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Recent Advances of Biotechnological Tools on Diverse Species of Citrus: Current Applications and Future Prospects

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Based on the long term conservation of several endangered and indigenous species of *Citrus*, significant impact of biotechnological tools particularly in terms of *in-vitro* micropropagation methods in addition to synthetic seed production using encapsulation of plant propagules including shoot tips, nodal segments, androgenic embryos, embryogenic callus, etc. in sodium alginate has been highlighted in this manuscript. When seed is not available in enough quantity for raising seedlings for rootstock or have low levels of polyembryony and do not produce adequate quantities of nucellar seedlings, then micropropagation techniques could quickly supply *in vitro* regenerated rootstock or budwood. Rapid, mass-production and cost-effective biotechnological tools for propagation of citrus rootstocks and budwood would be of great importance in this regard. Reports on another aspect of long term conservation particularly based on storage of cells, tissues and organs of drought tolerant species of *Citrus* at ultra-low temperature preferably at -196 ºC via applications of cryopreservation techniques using vitrification and encapsulation or dehydration methods has been highlighted in this manuscript. In addition, several research on techniques of *in-vitro* micrografting using superior scion and rootstocks of two different species of *Citrus* with an objective of eradication of virus infected citrus stocks for successful production of grafts have been

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reviewed. Furthermore, effects of explants either through direct and indirect regeneration and conversion into a complete disease free plantlet using suitable synthetic nutrient media along with plant growth regulators at various concentrations and combinations have been highlighted in this manuscript. Hence, the current review is primarily focused on the applications and its effects of superior biotechnological tools for long term conservation of diverse species of citrus for further increasing the potentiality of *Citrus* industries in addition to genetic improvement and genetic resource conservation.

Keywords: In vitro micro-propagation; plant growth regulators; callus induction; regeneration, cryopreservation; conservation.

1. INTRODUCTION

Citrus (*Citrus* spp.) is grown throughout tropical and sub-tropical regions of the world [1] and it is grown in nearly 49 countries around the world. North-eastern region of India is confirmed to be the centre of origin and rich in diversity of *Citrus* (L.) species, where some wild and endangered species namely *Citrus indica*, *Citrus macroptera*, *Citrus latipes*, *Citrus ichagensis* and *Citrus assamensis* exist in their natural and undisturbed habitat. The genus includes several commercially important fruits viz. mandarin (*Citrus reticulata* Blanco), sweet orange (*Citrus sinensis* (L.) Osbeck), grapefruit (*Citrus paradisi* Macf.), lemon (*Citrus limon* (L.) Burm. f.) and lime (*Citrus aurantifolia* (Christm.) Swingle). *Citrus* fruits are reported to be one of the most significant fruits, ranking first with respect to fruit production in the world. Altogether 17 *Citrus* species, 52 cultivars and 7 natural hybrids have been reported to be originated in the North-eastern region of India (Bhattacharya and Dutta 1956). Those species along with its common name, distribution and its uses is described below in Table 1. *Citrus* plants particularly growing in deep forests untouched by abiotic factors have also been confined from the region, thus endowing this region with an exceptional status of “treasure house” of *Citrus* germplasm [2].

The role of tissue culture in genetic engineering and biotechnology was first time exemplified by Kanta and Maheshwari [3]. Gene transfer, selection and regeneration of transformants are nowadays employed by the plant tissue culture techniques [4]. Tissue culture can be apparently encouraged as a substitute to conservative methods such as *in vitro* propagation with the purpose of increasing developmental rate of preferred genotypes and commercial micropropagation [5]. Application of tissue culture biotechnology in the field of agriculture seems very crucial so as to increase agricultural productions including citrus for the purposes of feeding the population without any requirement of international aids. Traditional breeding techniques have several limitations, such as access to a limited gene pool, crossing barriers, polyembryony, parthenocarpy and inefficient selection. Recent developments in biotechnology have opened opportunities to create new cultivars and rootstocks. For successful application of tissue cultural techniques in crop breeding, callus growth and plant regeneration potential of each crop must be determined [6]. Development of an efficient tissue culture and plant regeneration protocol for citrus rootstocks is the first step towards application of transgenic technology to improve *Citrus* breeding and is thus, regarded as the foundation of *Citrus* biotechnological research program [7]. In recent years, techniques of plant tissue culture commonly known as micropropagation are extensively used for rapid clonal propagation of several economic plants as well as restoration of vigour and yield due to infection and preservation of germplasm. Hence techniques of micropropagation can be considered to be a very efficient tool for production of large number of planting materials. In addition, this technique is particularly useful for further protection and conservation of diverse species of *Citrus* from threat of extinction. The importance of tissue culture in *Citrus* research was recognized long back and emphasised by Britters and Murashige (1967) and Kochba and Spiegel Roy [8]. The future attainment of consequence of tissue culture in *Citrus* breeding for improvement and augmenting production was discussed by Kochba and Spiegel Roy [9] and various other aspects of citrus tissue culture by Button and Kochba [9] and Spiegel Roy and Kochba [10]. Current objective of micropropagation is to acquire a large number of genetically identical, physiologically uniform and developmentally normal plantlets preferably with a high potential to survive extreme adverse *ex vitro* conditions in a reduced time period and at a lower cost.

Table 1. Edible Species of Citrus distributed in the regions of North Eastern India

Sl. No.	Botanical Name	Common Name	Distribution	Uses
1	*Citrus medica*	Citron	Garo hills of Meghalaya and Siang districts of Arunachal Pradesh	Table use, rootstock
2	*C. lemon*	Assam lemon	Assam, Meghalaya	Table purpose, juice, cordial, rootstock
3	*C. jambhiri*	Kata jumiri, Rough lemon	Assam	Citric acid extraction, Table purpose by tribals, rootstock
4	*C. karna*	Soh Sarkar	-	Rootstock
5	*C. aurantifolia*	Abhayapuri lime, Acid lime	Assam, Arunachal Pradesh, Sikkim	Table purpose, juice, Pickle
6	*C. limetta*	Mitha, Kagzi	Jaintia Hills (Meghalaya)	Table purpose
7	*C. reticulata*	Mandarin	Meghalaya Arunachal Pradesh, Sikkim	Table purpose, juice, squash
8	*C. nobilis*	King orange	Upper Assam	-
9	*C. indica*	Indian wild orange	Assam, Meghalaya and Garo Hills of Meghalaya	Medicinal value
10	*C. sinensis*	Sweet orange	Meghalaya, Arunachal Pradesh	Table purpose, juice,squash
11	*C.aurantium*	Sour orange	Mokokchung of Nagaland, Khasi Hills of Meghalaya	Oil extraction, rootstock
12	*C. maxima*	Pummelo	Assam, Meghalaya	Table purpose
13	*C. megaloxycarpa*	Bor Tenga	Assam	-
14	*C. ichangensis*	Ichang papeda	Barail range of Naga hills	Inedible, cold hardy
15	*C. macroptera*	Satkara	Shella area of Meghalaya, Manipur, Mikir and North Cachar Hills of Assam, Mizoram and Jampui Hills of Tripura	Used by local tribals for medicinal purpose and in cooking
16	*C. latipes*	Soh Shyrkhoit	Shillong, Mawflong, Pynurslee and Cherapunji of Meghalaya	Cold tolerant rootstock
17	*C. assamensis*	Ada jamir	Karimganj, North Cachar of Assam, Shella and Cherapunji of Meghalaya	Consumed by local people.

[11]

2. ASSESSMENT OF GROWTH REGULATORS ON MULTIPLE SHOOT FORMATION

Multiplication rate of shoot tips is an essential criterion for reduction of cost and genetic purity of micro propagated plants. Plant growth regulators used in MS medium plays a crucial role in achieving desired rate of multiplication and in order to justify the above mentioned findings, it was specifically confined by the researcher [12] thereby indicating an appearance of multiple shoot buds from shoot tip explants of *Citrus megaloxycarpa* Lush cultured on agarized Murashige and Skoog's medium supplemented with 0.25 to 2 mg/L N6 benzyl adenine (BA) alone, and in combination with 0.50 mg/L naphthalene acetic acid (NAA) or with 0.50 mg/L kinetin. Meanwhile, for achieving maximum number of shoots, excised explants were induced on MS medium containing 0.25 mg/L BA along with 0.50 mg/L NAA or 1 mg/L BA with 0.50 mg/L kinetin. In case of micropropagation in *C. aurantifolia* (lime) by using nodal explants of matured tree, nodes were found to be the potent explants for multiple shoot formation resulting in 8.0 shoots per node on MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L kinetin [13]. Considering the effects of plant growth regulators, a concentration of 0.25 mg/L BA in combination with 0.50 mg/L NAA had proven to be most effective, followed by 1 mg/L BA with 0.50 mg/L KN and 0.25 mg/L BA [14]. However, for inducing multiple buds from species *Citrus jambhiri*, 2 mg/L BA along with 0.50 mg/L NAA was found to be most effective. BAP is the most commonly used cytokinin in tissue culture for genus citrus, but the optimum concentration for maximum proliferation varies among species. For instance, shoot tips of *Citrus mitis* requires 4.44µM BAP and *Citrus grandis* requires 1.8µM BAP whereas *Citrus depressa, C. jambhiri* and *Citrus reshni* requires 4.44µM BAP for maximum shoot proliferation [7]. With regards to the effects of elongation of shoots in *Poncirus trifoliate,* maximum shoot elongation was derived from MS

Fig. 1. *In vitro* shoot multiplication and synthetic seed preparation. A)*In vitro* grown seedlings; B) Multiple shoots regeneration from the nodal segments on MS medium supplemented with 2 mg/L BAP; C) Multiple plantlets regeneration from the shoot tips on MS medium supplemented with 2 mg/L BAP; D) Inoculation of synthetic seeds on MS basal medium for germination; E) Geminated synthetic seeds on MS basal medium; F) Acclimatized plantlet from synthetic seeds *[20]*

Table 2. Reports on *invitro* micropropagation of citrus species that has worked on by several researchers

Species	Common Name	Explant Source	Shoot regeneration medium		Root regeneration medium		References
			Basal medium (and % source)	Growth component (mg/l)	Basal medium (and % source)	Growth component (mg/l)	
C. aurantjfolia	West Indian acid lime	Stem, root	Mod. MS (5%)	BA (0.5%)+ ME (1000)	Mod. MS (2%) (C/M)	NAA(1-2) IAA(2), IBA (1-2) NAA(3)	Raj Bhansali and Arya [21,22] Thirumalai & Thamburaj, [23]
C. *jambhiri*	Sohmyndong	Stem	MS	BAP(0.75)	-	-	Parthasarathy & Nagaraju, 1996
C. *aurantium*	Sour orange	Stem	MS (3%)	K(l)+NAA(l)	No Change	No change	Bouzid [24]
C. *grandis*	Shaddock Pummelo	Stem, leaf Stem	Mod. MS (5%) MS	BA(0.25), NAA(0.1), +ME(500), BAP(0.75)	No change -	NAA (0.1-0.5) -	Chaturvedi and Mitra [25] Parthasarathy & Nagaraju, 1996
C. *volkameriana*	Volkamer lemon	Stem	MS	BAP 0.75	-	-	Parthasarathy & Nagaraju, 1996
C. limettioides	Sweet lime	Stem	Mod. MS (3%) (C/M)	BA(0.5), K(0.25),NAA (0.2)+ME(500)	Mod. MS (2%)	NAA (1-2-5)+K(0.1)	Raj Bhansali and Arya [26]
C.limon	Lemon	Stem Root	MS (3%) Mod. MS (3%)	K(l)+NAA(l) BA(l)+2,4-	No change MS (3%)	No change	Bouzid [24] Sauton et al.[27]
	Assam lemon	Shoot tips	MS (3%)	BA(l)+Kinetin(0.5)+ NAA (0.5)	MS (3%)	BAP (0.25) +NAA(0.5)+IBA (0.5)	Singh et al.,[17]
C. *madurensis*	Calamondin	Stem Stem	MS (5%) White 1943(2%) Tukey,1938 (0.5%glucose)	BA(0.1-10)NAA(0.1) +ME (500) Barbitone (5) or ME (50)	MS (5%) No change	NAA(0.1)+ME (500) No change	Grinblat [28] Rangaswamy (1975)
C. paradisi	Grape fruit	Stem, leaf	Mod. MS(5%)(Mrn	BA(0.5),NAA(0.15), +ME(l000)	No change	No change	Raj Bhansali &Arya [28]
Species	**Common Name**	**Explant Source**	**Shoot regeneration medium**		**Root regeneration medium**		**References**

Species	Common Name	Explant Source	Shoot regeneration medium		Root regeneration medium		References
			Basal medium (and % source)	Growth component (mg/l)	Basal medium (and % source)	Growth component (mg/l)	
C. reticulata	Mandarin	Stem	MS (3%)	K (1)+NAA(I)	No change	No change	Bouzid [24]
	Khasi mandarin	Shoot tips	MS (3%)	BAP(I)+ Kinetin	MS (3%)	BAP 0.25+ NAA(0.5)+ IBA(0.5)	Singh et al., [17]
C. limonia	Rangpur lime	Stem	MS (3%)	BA(2)	White 1943 (2%)	NAA(2-5)	Barlass and Skene [13]
C. sinesis	Sweet Orange	Stem, leaf	Mod. MS (5%)	BA (0.25) NAA (0.1)+ME (500)	No change	NAA (0.1-0.5)	Chaturvedi and Mitra [25]
	cv. Mosambi	Nodal segment	MS	BAP(0.25)+ 1M (1.00)	MS (3%)	NAA(I)+IBA (2)	Mohanty et al., [29]
C. sinesis x P. trifoliate	Citrange	Stem	Mst Nitschand Nitsch, 1965 (5%)	BA(10)+ NAA (10)	No change	NAA(I)	Primo Millo and Harada [30]
P. trifoliate x C. sinensis	Troyer citrange	Stem	MS (3%)	BAP(0.08)	-	-	Lukman et al. [31]
	Troyer citrange	Epicotyl Segment	MS (5%)	BAP(I)+ NAA(I)	MS	NAA(2)	Edriss and Burger, [32]
Poncirus trifoliate	Trifoliate orange	Root	Mod.MS (3%)	BA (1)+2,4-0 0.1)	MS (3%)	-	Sauton et al. [27]
C. *indica*	Indian wild Orange	Stem	MS (3%)	BAP 0.75	-	-	Parthasarathy et al.1996
		Stem	MS (3%)	BAP 0.5	-	Soil rite	Baruah et al., [33]
C. latipes	Khasi papeda	Stem	MS (3%)	BAP 0.75	-	-	Parthasarathy et al. 1996
C. *assamensis*	Ada Jamir	Stem	MS (3%)	BAP(0.5)	-	Soil rite	Baruah et al., [33].
C. unshiu	cv. Aoshima Unshiu	Shoot tips	MS (3%)	GA 3(50µm)+ 1 µm BA+ 0.1µmNAA	MS	NAA (0.1µm) or IBA (10µm)	Omura and Hidaka [34].

Source: [35 and 36]

Table 3. Regeneration of shoots from shoot tips and nodal segments of citrus species

Citrus species	Common name	Explant	Response	References
Citrus aurantifolia	Lime	Nodal segments	Auxillary shoot proliferation, Plants *exvitro*	Chaturvedi and Sharma [37,38]
Citrus aurantifolia	Lime	Tiny shoot apices	Shoot growth, plant *exvitro*	Chaturvedi and Sharma [37,38]
Citrus aurantifolia	Lime	Nodal segments	Auxillary shoot proliferation, Plants *exvitro* Attained reproductive phase after one year of transplantation	Singh and Chaturvedi [39]
Citrus jambhiri	Rough lemon	Nodal segments	Auxillary shoot proliferation, Plants *exvitro*	Singh and Chaturvedi [39]
Citrus karna	Karna khatta	Nodal segments	Auxillary shoot proliferation, Plants *exvitro* Attained reproductive phase after two years of transplantation	Singh and Chaturvedi [39]
Citrus limonia	Rangpur lime	Nodal segments	Multiple shoots, Rooted shoots	Barlass and Skene [13]
Citrus limon	Lemon	Shoot tips	Multiple shoots, Plants *exvitro*	Singh et al., [17]
Citrus mitis	Calamondin	Shoot tips and nodal segments of glasshouse grown plants	Shoot regeneration, Plants *exvitro*	Sim et al., [40]
Citrus reshni	Cleopatra mandarin	Nodal segments of glasshouse grown plants	Multiple shoots	Barlass and Skene [41]
Citrus reticulata	Khasi mandarin	Shoot tips	Multiple shoots, Plants *exvitro*	Singh et al., [17]
Citrus sinensis	Sweet orange	Dormant buds	Auxillary shoots	Altman and Goren [42]
Citrus sinensis	Sweet orange	Nodal segments of glasshouse grown plants	Multiple shoots, Rooted shoots	Barlass and Skene [41].
Citrus sinensis	Sweet orange	Nodal segments	Auxillary shoots, Plants *exvitro*	Chaturvedi and Sharma [37,38]
Citrus sinensis x *P.trifoliata*	Carrizo citrange	Shoot apices and nodal segments of glasshouse grown plants	Multiple shoots, Plants *exvitro*	Barlass and Skene [41]

[Source: 43]

Table 4. Regeneration of roots and shoots from diverse species of Citrus

Species	Common name	Explant	Callus Induction medium	Shoot Regeneration Medium	Root Regeneration Medium	Reference
C. acida	Wild orange	Epicotyl	MS + 4.5 µM BA	MS + 4.5 µM BA + 5.8 µM GA3	MS + 5.4 µM NAA	Chakravarty and Goswami, [44]
C. assamensis		Shoot tips	-	MS+22µMBA	Soirlite	Baruah et al.,[33, 45]
C. aurantifolia	Mexican lime	Nodal stem segments		MS + 0.4 µM BA	MS + 16.1 µM NAA	Duran-Vila and Navarro., [46]
C. aurantifolia	Mexican lime	Internodal Stem segments		MS + 33.3 µM BA + 5.4 µM NAA	½ MS + 2.7 µM NAA	Pérez- Molphe- Balch and Ochoa-Alejo [47]
C. aurantium	Sour orange	Internodal seedlings stem sections		MT + 22 µM BA + 5.4 µM NAA	½ MT + 5.4 µM NAA	Moore, [48]
C. aurantium	Sour orange	Mature embryos	MT + 9 µM 2,4-D	MT + 44.4 µM BA + 5.4	MT + 5.4 µM NAA	Beloualy, [49]
C. grandis	Pummelo	Root segments		MS + 0.089 µM BA	MS + 2.5 µM IBA	Goh et al., [50]
C. grandis	Pommelo	Shoot-tip		MS + 1.8 µM BA	½ MS + 5.4 µM NAA	Paudyal and Haq, [51]
C. halimii		Hypocotyl segments		MS + 4.4 µM BA	MS + 2.7 µM NAA	Normah et al., [52]
C. indica		Shoot tips		MS + 2.2 µM BA	Soilrite	Baruah et al., [33,45]
C. jambhiri		Stem and root segments	MS + 0.9 µM KIN + 53.7 µM NAA	½ MS + 22.2 µM BA	½ MS + 5.4 µM NAA	Raman et al., [53]
C. latipes				MS + 2.2 µM BA	Soilrite	Baruah et al., [33,45]
C. limon	Lemon	Stem and root segments	MS + 0.9 µM KIN + 53.7 µM NAA	½ MS + 22.2 µM BA	½ MS + 5.4 µM NAA	Raman et al., [53]
C. limon	Lemon	Shoot tips		MS + 4.4 µM BA + 4.6 µM KIN + 2.7 µM NAA	MS + 1.1 µM BA + 2.7 µM NAA + 2.5 µM IBA	Singh et al., [17]

Species	Common name	Explant	Callus Induction medium	Shoot Regeneration Medium	Root Regeneration Medium	Reference
C. limon	Lemon	Shoot-tip and node		DKW + 0.76 µM ABA + 8.87 µM BA	DKW + 19.6 µM IBA	Kotsias and Roussos, [54]
C. limonia	Rangpur lime	Nodal and intermodal Stem segments		MS + 10 µM BA	W+ 10 µM NAA	Barlass and Skene, [41]
C. medica	Citron	Nodal stem segments		MS + 4.4 µM BA	MS + 16.1 µM NAA	Duran-Vila and Navarro, [46]
C. mitis	Calamondin	Epicotyl, shoot tip and nodal stem segments		MS + 2.2-4.4 µM BA	½ MS + 4.9 µM IBA	Sim et al., [40]
C. mitis	Calamondin	From root of whole seedlings		MS + 2.2 µM BA	½ MS + 4.9 µM IBA	Sim et al., [40]
C. mitis	Calamondin	Leaf from seedlings		MS + 8.9 µM BA	½ MS + 4.9 µM IBA	Sim et al.,[40]
C. paradisi xP. trifoliata	Swingle citrumelo	Epicotyl stem segments		MT + 150 µM Cumarin	MT + 150 µM Cumarin	Grosser and Chandler, [55]
C. reshni	Cleopatra mandarin	Internodal seedlings stem sections		MT + 22 µM BA+ 5.4 µM NAA	½ MT + 5.4 µM NAA	Moore, [48]
C. reticulata	Mandarin	Shoot tips		MS + 4.4 µM BA+ 4.6 µMKIN + 2.7µM NAA	MS + 1.1 µM BA + 2.7 µM NAA + 2.5 µM IBA	Singh et al., [17]
C. reticulata	Mandarin	Internodal stem segments		MS + 33.3 µMBA + 5.4 µM NAA	½ MS + 2.7 µM NAA	Pérez- Molphe- Balch and Ochoa-Alejo [47]
C. sinensis	Sweet orange	Nodal and internodal Stem segments		MS + 10 µM BA	W+ 10 µM NAA	Barlass and Skene, [41]
C. sinensis	Sweet orange	Nodal stem segments		MS + 4.4 µM BA	MS + 16.1 µM NAA	Duran-Vila and Navarro, [46]
C. sinensis	Sweet orange	Nodal and internodal Stem segments		MS + 4.4 µM BA or 13.3 µM BA	MS + 54 µM NAA	Duran-Vila et al., [56]
C. sinensis	Sweet orange	Epicotyl and hypocotyl		MS + 8.89 µM BA +0.76 µM ABA	Not reported	Maggon and Singh, [57]
C. sinensis	Sweet orange	Nodal stem segments		MS + 2.2 µM BA + 0.5 µM NAA	½ MS + 2.7 µM NAA + 2.5 µM IBA	Tapati et al., [58]
C. sinensis xP.	Carrizo	Shoot tips and nodal		MS-KNOP + 22.2	MT + 5.4 µM NAA	Kitto and Young, [59]

Species	Common name	Explant	Callus Induction medium	Shoot Regeneration Medium	Root Regeneration Medium	Reference
trifoliata	citrange	sections		µM BA		
C. sinensis xP. trifoliata	Carrizo citrange	Nodal and internodal Stem segments		MS + 10 µM BA	W+ 10 µM NAA	Barlass and Skene, [41]
C. sinensis xP. trifoliata	Troyer citrange	Epicotyl stem segments		MS + 2.2 µM BA + 0.5 µM NAA	MS + 10.7 µM NAA	Edriss and Burger, [32]
C. sinensis xP. trifoliata	Carrizo citrange	Internodal seedlings stem sections		MT + 22 µM BA + 5.4 µM NAA	½ MT + 5.4 µM NAA	Moore, [48]
C. sinensis xP. trifoliata	Carrizo citrange	Shoot-tip		MS + 4.4 µM BA + 2.5 µM IBA + 296µM AD	MT + 5.4 µM NAA	Starrantino and Caruso [60]
C. sinensis xP. trifoliata	Troyer citrange	Shoot-tip		MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD	MT + 5.4 µM NAA	Starrantino and Caruso, [60]
C. sinensis xP. trifoliata	Carrizo citrange	Nodal stem segments		MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD	MS + 5.4 µM NAA	Starrantino and Caruso, [61]
C. sinensis xP. trifoliata	Troyer citrange	Nodal stem segments		MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD	MS + 5.4 µM NAA	Starrantino and Caruso, [61]
C. sinensis x P. trifoliata	Carrizo citrange	Mature embryos	MT + 9µM 2,4D+22.2 µMBA	MT + 22.2 µM BA + 5.4 µM NAA	MT + 5.4 µM NAA	Beloualy, [49]
P. trifoliata	Trifoliate orange	Nodal and internodal Stem segments		MS + 10 µM BA	W+ 10 µM NAA	Barlass and Skene, [42]
P. trifoliata	Flying Dragon	Shoot-tip		MS + 2.2 µM BA + 1.2 µM IBA + 296 µM AD	MS + 5.4 µM NAA	Starrantino and Caruso, [60]
P. trifoliata	Flying Dragon	Nodal stem segments		MS + 2.2 µM BA + 1.2 µM IBA + 296 µM AD	MS + 5.4 µM NAA	Starrantino and Caruso, [61]
P. trifoliata	Trifoliate orange	Mature embryos	MT + 9 µM 2,4-D	MT + 22.2 µM BA + 5.4 µM NAA	MT + 5.4 µM NAA	Beloualy, [49]

Species	Common name	Explant	Callus Induction medium	Shoot Regeneration Medium	Root Regeneration Medium	Reference
			+ 22.2 µM BA			
P. trifoliata	Trifoliate orange	Hypocotyl		MS + 44.4 µMBA	½ MS + 0.5-5.0 µM IBA	Harada and Murai, [62]
P. trifoliata	Trifoliate orange	tTCL from stem internodes		MS + 10 µM BA + 1 µM TDZ	MS + 5 µM NAA	Van Le et al.,[63]

[Source: 64]

Abbreviations used: AD-Adenine; BA - 6-benzylaminopurine; DKW -Driver-Kuniyuki medium; IBA - indole- 3-butyric acid; KIN - kinetin; MS - Murashige and Skoog medium; MS-KNOP - medium based on MS microelements and Knop's macroelements and vitamins; NAA - naphthaleneacetic acid; TDZ - thidiazuron; tTCL - transverse thin cell layer; W - White medium

medium containing 0.1 mg/L BA alone or with 0.1 mg/L NAA [15]. For achieving highest number of shoots per explants in *C. jambhiri*, response of *in vitro* multiplication through nodal segments on MS medium supplemented with BAP (1.5 mg/L) and malt extract (500 mg/L) was found to be the highest and established well [16]. Shoot tips of size 2-3 mm derived from matured trees of 5-6 years old are responsible for proliferation of multiple shoots in several species of citrus such as *C. reticulata*. Blanco cv. *Khasi mandarin* and *C. limon* Burm.cv. Assam lemon when cultured on MS medium supplemented with 1mg/L BAP, 0.5mg/L kinetin and 0.5mg/L NAA [17]. Considering the importance of shoot number, shoot length and leaf number, highest percentage of multiple shoots were obtained when shoots were cultured on MS medium supplemented with 2.0 mg/L of BAP and 30 g/L sucrose in musambi and lemon. Maximum number of shoots and longest leaf was observed in lemon as compared to musambi (Pérez-Tornero et al. 2010). For regeneration of shoots in several species of citrus, MS medium supplemented with BA alone was considered to be the best treatment [18] whereas on the other hand, kinetin was found to be an effective plant growth regulator for regeneration of shoots (Rahman et al. 1996). In terms of *in vitro* shoot regeneration of kinnow mandarin (*C. reticulata* Blanco) through shoot tip explants obtained from *in vitro* germinated seedlings, it was in conformity by application of MS medium supplemented with 2.5 mg/L BAP and 30 g/L sucrose that supported maximum shoot proliferation at the rate of 2.45 shoots per explant [19]. Another report on multiple shoot formation indicates that when nodal segments of *C. jambhiri* Lush. were inoculated on MS medium supplemented with BAP 2mg/L, regeneration of micro multiple shoots were observed as shown in Fig. 1. [20]. Additional reports on regeneration of *in vitro* rooting and shooting from explants of diverse species of citrus by several researchers have been mentioned in Table 2, 3 and 4.

3. CALLUS INDUCTION

The callus is a rapidly proliferating undifferentiated mass of cells, which can be obtained by culturing explants on nutrient media supplemented with auxins and cytokinins of various combinations [20]. Callus cultures are needed to be sub cultured every 3-5 weeks because nutrients depletion takes place in course of cell growth and medium drying. Establishment of culture for micro propagation or callus induction also depends on the plant part, age of the plant, growing environment of the stock plant and finally the composition of the culture medium. Commonly used explants for callus induction are leaves, stalk, inner node, portion of young twigs, plumule region of germinated seeds, root, rachila, immature inflorescence, anther, microspore, coleoptiles, ovary, ovules, matured seeds, immature seeds, seed embryo, immature embryo etc. 2, 4-Dichlorophenoxyacetic (2,4-D) acid is an organic compound with the chemical formula of $C_8H_6Cl_2O_3$. It is the most commonly used auxin for callus induction and is extremely stable in most conditions. It mimics the action of the plant growth hormone auxin, thus it has been classified as an auxin. It causes cells and the tissues to divide and grow without stopping thereby leading to the formation of callus. In terms of 6-Benzylaminopurine (BAP), it is a first-generation synthetic cytokinin that promotes responses of plant growth and development. BAP is used to induce sprouting in plant explant, induction of shoots, multiple-shoot initiation etc. Plant growth regulator particularly 2, 4-D in combination with BAP is one of the best combinations for induction and development of callus in *Citrus acida* [65] whereas 2, 4-D in combination with BAP was also tested and observed that 5mg/L 2,4D and 1 mg/L BAP had confirmed to be effective in callus induction. At this concentration, response for primary callus induction was 88% (Al Taha et al. 2013). Optimal callus induction response was observed on MS medium, supplemented with1.5 mg/L 2, 4-D from all types of explants, with highest response (92%) and maximum shoot regeneration response (70 %) from callus incubated on MS medium supplemented with BA 3 mg/L [67 and at the same time in genus *C. sinensis*, MS medium supplemented with orange juice stimulated callus growth [68] whereas in Mexican lime, embryogenic calli was induced by 0.5mg/L 2,4 D supplemented MS medium [69]. Among the three explants such as nodal segments, leaf and root segments, nodal segments had been considered as the best explants for induction of callus since calli derived from nodal segments were green and friable as compared to leaf and root segments while callus derived from the leaf segments appeared to be brown and necrotic and therefore it had been confirmed that response to callus induction depended on the type of explants as well as concentration and the type of plant growth regulator used [70]. Consequently, in terms of organic adjuvant such

as Casein hydrolysate, it was significantly considered to be the best medium composition for regeneration protocol developed from suitable explant for callus induction of mandarin and has been confined that mixture of amino acids like Casein hydrolysate, rather than a single amino acid, as very supportive for shoot multiplication even in prolonged cultures [71]. Casein hydrolysates can be a source of calcium, phosphate, several microelements, vitamins and most importantly, a mixture of up to 18 amino acids. Casein hydrolysate overcomes the shortage of glutamine when there is insufficient phosphorus for adequate biosynthesis, however several investigators have concluded that casein hydrolysate itself is more effective for plant tissue culture than the addition of the major amino acids. Another important plant growth regulator particularly known as Indole-3-butyric acid (1*H*-indole-3-butanoic acid, IBA) in the auxin family, with the molecular formula of $C_{12}H_{13}NO_2$ is commonly used for multiple shoot formation in different combinations with other plant growth regulators. For induction of callus and regeneration of shoots in various species of citrus, MS medium supplemented with combination of IBA and NAA had been observed to be favourable for clonal propagation of *C. sinensis* and hence considered IBA (2.64 μM/L) as best treatment with 100 % of the explants producing roots among different concentration of IBA (0.98 to 4.9 μM/L) [71]. A protocol was developed for micropropagation of elite plants of sweet orange (*Citrus sinensis*) through nucellar embryo culture and confined NAA (1.0 mg/L) or 2, 4-D 1 mg/L) supplemented MS medium had encouraged callus development in both nucellar and zygotic embryos [72]. At lower concentration, NAA stimulates cell elongation rather than those required to stimulate cell division [73]. NAA takes part both in plantlet regenerations and root initiation. Establishment of an association between embryo formation and endogenous hormone levels in sweet orange and satsuma mandarin from liquid culture callus was developed where endogenous auxin level was found to decrease in callus that underwent embryo formation and remained relatively high in callus that had no embryo formation [74]. During conduction of an experiment on *in vitro* micropropagation and callus induction in acid lime (*C. aurantifolia*) cv. Sai Sarbati, highest callus induction with epicotyl was obtained when cultured on half-strength MS medium supplemented with NAA (10.0 mg/L) and BAP (0.5 mg/L) (Kamble et al. 2012). An efficient protocol was developed for *in vitro* embryogenic callus induction and regeneration of rough lemon (*C. jambhiri* Lush.) indicating that MS medium fortified with NAA (0.5mg/L) in combination with BAP (3.0 mg/L) and kinetin (1.0 mg/L) had good regeneration potential, highest number of shoots and shoot length and also took minimum number of days for regeneration [75]. In epicotyls segments, maximum callusing was obtained when MS medium was supplemented with NAA (10.0 mg/L) in combination with BA (1.0 mg/L), KN (0.5Mmg/L), sucrose (6%) and galactose (3%) [76]. In contrary, based on the combination of reports in terms of hormones, it had shown that the best combination of hormones is treatment D2B2 (2 mg/L, 2, 4-D, Dan 2 mg/L BAP) producing embryogenic callus of *Citrus microcarpa* [77]. Among excised explants such as stem, leaf and root for induction of green callus, after 20 days of culture, stem explants of *C. assamensis* were most responsive as compared to leaf explants and exhibited very less percentage of green calluses after 40-50 days of culture. However, root explants were found to yield a mixture of yellow and green callus [78]. When shoot segments of lime were cultured on MS medium containing 2, 4-D and coconut milk, maximum percentage of callus was induced [79]. Further, embryo proliferation was greatest on MS medium supplemented with kinetin (1.5 mg/L). In addition, shoot induction was highest on MS medium along with BAP (2.0 mg/L). MS medium fortified with higher concentrations of 4 mg/L 2, 4-D induced maximum percentage of callus from leaf segments (98.66%) and at lower concentration such as 1.0 mg/L produced maximum percentage of callus from nodal segments (96.00%), whereas in case of root segments, callus induction performance was lower (48.66%) when MS medium was supplemented with 2, 4-D at the rate of 2.0 mg/L [80]. Relatively, callus induction and somatic embryogenesis formation was observed from undeveloped ovules in citrus by the use of 500 mg/L malt extract on MS medium [81]. In *vitro* plant regeneration of *C. aurantifolia* through callus culture, shoot tip, epicotyls and hypocotyl segments reported callusing on MS medium enriched with BAP (5.0 mg/L) and observed highest per cent of callus and shoot regeneration with 5.0 mg/L BAP [82]. Callus induced from cotyledons of *C. jambhiri* could be maintained in culture for more than a year and was found to regenerate in 58 % of cultures even after 420 days of culture. However, regeneration capacity of the callus decreased with increasing age of the callus (Chakraborty and Goswami 1999). Callus obtained from

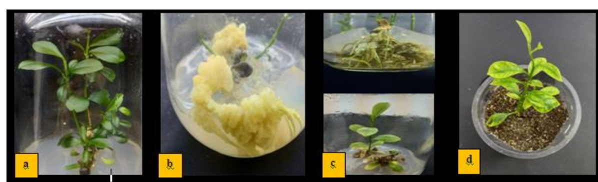

Fig. 2. (a-d). Germination and regeneration of plant of *Citrus jambhiri* Lush.from callus of cotyledonary explants on plant growth regulators supplemented MS medium

a.Germination of matured seeds of Citrus jambhiri Lush. b.Callus induction from cotyledonary explants of Citrus jambhiri Lush.c. Regeneration of roots and shoots from cotyledonary derived callus d. Transplanted plantlet into a plastic cup for acclimatization

(Source: Unpublished photographs of Priyanka Sharma and Bidhan Roy)

cotyledons of *C. jambhiri* Lush. was induced on regeneration medium for proliferation of shoots and roots and the well-established plantlet after complete formation of roots and shoots were further transferred into plastic cups for hardening as shown in Fig. 2(a-d). Since, several researchers have used different species, genotypes, explants and different concentrations along with different combinations of plant growth regulators for callus induction in addition to *in vitro* mass-multiplication of citrus, hence in terms of achieving highest percentage of callus, the best part of explants could be cotyledons and nodal segments supplemented with 1.0 mg and 2.0mg/L of 2,4D. These findings are in agreement with the findings of Badr-Elden [83] who also reported that cotyledons and stem explants had given the best results in terms of callus induction by inoculating it in 1.0 mg and 2.0 mg/L of 2,4D.

4. REGENERATION OF ROOTS

Several factors associated with rooting of micro shoots have been observed and taken into consideration which includes nature of cuttings, rooting co-factor, synergistic role of exogenously applied growth hormone and endogenously present co factors in the rooting, relative efficiency of different auxins as well as their combination and methods of application [84,85]. Another essential factor such as high light intensity helps in better proliferation of roots thereby encouraging it for further acclimatization which renders them more tolerant to moisture stress and diseases [86]. Lower concentration of salt medium has also been proven satisfactory for initiation of roots from shoots in a large number of plant species. Although shoot multiplication was established satisfactorily in full strength MS medium, however salt concentration was reduced to half during root initiation [87]. Mostly some auxin induces the rhizogenesis by prompting division of meristematic cells, cell elongation and differentiation into root primordial [88]. An experiment with *C. grandis* resulted in maximal (number) rooting at 2 mg/L NAA, and a decrease in frequency of rooting with NAA concentration @ 2 mg/L [89]. Rooting of micro shoots regenerated particularly from nodal explants had been considered to be the best among all the excised explants cultured. Treatments including MS medium with IBA at 0.0, 0.5 and 1.0 mg/L and NAA at 0.0, 0.5 and 1.0 mg/L were evaluated for rooting and NAA at 0.5 mg/L resulted in best rooting response among all the treatments (El-Sawy et al. 2006). Epicotyls have been proven to be the most beneficial explants for standardization of regeneration protocols because of its apparent *in vitro* morphogenic response and therefore, transfer of healthy shoots to rooting medium containing IBA gives most appreciable percentage of rooting in citrus. For regeneration of roots in species *C. acida*, among two plant growth regulators used specifically NAA and IBA, NAA was being considered as the best treatment when supplemented with MS medium as compared to IBA [90]. MS medium supplemented with 0.05 mg/L NAA was found to be suitable for rooting in many Citrus species, excepting Musambi for which the best concentration was 0.2 mg/L NAA [91]. A study on *in vitro* propagation and rooting in some citrus rootstock through tissue culture was carried out in Troyer citrange and Carrizo on MS medium supplemented with BAP (1.0 mg/L), NAA (1.0 mg/L) and GA3 (1.0 mg/L) and had obtained optimum growth and development other than MS supplemented with BA (1.0 mg/L) and NAA (1.0 mg/L) in Sour orange cv. Trunk [92].Good response for *in vitro* rooting in Mosambi (Jaffa) was obtained and further recorded longest regenerated roots of 5.33 cm on half strength MS medium supplemented with NAA (0.5 mg/L) combined with IBA (0.5 mg/L) [93]. Percentage of

shoots that produced roots in sweet orange variety 'Para' was considerably higher in media with NAA and IBA than with NAA alone [94] and also reported highest rooting percentage of 77 % on MS medium containing NAA (1.0 mg/L) combined with IBA (1.0 mg/L) in *C. jambhiri* [95]. Meanwhile, maximum rooting of shoots (1.11 %) was derived in rootstock Rough lemon followed by Cleopatra mandarin for MS media (half strength) supplemented with IBA @ 10 mg/L [7]. Based on the results of effect of bio-regulators on rooting of *in vitro* raised micro shoots in two *Citrus* species, namely, Khasi mandarin and Sweet lime, it was significantly observed that medium having NAA at 0.1 mg/L resulted in the maximum rooting (87.71 %) with longer root length of 46.79 mm [96]. Paclobutrazol increased root diameter but reduced root length and the growth regulators used in culturing of Sweet lime accounted a lower rooting percentage (6.83 %) than mandarin (51.75 %). Considerably, among all the plant growth regulators used, IAA, IBA and NAA in particular have been apparently considered as rooting hormones in plant tissue culture. Comparatively, NAA had been found to be superior to IBA for *in vitro* root induction (75%) in Pummelo when shoots were transferred into half strength MS medium supplemented with 1.3, 2.7 and 5.4 µM of NAA [97].While conducting a research based on an efficient plant regeneration protocol from callus cultures of *C. jambhiri* Lush. resulted in maximum rooting response (91.67 %) on half strength MS medium supplemented with 0.5 mg/L NAA [98]. Reports on regeneration of *in vitro* rooting and shooting on assorted species of citrus have been presented in Table 2, 3 and 4. Standardization of an interesting protocol for root initiation [99] using 1/2 MS medium added with 1.0 mg/L of NAA for *in vitro* propagation of *C. limon* was developed. *In vitro* regenerated micro-shoots were rooted best when MS medium was supplemented with 100µM NAA [100]. Hence from the existing results in terms of root induction from *invitro* grown seedlings, inoculation of epicotyl segments on 1.0 mg/L NAA in combination with 1.0 mg/L IBA can be considered as the best protocol for root initiation.

5. *In vitro* MICRO-GRAFTING

It is a potential technique of combination of superior scion and rootstock resulting from an *in vitro* graft multiplication obtained from rapid *in vitro* multiplication of plantlets [101]. This technique was first developed by Murashige et al. [86] in order to obtain virus free citrus plants. Later on this technique of micrografting was further modified and applied in diverse species of citrus by several researchers [57]; Navarro and Juarez [102]; Roistacher et al. [103] and Roistacher and Kitto [104]. Propagules used for micrografting in order to obtain virus free planting materials in citrus includes nucellar seedlings of polyembryonic cultivars [105]. Since this method of obtaining mass of nucellar seedlings requires a longer period of time for conversion from juvenile to adult phase the method has become a limitation for further propagation [106]. Hence the method of *in vitro* micrografting in the form of *invitro* multiplication technique has proven to be very practical in the regeneration of entire orchards of citrus infected by viruses [107]. Standardisation of protocols for conducting experiments on shoot tip grafting in Nagpur mandarin, Darjeeling oranges [108], Khasi mandarin (*C. reticulata* Blanco.) [109], sweet oranges (*C. sinensis* Osbeck.) [110] for elimination of virus infected citrus stocks and increasing the efficiency of this technique was developed by several researchers [111,112]. For eradication of virus infected citrus plants and successful production of graft, size of the shoot tips has to be taken into consideration. Through this technique, 30% to 50% successful grafts were obtained which were further transferred under the field conditions and established a survival rate of 95% [113].Well established micrografted plants were observed to be free of virus and virus like diseases as well as absence of juvenile characters which were previously present in the explants source [114]. Techniques of *in vitro* micro-grafting involving incision of apical meristem on the seedling rootstock is difficult and time consuming and hence incision of shoot tip in contact with the vascular ring or in the cortical surface in an inverted T position have been the most successful technique [115. Increase in production of micrografted plants increases with the pre-treatment of shoot tip and seedling rootstock. Relatively, it was observed that pre-treatment of apex and decapitated seedlings for ten minutes in a solution of 0.5 ppm BAP before micro-grafting increased the emergence of microshoots from 73% to 91% [116]. Application of an advanced and modified technique of rectangular and triangular hole [117] instead of inverted cut in terms of incision of scion in micrografting had increased the percentage of healthy grafts upto 60% whereas incision of scion in an inverted T cut method had produced only 20% of successful grafts. Techniques of *in vitro* micrografting in citrus

species provides micro shoot mersistems of less than 1mm which can be easily regenerated where such small excised shoot meristems cannot be regenerated despite of time consuming and tedious. In Spain, 31 million healthy grafts and plants were produced successfully with an increase in fruit production in citrus orchards through micrografting techniques [118]. Apart from using rootstocks of several citrus species such as Carrizo citrange, Trifoliate orange, rough lemon, Rangpur lime and Karna khatta, the most potent cultivar in which its seedlings can be utilised for micrografting is Troyer Citrange. Incision of excised shoot meristems of length 200µm to 1 mm of two citrus species namely *Citrus aurantifolia* and *C. sinensis* in addition to using *invitro* raised seedlings of 2-3 weeks old of some particular species namely *C. jambhiri*, *Citrus karna* and *Citrus limonia* or *C. limon* had been proven to be successful and proficient in terms of micrografting [119]. Among the diverse standard protocol in which a shoot meristem was incised on the cut face of epicotyl, its incision in contact with the cambial tissue of epicotyl gave the most excellent results [120]. Use of shoot meristems of length 500µm derived from scion of rootstock species *Citrus limonia* or *C. limon* resulted in 57.14% success in *C. aurantifolia* and 42.85% success in *C. sinensis* [121]. The percentage of successful micrografts obtained in *Citrus limonia* or *C. limon* was considerably lesser than the percentage obtained in *C. aurantifolia* (65%) and *C. sinensis* (50%) using seedlings (70% and 60%) correspondingly. Meanwhile the enhancement in increased percentage was due to the utilisation of bigger sized explants of shoot meristem culture which clearly determines that the increase in percentage of successful micro-grafting differed not only with two scion species but also with their respective rootstock combinations [16]. Considering the importance and its advantages of using nucellar and zygotic seedlings in terms of maintaining genetic uniformity and free from pathogens, it was being apparently observed that despite of obtaining slow growth of grafted shoot meristems from nucellar seedlings as compared to zygotic seedlings in *Citrus karna*, emphasis was mainly laid in using nucellar seedlings of rootstocks due to its genetic uniformity and pathogen free plants [122]. A research was conducted on effect of rootstock and its age on achievement of shoot tip grafting. Three types of rootstocks were used viz., Rough lemon, Troyer citrange and Carrizo citrange of different ages and incised with shoot tip scion of Nagpur mandarin. The success percent of *in vitro* micrografting was dependent on the type of rootstocks used. Apparently, successful rate of *in vitro* shoot tip grafting was relatively more in Troyer citrange followed by Carrizo and Rough lemon. In terms of age of rootstock, it did not show any development on success. In rough lemon, rate of success was increased at the age of 10-12 days, whereas in Troyer citrange, it increased at the age of 8-13 days and in Carrizo, between 10-12 days. Hence, highest rate of success for all the three rootstocks were derived at the age ranging from 8-13 days [122]. By the application of an advanced technique of micro-grafting, virus free plants were produced by *in vitro* grafting or incision of *Eremocitrus glauca* and *Feronia limonia* (*Limonia acidissima*) apices on Troyer citrange rootstocks [123]. In addition, a particular method for obtaining virus free plants was developed thereby attaining a successful rate of 100 per cent by culturing rootstock seedlings of Troyer citrange *in vitro* which further stored at 4 degree Celsius in darkness for up to 14 months after attaining a size of 30-40 mm long to overcome the problem of obtaining fresh seeds throughout the year [124].

6. SYNTHETIC SEED PRODUCTION

Synthetic seed was produced first time by Kitto and Janick [125] involving carrot somatic embryos. They used polyoxyethylene, which is readily soluble in water, dries to form a thin film, does not support the growth of microorganisms and is non-toxic to the embryo, leading to the production of desiccated synthetic seed. Synthetic seed is produced by enclosing viable plant materials such as somatic embryos, androgenic embryos [124], pro-embryos, embryos-like-structure [125], protocorms [126], protocorm-like-bodies [127], axillary buds [128], meristem [129], shoot segments [130], shoot tips [87] etc. in alginate with nutrient sources. For encapsulation, plant propagules are mixed with sterilized sodium alginate (3% w/v), which is prepared in suitable tissue culture basal medium supplemented with sucrose. Propagules are then picked up individually and dropped into sterilized aqueous solution of 3% (w/v) calcium salt solution [$CaCl_2$ or $(CaNO_3)_2$] with occasional agitation [124]. Calcium alginate beads are formed within 15-30 minutes. The size of the beads depends upon the inner diameter of the pipette nozzle. Shoot tips are considered to be most suitable for encapsulation and preparation of synthetic seeds as it produces true-to-type planting materials. Gholami and Kavani [131],

used 3-4% sodium alginate and 100 mM of $CaCl_2.2H_2O$ for preparation of synthetic seeds. They found that 4% sodium alginate and 100 mM of CaCl2.2H2O was suitable for maximum conversion of synthetic seeds of hybrid citrus [*C. paradisi* Macf. (Duncan) × *C. reticulata* Blanco. (Dancy)] into plantlets. Encapsulation of shoot tips derived from pre-existing meristematic tissues is considered to be the most efficient method for development of plantlets. Nodal segments were more appropriate than the shoot tips for *in vitro* multiplication of plantlets in *C. jambhiri* Lush. In terms of *in vitro* mass multiplication of plantlets, inoculation of nodal segments on MS medium fortified with 1 and 2 mg/L of BAP (6-bezylaminopurine) was found to be appropriate resulting in (10.18 and 13.05 plantlets/explants) respectively. Synthetic seeds were prepared using 2.5% sodium alginate dropping into 3.0% $CaCl_2$ solution. Maximum germination was recorded when beaded shoot tips were cultured on MS medium fortified with 1 and 2 mg/L of BAP (96.67 and 100.00%) (Figure 2.[a-f]). However, the germination of synthetic seeds was found to be comparatively high than the earlier findings. The results support the use of encapsulated unipolar explants for synthetic seed preparation.

7. CRYOPRESERVATION

Storage of plant cells, tissues and organs at ultra-low temperature, preferably in liquid nitrogen (-196 ºC) via cryopreservation is the most indispensable technique in order to provide safest and cheapest conservation of germplasm in terms of long term conservation techniques. For long term conservation of plant genetic resources, concept of advanced and developed cryopreservation techniques can be used as an efficient tool in many laboratories globally. Commonly used cryopreservation techniques like vitrification and encapsulation or dehydration method are preferably used in terms of germplasm conservation. Techniques of vitrification involving cryogenic storage can be efficiently combined with several vitrification methods such as droplet vitrification, encapsulation or vitrification and cryo-plate. At the same time, maintenance of genetic stability of different plant species after cryo-storage has become a major concern for cryo-bionomists. Culturing of plant species under standard culture conditions have resulted in no or small variations among mother plants and cryo-preserved plants [88]. Cryopreservation of embryogenic callus and somatic embryos [132] is not worth to be an ideal germplasm preservation method because of its probability of genetic infidelity [133]. Similarly a distinct endeavour to preserve *C. aurantifolia* through its regenerative excised root culture may hardly value being a practicable method in no less than two counts. First it has been established for only a period of 3 years and secondly a low frequency of shoot regeneration [134]. Development of standard protocols involving long term conservation of several species of citrus particularly *Poncirus* includes cryopreservation followed by air desiccation, vitrification and encapsulation dehydration can be efficiently used. Explants of 17 species of North Eastern states of India involving seeds, zygotic embryos, embryonic axes etc. have been cryopreserved as per the reports of several researchers [135, 136].Due to the economic importance of commercially important drought tolerant species like *C. reticulata, C. grandis, C. medica* and *C. aurantifolia* and *C. jambhiri* (rootstock), the need has been recognized for the establishment of a cryo-gene bank base collection for the long-term conservation of a substantial fraction of genetic variability [2]. As per the evidence of published reports concluded so far in terms of commercially important species; *C. aurantifolia, C. grandis*, *C. reticulata* and *C. medica*, it has been confined that seeds are partially desiccation tolerant indicating intermediate or Type II whereas *C. jambhiri* is under the category of desiccation sensitive indicating recalcitrant or Type III [137].

8. *In vitro* CONSERVATION OF *CITRUS* GERMPLASM

Failure of conservation of Citrus genetic resources under natural habitat is increasing due to diverse biological and environmental factors that have apparently resulted in genetic erosion from nature and collection centres. Factors such as cutting of trees for urbanization, several activities such as some cash crops introduced by the farmers and large scale deforestation are the primary reasons for genetic diversity losses [138]. Citrus being a heterozygous and open pollinated tree, its germplasm cannot be preserved through seed storage. As a conventional practise therefore preservation of citrus genotypes is done through maintenance of field collections of specimen plants which however is not advisable since it is attacked by a number of pathogens. Genetic improvement of a species depends on safe conservation and efficient utilization of the indigenous genetic diversity available. Many of the Citrus species

are found growing in their natural habitats in a wild or semiwild state [139]. In view of the rapid destruction of these natural habitats and biotic and abiotic threats foreseen in the field gene banks, there is an urgent need to conserve the vast genetic resources of Citrus for effective utilization in improvement of existing varieties and rootstocks through conventional and biotechnological methods. Thus Germplasm preservation can effectively be done through long term culture of proliferating shoots under normal *in vitro* growth conditions. Cultures of proliferating shoots derived from nodal stem explants of mature trees of *C. aurantifolia, C. sinensis, C. jambhiri, Citrus karna* and *Citrus limonia* or *C. limon* had been maintained as normal growth cultures without any declination in their regeneration potentiality for the past 7 years experimented so far. Meanwhile, cultures of proliferating shoots obtained from meristematic tissues are apparently suitable since the germplasm preserved would be both true to type in genetic make-up and free from all pathogens. Such cultures comprise a true tissue bank or gene bank which would also encourage free exchange of germplasm across the phytosanitary boundaries in the plant quarantine system. Semi wild *Citrus* species in addition to wild species which are protected from human destruction with very restricted distribution are needed to be preserved immediately and all of them should be investigated and screened against biotic and abiotic stresses for further utilization. Development of an advanced propagation and cultivation procedures should be highly encouraged for maintenance and protection of plant genetic diversity which are required to be immediately characterized both morphologically and at molecular level by using molecular markers [140]. *In vitro* conservation strategies include several techniques involving short, medium and long term preservation of *Citrus* germplasm particularly consisting of embryos, somatic embryos, meristems, shoot tips or embryogenic calli on liquid or semisolid synthetic culture media. At the same time, each species requires specific procedures, each *in vitro* collections including some general process such as culture initation, clonal multiplication and maintenance, followed by medium or long term (cryopreservation) conservation [141]. Newly developed procedures are literally endowed with alternative choices and simplify preservation in the form of *in vitro* cultures, embryos, seeds and pollens. In terms of storage behavior of citrus species, long term storage in the form of gene banks and cryo-banks can be considered as the most efficient and cheapest method of ex-situ conservation of citrus germplasm because of easy handling and accessibility. Hence, cryopreservation is the only available alternative for long-term conservation of Citrus species. It offers long-term storage potentiality, maximal stability of phenotypic and genotypic behavior of stored germplasms in addition to minimal storage space and maintenance requirements [142].

9. CONCLUSION

Since north eastern region of India is abundantly endowed with diverse indigenous species of citrus particularly *C. indica*, *C. assamensis*, *C. latipes*, *C. ichagensis*, *C. macroptera*, *C. aurantium*, *C. reticulata*, *C. megaloxycarpa*, *C. jambhiri*, *C. aurantifolia*, *C. grandis*, *C. limonand* and *C. karna*, hence continuous efforts have been made by several researchers in terms of *invitro* micropropagation techniques in addition to cryopreservation via somatic embryos, embryogenic callus following vitrification, encapsulation or dehydration for long term conservation of these species. Apart from it, techniques of *invitro* micrografting has also been considered as a potent biotechnological tool for obtaining virus free planting materials in addition to maintaining genetic uniformity in citrus genotypes. Based on the previous results of the several researchers reviewed so far, it can be evidently concluded that several plant growth regulators at varying concentrations and combinations with respect to *in vitro* micropropagation has an individual and specific impact on conservation of citrus diversity. In addition to it, plant growth regulators particularly BAP, Kinetin, IBA, IAA and NAA are extremely suitable in formation of multiple shoots leading to further resulting in plantlet regeneration. Similarly in case of induction of callus, certain synthetic medium particularly Murashige and Skoog (1962) medium supplemented with auxins viz., 2,4-D, 2,4,5-T, TDZ and NAA helps in formation of undifferentiated mass of cells. On the other hand, rooting medium consisting of IBA in combination with NAA gives most appreciable results in case of regeneration of roots in citrus. Therefore, in order to conserve horticultural and woody species on long term basis such as citrus, techniques of tissue culture and cryopreservation are regarded as the most potent biotechnological tool for further mass multiplication as well as improving the growth of citrus industries on a large scale.

10. FUTURE PROSPECTS

Tissue culture has created a significant impact on both industry and agriculture as an emerging technology for mass propagation of plants on a large scale in order to accomplish human needs. For improving the quality of the crop varieties in terms of yield potential and resistance to insects, pests and diseases, application of genetic engineering has proven to be an imminent prospect. Techniques of genetic transformation in addition to molecular biology would also help in further production of disease free planting materials having a potentiality in tolerance to drought, salinity and heat stresses. Moreover, several biotechnological tools particularly genetic engineering, haploid culture, in addition to somaclonal variation entirely depends on *in vitro* plant regeneration system. Production and development of transgenic plants through the method of plant cell culture would be an emerging tool for further use. Relatively, slow growth techniques and cryopreservation would also prove to be an alternative technique of field gene banks for collection and conservation of germplasm as well as for conventional breeding programme. Another prospect of applications of plant tissue culture is the use of liquid culture systems based on shoot cultures or somatic embryos that would become of increasing interest to commercial micropropagation for some stages of the plant propagation cycle for future purpose. A wide variety of vessels have been examined and prepared at lowest possible price for liquid cultures, from simple devices supplying an arbitrary amount of oxygen, to complex computer-controlled bioreactors that have been especially designed for plant cell multiplication and regeneration. The primary objective behind the choice of liquid systems for micropropagation is to simplify handling and reduce labour costs.

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ETHICAL APPROVAL

This manuscript does not contain any studies in relation with human participants or animals performed by any of the authors

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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