EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES

http://www.ejbps.com

ISSN 2349-8870
Volume: 3
Issue: 5
573-578
Year: 2016

IN VITRO CONSERVATION OF *CITRUS PARADISI* MACF. MARSHSEEDLESS THROUGH MICROPROPAGATION

***Chandra Gurnani[1], Kamlesh Choure[2], Shinam Mukhija[1] and Vikram Kumar[1]**

[1]Department of Biotechnology, IASE (D) University, Sardarshahar, Churu, Rajasthan.
[2]Department of Biotechnology and Microbiology, Faculty of Life Sciences & Technology, AKS University, Satna, Madhya Pradesh.

***Corresponding Author: Dr. Chandra Gurnani**
Department of Biotechnology, IASE (D) University, Sardarshahar, Churu, Rajasthan.

Article Received on 14/03/2016 **Article Revised on 04/04/2016** **Article Accepted on 25/04/2016**

ABSTRACT
The present investigation was carried out with several aspects such as establishment of culture, shoot & root regeneration, shoot & root length, hardening & acclimatization in Citrus paradisi Macf. Marshseedless, belongs to family Rutaceae also known as Grapefruit. The impact of PGR's on adventitious shoot regeneration in C. paradisi Macf. Marshseedless by carried by using MS basal medium added with BAP & Kinetin (0.5 -4 mg/l) for shoot regeneration and IBA & NAA (0.5-4 mg/l) for rooting either alone or in combination were used. The combination of BAP (0.5mg/l) and Kinetin (2mg/l) had good results in maximum shoot regeneration (70%) and maximum shoot length also. Best rooting (70%) obtained in 3 mg/l IBA. Maximum Root regeneration & length obtained in this medium. In green house, maximum survivals of plantlet were recorded in pots containing sterilized soil, vermiculite and perlite in equal proportion.

KEYWORDS: C. paradisi Macf. Marshseedless, Hardening, shoot regeneration, MS medium.

ABBREVIATIONS
MS: Murashige & Skoog Media; BAP: Benzyl Adenine Purine; NAA: Naphthalene Acetic Acid; IBA: Indole Butyric Acid.

INTRODUCTION
Plant tissue culture techniques have been increasingly applied to many medicinal plants in particular for mass propagation, conservation of germ-plasm and production of bioactive compounds and for genetic improvement. Large scale plant tissue culture is found to be an attractive approach to the traditional methods of plantations because it offers controlled supply of biochemical independent of plant availability and more consistent product quality.[1]

Citrus is the leading tree fruit crop of the world and refers to all edible and rootstock species. The genus citrus includes more than 162 species belonging to the Order Geraniales family Rutaceae and sub family Aurantoideae. Citrus fruits are grown throughout the world and are known for their fine flavor and quality.

Citrus fruits lack a firm pulp. It is heterozygous in nature and thus exhibits a great variability in seedling population. These elite chance seedlings possess desirable horticultural traits can be selected as variety/strains after their evaluation under particular agro-ecological zone. In India collection and conservation of citrus species started long back, however, in the middle of nineteenth century it received major emphasis. In early part, collection and conservation were primarily made for the quality fruits, while current research efforts are for collection of gene pool with distinct desirable traits, which can be utilized for improvement of cultivars.

Citrus originated from south-eastern Asia, China and the east of Indian is archipelago from at least 2000 BC.[2,3,4] Currently, Citrus is cultivated in the subtropical and tropical regions of the world between 40° north and south latitude in over 137 countries on six continents and generates about 105 billion US dollar per year in the world fruit market.[5]

The grapefruit, *Citrus paradisi* Macf., is known today to be an apomictically stabilized hybrid between the pummelo, C. grandis (L.) Osb, also known as C. maxima (Burm.) Merrill and the sweet orange, C. sinensis (L) Osb.[6] The grapefruit (*Citrus paradisi*) is a subtropical citrus tree known for its bitter fruit, an 18th-century hybrid first Red in Barbados.[7] The grapefruit was known as the shaddock or shattuck until the 19th century.

The first known recorded use of the word grapefruit is in Jamaica, where a fruit was grown that was commonly known as the "Barbadoes grapefruit".[8] This early name for the fruit provides a strong clue in pursuing information on the early history of this hybrid. Today's grapefruit, however, has maliform fruits and alate petioles.[9]

Marsh Seedless fruit is medium in size with medium-size oil glands, mildly aromatic extremely juicy and rich in flavour and seeds absent. This variety grown in Florida, California, Texas, Arizona, South America, Australia, South Africa, Israel and India. A local selection, presumably of a seedling 'Marsh', in Surinam is known there as 'Hooghart'.

Grapefruit is an effective aid in the treatment of urinary disorders and cancer. Its inhibiting effect on the metabolism of some drugs may allow smaller doses to be used, which can help to Reduce costs.[10] These are known to have curative value for various diseases of bones and joints, bilious diseases, prevention of capillary bleeding, piles, dysentery, cold, influenza, habitual constipation and scurvy.[11]

Plant Tissue culture techniques are widely applied for the improvement of field crops, forests, horticulture and plantation crops for increased agricultural and forestry production. This technique has been commercialized globally and contributed significantly towards the enhanced production of high quality planting material. *In vitro* cultures are now being used as tools for the study of various basic problems in plants of economic importance in large numbers by tissue culture. In this perspective present investigation will be taken to standardize the protocol through micro-propagation technique in C. paradisi Macf. Marshseedless.

MATERIALS AND METHODS

Collection of Explant

Seeds were taken from the fruit of *Citrus paradisi* Macf. Marshseedless plant which was growing in the Lyallpur Nursery, Teen Puli Road, Sriganganagar (Rajasthan).

Surface Sterilization

Collected seed explants of *Citrus paradisi* Macf. Marshseedless soaked in water overnight, washed with Bavistin (0.2% for 10 minutes) followed by quick rinsing with 70% ethanol. These explants were surface sterilized with 0.1% mercuric chloride for 4 minutes. Finally these seeds were washed with autoclaved distilled water 3-4 times.

Shoot and Root induction

The seeds were extracted from the fruits of *Citrus paradisi* Macf. Marshseedless were cultured on Murashige and Skoog medium supplemented with 3% w/v Sucrose, different concentrations of BAP, Kinetin, IBA, and NAA which was solidified with 0.8% Agar. The regenerated shoots were separated individually and transferred on MS media containing different concentrations of NAA (0.5-4mg/l) or IBA (0.5-4mg/l) for proliferation of roots. The pH of media adjusted to 5.8 with 1N NaOH, 1N HCl and autoclaved at 121°C temperature with 15 lbs pressure for 20 minutes. Inoculated explants were kept under control environment with 2500 lux light intensity at temperature of 25±2˚C for 16 hours photoperiod.[12] Data were collected after two weeks including response of shoot and root regeneration (Table 1 & 2).

Hardening and Acclimatization

Rooted plantlets were carefully removed from the culture tubes and their roots were thoroughly washed under running tap water and cleaned with fine brush to remove adhered agar. After that the plantlets were covered with sterilized cotton wetted with half strength MS medium for 24 hours in culture room followed by the treatment of Bavistin (0.2% for 10 minutes) to prevent fungal contamination. Finally plantlets were transferred to pots containing cocopeat. Pots were kept in greenhouse with 90% humidity and temperature 26±2˚C. [13]

The observations were recorded for number of plantlets survival after 15, 30 and 60 days of planting in pots incubated in greenhouse**.**

STATISTICAL ANALYSIS

The experiments were performed with five replicates and were repeated thrice. The results were subjected to an analysis of variance (ANOVA) and the means were compared using Tukey's Test ($p < 0.05$) between each pair of data. The analysis was performed using SPSS 18.0.

RESULTS AND DISCUSSION

This work sought to assess shoot and root regeneration in C. paradisi Macf. Marshseedless. In this work growth regulators which affects shoot and root regeneration were BAP, Kinetin, IBA and NAA (Figure A to L).

Shoot Regeneration

Table 1 shows the effects of cytokinins types and concentrations on adventitious shoot production. Shoot regeneration and length frequency ranging from 10-70% and 2.3-7.8 cm respectively obtained in the treatments excluding MS Medium without any PGR. Combined effect of BAP (0.5 mg/l) and Kinetin (2 mg/l) showed highest shoot regeneration (70%) and shoot length (7.8 cm) compared to other treatments while minimum shoot regeneration (10%) obtained in 0.5 mg/l Kinetin containing medium (Fig B to F). These results are in agreement with the earlier findings of Rana and Singh[14] in Kagzi lime and Parthasarthy et al[15] in Citrus. They reported that 2mg/l BAP or above suppressed length of shoot. Similar reports were also given by Lane.[16] Baruah et al reported that BAP was superior to kinetin for shoot proliferation in all Citrus species.[17] Vashist et al reported maximum survival of explants (82.30%) on

2mg/l BAP.[18] Otoni and Teixeira reported BAP as the best cytokinin for Citrus shoot proliferation.[19]

Root Regeneration

Table 2 showing the effects of IBA and NAA on the rooting of regenerated shoots obtained from different cytokinin treatments. After this successful establishment, the culture of *Citrus paradisi* Macf. Marshseedless the regeneration of roots from the micro shoot was most important part in present study. Proliferated micro-shoots were subjected to the root formation with different levels of IBA & NAA (0.5-4 mg/l). The root formation was significantly influenced by the concentration of IBA and NAA. All the treatments resulted in root regeneration with frequency ranging from 15-70%. A higher percentage of rooting (70%) with higher shoot length (2.5 cm) obtained in 3 mg/l IBA (Fig. G) whereas no root formation was observed in Control. Karwa reported maximum roots in MS media supplemented with 4.92 µm IBA & 1.11µm BAP in Nagpur Mandarin.[20] The length of root was significantly influenced by different concentrations of NAA & IBA. The lower concentration of Auxin produced lesser roots, medium levels produced more and healthy root. Singh et al reported the maximum root length of micro-shoots in MS medium containing 0.5 mg/l IBA & 0.25 mg/l BAP.[21] Al-Bahrany observed maximum length of root (4.80 cm) in Lime with similar composition in MS medium[22]

Establishment of Plantlets in the Greenhouse

The success rate was recorded by the emergence of two and three new leaves (Fig. H-L). The survival rate was 40%. Singh et al reported 92% success of *In vitro* plantlets in in vivo condition when vermiculite and cocopeat (3:1) ratio used as potting medium.[23]

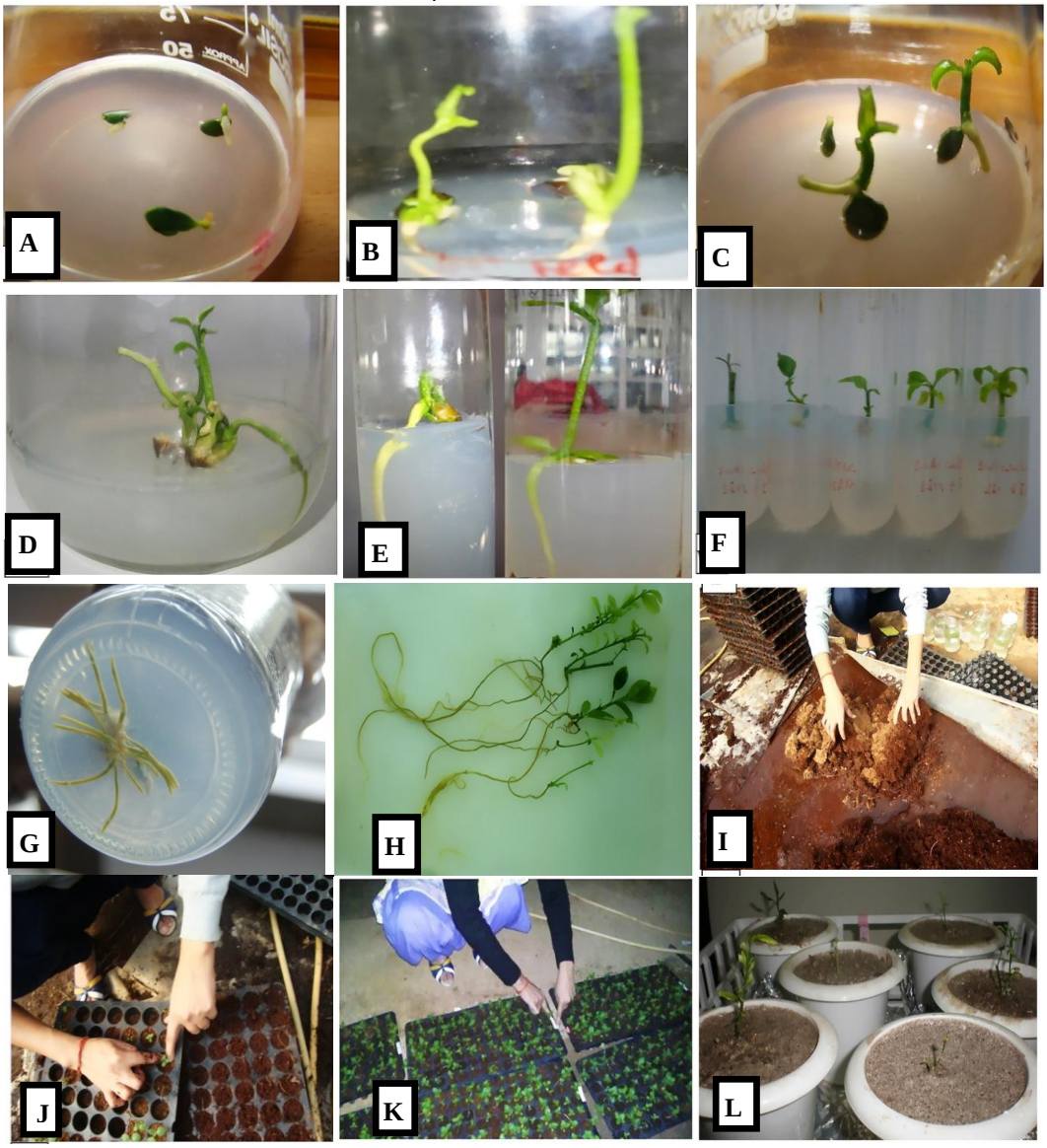

Figure A to L showing *In vitro* propagation of *Citrus paradisi* Macf. Marshseedless (seed explants). A is in Control medium (Without any PGR), B & C showing shoot regeneration, D-F showing Multiplication of Shoot, G showing Root Regeneration, H showing the Hardening and I-K showing the preparation of Cocopeat for hardening and L showing 2 month old Acclimatized plant in Greenhouse.

Table 1 showing Shoot Regeneration

Growth Regulators	Concentration (mg/l)	Shoot regeneration frequency*	Shoot length (cm ± S.E.)	Time taken in days for regeneration
MS	0	0	0 ± 0	40
BAP				
B1	0.5	39.21a	3.5 ± 0.41bc	35
B2	1.0	44.98bc	4.1 ± 0.37a	29.6
B3	2.0	33.19ac	3.8 ± 0.98ab	21.8
B4	3.0	26.55bd	3.5 ± 0.24b	29.8
B5	4.0	0	0	28
Kinetin				
K1	0.5	18.42cd	2.3 ± 0.32c	39.8
K2	1.0	39.21abc	3.4 ± 0.75ce	38.2
K3	2.0	50.74bd	5.9 ± 0.55a	33.6
K4	3.0	33.19ac	4.7 ± 0.19bc	34.6
K5	4.0	26.55d	3.9 ± 0.53ad	29.4
BAP + Kinetin				
T1	0.5 + 0.5	39.21cde	4.8 ± 0.68dfg	40.1
T2	0.5 + 1.0	44.98adf	5.1 ± 0.87abcde	37.8
T3	0.5 + 2.0	56.76abcd	7.8 ± 0.41adg	35.1
T4	0.5 + 3.0	50.74b	7.0 ± 0.21df	38.08
T5	0.5 + 4.0	39.21ef	6.2 ± 0.35cg	40.20
T6	1.0 + 0.5	36.25cf	4.5 ± 0.74be	40.1
T7	2.0 + 0.5	33.19ac	4.4 ± 1.10bde	40.04
T8	3.0 + 0.5	22.73df	3.5 ± 0.29dg	29.14
T9	4.0 + 0.5	0	0 ± 0	30.12

*Shoot regeneration % calculated as angular values. Shoot length values are in Mean ± S.E. On the same column means followed by different letters are significantly different at ($p < 0.05$) (Tukey's Multiple Range Test)

Table 2 showing Root Regeneration

Growth Regulators	Concentration (mg/l)	Root regeneration frequency	Root length in cm	Time taken in days for regeneration
Control	0	0	0 ± 0	40
IBA				
I1	0.5	33.21a	1.1 ± 0.35bc	35
I2	1.0	39.21c	1.2 ± 0.43c	29.6
I3	2.0	42.11b	2.3 ± 0.37a	21.8
I4	3.0	56.76bc	2.5 ± 0.30ac	29.8
I5	4.0	40.38ab	1.8 ± 0.29d	28
NAA				
N1	0.5	22.77cd	0 ± 0	39.8
N2	1.0	26.55a	1.6 ± 0.41a	38.2
N3	2.0	29.98bc	1.8 ± 0.64bcd	33.6
N4	3.0	25.09ad	1.7 ± 0.74ac	34.6
N5	4.0	25.09c	1.6 ± 0.29acd	29.4
IBA + NAA				
T1	0.5 + 0.5	0	0 ± 0	40.1
T2	0.5 + 1.0	39.21abg	1.9 ± 0.16bd	37.8
T3	0.5 + 2.0	42.11abcd	1.8 ± 0.25a	35.1
T4	0.5 + 3.0	44.98c	1.5 ± 0.18cf	38.08
T5	0.5 + 4.0	46.12dg	1.1 ± 0.45ad	40.20
T6	1.0 + 0.5	47.85bdf	1.9 ± 1.02dg	40
T7	2.0 + 0.5	50.74ag	4.5 ± 0.21cf	40.04
T8	3.0 + 0.5	44.98cdf	2.0 ± 0.20aef	29.14
T9	4.0 + 0.5	0	0 ± 0	30.12

Root regeneration % calculated as angular values. Shoot length values are in Mean ± S.E. On the same column means followed by different letters are significantly different at ($p < 0.05$) (Tukey's Multiple Range Test).

CONCLUSION

On the basis of results it is concluded that 0.5 mg/l & 2 mg/l kinetin was better for maximum shoot regeneration and length. The higher levels of both PGRs singly or in combination had negative effect on all parameters. In rooting 3 mg/l IBA is best for root regeneration and length. For hardening treatment plantlets were transferred to pots containing cocopeat.

ACKNOWLEDGEMENT

We are thankful to Dr. O. P. Jangir, IASE University, and Sardarshahar (Raj) India for extending laboratory facility for this work.

Compliance with Ethical Standards

The authors declare that no animal experimentation is conducted during this research.

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Sajc L, Grubisic D & Vunjan-Novakovic G. Bioreactors for plant engineering: an outlook for further research. Biochem. Eng J., 2000; 4: 89-99.
2. Swingle WT. The botany of citrus and its wild relatives of the orange subfamily (family Rutaceae, subfamily Aurantiodeae) In: Webber, H.J., Batchelor, L.D. (Eds.), The citrus industry, University of California Press, Berkeley, 1943; 1: 129-474.
3. Webber JH, Reuther W, Lawton HW. History and development of the Citrus industry. In: Davies, F.S., Albrigo, L.G. (Eds.), Crop production science in Horticulture: Citrus, Cab International, and Wallingford, UK., 1967; 1-242.
4. Gmitter FG, Hu JX. The possible of Yunnan, China in the origin of contemporary citrus species (Rutaceae). Econ. Bot., 1990; 44: 267-277.
5. Ismail M, Zhang J. Postharvest citrus diseases and their control, Outlooks Pest Manag., 2004; 1: 29-35.
6. Robinson T. The organic constituents of higher plants 4. Cordus Press, Massachusetts, USA, 1980; 54-86.
7. Carrington S, Fraser, Henry C. Grapefruit". A~Z of Barbados Heritage. Macmillan Caribbean, 2003; 90–91.
8. Macfadyen J. Some Remarks on the species of the genus Citrus which are cultivated in Jamaica", Hooker's Bot. Misc., 1830; 1: 295-304.
9. Hume HH. The cultivation of Citrus Fruits, New York, The Mac Millan Co., 1926.
10. Gandey A. Cut Cancer Drug Costs by exploring food Interactions, Medscape medical News, 2007; 12.
11. Singh NP, Gill PS, Jawandhwa SK & Kaur H. Genetic Variability in Hill Lemon Strains (Citrus pseudolimon Tanaka) under Punjab Conditions. Nat. Bot Agrobot. Cluj, 2009; 37(1): 238-243.
12. Murashige I, Skoog F. A revised medium for rapid growth and bioassays with tobacco culture Physiol. Plant., 1962; 15: 473–9.
13. Nagpal A, Virk GS, Vijay, Savita. Effect of Explant Type and Different Plant Growth Regulators on Callus Induction and Plantlet Regeneration in Citrus jambhiri Lush. Environ, Wo Int. J. Sci. Tech, 2010; 5: 97-106.
14. Rana JS & Singh R. *In vitro* clonal propagation of Kagzi lime (Citrus aurantifolia swingle) through shoot tips. Prog. Hort, 2002; 34: 27-34.
15. Parthasarathy VA, Barus A, Nagaraju V & Parthasarathy V. Quadratic response of Citrus species to cytokinins and comparative efficiency on morphogenetic characters of *In vitro* proliferated shoots. Indian J. Horti, 2001; 58: 336-41.
16. Lane WD. Regeneration of Pear plants from shoot meristem tips. Plant Science Letters, 1979; 16: 337-42.
17. Baruah V, Nagarajun, & Parthasarathy VA. Micro-propagation of three endangered Citrus species-1 & 2, rooting ex vitro, Ann Plant physiol, 1996; 10: 129-32 and 124-28.
18. Vashist U, Yadav NR, Yadav RC & Yadav OP. *In vitro* micro-propagation in Gurmar (Gymnema sylrestre R. Br) Haryana. J. Hortic. Sci., 2006; 35(1&2): 60-62.
19. Otoni WC & Teixeira SL. Influence of the length and position of juvenile nodal segments obtained from Citrus sinensis (L.) Osb. Cv. Pera moter plants on axillary bud multiplication. Revista Ceres, 1991a; 38: 474-84.
20. Karwa A. *In vitro* propagation of Citrus reticulate Blanco (Nagpur mandarin), Ind. J. Genet, 2003; 63(2): 187-188.
21. Singh S, Roy BK, Bhattacharya S and Deka PC. *In vitro* propagation of Citrus reticulate Blanco and Citrus limon Burm, Hort. Sci., 1994; 29(3): 214-216.
22. Al-Bahrany AM. Effect of phytohormones on *In vitro* shoot multiplication and rooting of Lime (Citrus aurantifolia Christm) Swing, Scientia Horticulture, 2002; 95: 285-295.
23. Singh D, More TA, Awasthi OP, Niwas R, Srivastava S. *In vitro* morphogenesis in Mulberry, Nat. Seminar on "Oppurtunities and Challenges of Arid Horticulture for Nutrition and Live hood, March, 2008; 8-9.