus sinensis*), Fruits, 61, 2006, 367–371.
[22]. Al-khayri, J. M. and Aziz, A., In vitro micropropagation of *Citrus aurantifolia* (Lime), Curr. Sci., 81, 2001, 438–445.
[23]. Altaf, N., Khan and Ali, A. R., Bhattiia, L., Propagation of rough lemon (*Citrus jambhiri* Lush.) through in vitro culture and adventitious rooting in cutting, EJEAF Chem, 7, 2008, 3326–3333.
[24]. Omura, M. and Hidaka, T., Shoot tip culture of citrus. 1. Culture condition. Bull, Fruit Tree Res. Stat, 22, 1992, 23–26.
[25]. Parthasarathy, V. A. and Nagaraju, V., Rooting of micro-cutting of certain Citrus species, Indian J. Hortic., 53, 1996, 255-258.
[26]. Hyde, L., Phillip, G., Silver nitrate promotes shoot development and plant elongation of chile pepper (*Capsicum annuum* L.) via organogenesis, In Vitro Cellular and Developmental Biology Plant, 32, 1996, 72-80.
[27]. Sanatombi, K. and Sharma, G. J., In vitro propagation of *Capsicun chinense* Jacq, Biol. Plant, 52, 2008, 517–520.