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
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 transplants 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 | 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. megaloxyxcarpa* | Bor Tenga | Assam | - |
| 14      | *C. ichangensis* | Ichang papeda | Barail range of Naga hills | Inedible, cold hardy |
| 15      | *C. macroptera* | Satkara     | Shell 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, Mawfong, 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 medium supplemented with 2 mg/L BAP and 0.50 mg/L NAA.

![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]](image-url)
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. aurantifolia          | West Indian acid lime| Stem, root              | Mod. MS (5%)              | BA (0.5%) + ME (1000)     | Mod. MS (2%) NAA(1-2), IAA(2), IBA (1-2) NAA(3) |
| C. jambhiri              | Sohmyndong           | Stem                    | MS                        | BAP(0.75)                 |                                                 |
| C. aurantium             | Sour orange           | Stem                    | MS                        | BA(0.25), NAA(0.1), +ME(500), BAP (0.75) | No change NAA (0.1-0.5) |
| C. grandis               | Shaddock Pummelo     | Stem, leaf              | Stem                      | MS                        | No change                                       |
| C. volkameriana          | Volkamer lemon       | 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) |
| C. limettioides          | Sweet lime           | Stem                    | Mod. MS (3%)              | BA(l)+Kinetin(0.5)+ NAA (0.5) |                                                   |
| C. limon                 | Lemon                | Stem, Root tips         | MS (3%)                   | K(l)+NAA(l)               | No change                                       |
|                          |                      | Assam lemon             | MS (3%)                   | BA(l)+2.4-BA(l)+Kinetin(0.5)+ NAA (0.5) |                                                   |
| C. madurensis            | Calamondin           | Stem, Stem              | MS (5%) White 1943(2%) Tukey, 1938 (0.5%glucose) | BA(0.1-10)NAA(0.1)+ME (500) | MS (5%) NAA(0.1)+ME (500) |
| C. paradisi              | Grape fruit          | Stem, leaf              | Mod. MS(5%) (Mrn)         | BA(0.5), NAA(0.15), +ME(1000) | No change                                       |

References: Raj Bhansali and Arya [21,22], Thirumalai & Thamburaj, [23], Parthasarathy & Nagaraju, 1996, Bouzid [24], Chaturvedi and Mitra [25], Parthasarathy & Nagaraju, 1996, Raj Bhansali and Arya [26], Bouzid [24], Sauton et al.,[27], Singh et al.,[17], Grinblat [28], Rangaswamy (1975).
| 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. sinesis** | 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 |
| **C. latipes** | Khasi papeda | Stem | MS (3%) | BAP 0.75 | - | - | Baruah et al., [33] |
| **C. assamensis** | Ada Jamir Unshiu | 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 *ex vitro* | Chaturvedi and Sharma [37,38] |
| *Citrus aurantifolia* | Lime | Tiny shoot apices | Shoot growth, plant *ex vitro* | Chaturvedi and Sharma [37,38] |
| *Citrus aurantifolia* | Lime | Nodal segments | Auxillary shoot proliferation, Plants *ex vitro* | Singh and Chaturvedi [39] |
| *Citrus jambhiri* | Rough lemon | Nodal segments | Auxillary shoot proliferation, Plants *ex vitro* | Singh and Chaturvedi [39] |
| Citrus karna | Karna khatta | Nodal segments | Auxillary shoot proliferation, Plants *ex vitro* | 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 *ex vitro* | Singh et al., [17] |
| *Citrus mitis* | Calamondin | Shoot tips and nodal segments of glasshouse grown plants | Shoot regeneration, Plants *ex vitro* | 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 *ex vitro* | Singh et al., [17] |
| *Citrus sinensis* | Sweet orange | Dormant buds | Auxiliary 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* x *P. trifoliata* | Sweet orange | Nodal segments and apices of glasshouse grown plants | Auxillary shoots, Plants *ex vitro* | Chaturvedi and Sharma [37,38] |
| *Citrus sinensis* x *P. trifoliata* | Carrizo citrange | Nodal segments of glasshouse grown plants | Multiple shoots, Plants *ex vitro* | 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  | Nodal stem segments      | -                       | MS+22µMBA                  | Soirlite                 | Baruah et al.,[33, 45]                       |
| C. *aurantifolia* | Mexican lime | Internodal Stem segments | MS + 0.4 µM BA          | MS + 16.1 µM NAA           |                          | Duran-Vila and Navarro., [46]                |
| C. *aurantifolia* | Mexican lime | Internodal seedlings stem sections | 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 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*  | Pummelo     | Shoot-tip Hypocotyl segments | MS + 1.8 µM BA        | ½ MS + 5.4 µM NAA          |                          | Paudyal and Haq, [51]                       |
| C. *halimii*  | Shoot tips  | Hypocotyl segments      | MS + 4.4 µM BA          | MS + 2.7 µM NAA            |                          | Normah et al., [52]                         |
| C. *indica*   | Shoot tips  | Stem and root segments  | MS + 2.2 µM BA          | Soirlite                   |                          | 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*  | Stem and root segments | MS + 0.9 µM KIN + 53.7 µM NAA | MS + 2.2 µM BA | Soirlite                   |                          | Baruah et al., [33,45]                      |
| C. *limon*    | Lemon       | Stem and root segments  | MS + 2.2 µM BA          | ½ MS + 5.4 µM NAA          |                          | Raman et al., [53]                          |
| C. *limon*    | Lemon       | Shoot tips               | MS + 0.9 µM KIN + 53.7 µM NAA | MS + 4.4 µM BA + 4.6 µM KIN + 2.7 µM NAA | MS + 1.1 µM BA + 2.7 µM NAA | 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 x P. trifoliata* | Swingle citrusmelo | Epicotyl stem segments           | MT + 150 µM            | 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 µM KIN + 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 µM BA + 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 + 4.4 µM BA          | W+ 10 µM NAA              |                          | Barlass and Skene, [41]         |
| *C. sinensis*            | Sweet orange| Nodal stem segments                  | MS + 4.4 µM BA or 13.3 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 x P.*       | 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 \ C. sinensis x P. trifoliata | Carrizo citrange sections | MS + 10 µM BA | W + 10 µM NAA | Barlass and Skene, [41] |
| | Troyer citrange Nodal and internodal Stem segments | MS + 2.2 µM BA + 0.5 µM NAA | MS + 10.7 µM NAA | Edriss and Burger, [32] |
| | Carrizo citrange Epicotyl stem segments | MT + 22 µM BA + 5.4 µM NAA | ½ MT + 5.4 µM NAA | Moore, [48] |
| | Carrizo citrange Internodal seedlings stem sections | MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD | MT + 5.4 µM NAA | Starrantino and Caruso, [60] |
| | Carrizo citrange Shoot-tip | MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD | MT + 5.4 µM NAA | Starrantino and Caruso, [60] |
| | Troyer citrange Shoot-tip | MS + 4.4 µM BA + 2.5 µM IBA + 296 µM AD | MT + 5.4 µM NAA | Starrantino and Caruso, [61] |
| | 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] |
| | 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] |
| | Carrizo citrange Mature embryos | MT + 9 µM 2,4-D + 22.2 µMBA | MT + 22.2 µM BA + 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] |
| | 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] |
| | 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] |
| | Trifoliate orange Mature embryos | MT + 9 µM 2,4-D + 5.4 µM NAA | MT + 22.2 µM BA + 5.4 µM NAA | Beloualy, [49] |
| Species          | Common name | Explant                  | Callus Induction medium | Shoot Regeneration Medium | Root Regeneration Medium | Reference          |
|------------------|-------------|--------------------------|-------------------------|---------------------------|-------------------------|--------------------|
| *P. trifoliata*  | Trifoliate  | Hypocotyl                | + 22.2 µM BA            | MS + 44.4 µMBA            | ½ MS + 0.5-5.0 µM IBA   | Harada and Murai, [62] |
| *P. trifoliata*  | Trifoliate  | tTCL from stem internodes| MS + 10 µM BA + 1 µM TDZ| MS + 5 µM NAA             |                         | Van Le et al.,[63]  |

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 explant 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

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 C₆H₅Cl₂O₃. 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.

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
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 confirmed 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 (1H-indole-3-butanoic acid, IBA) in the auxin family, with the molecular formula of C_{12}H_{14}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 embryonic 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, epicotyl 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 (Chakraborthy and Goswami 1999). Callus obtained from
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-Eiden [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 in vitro 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 meristems 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 meristem of length 200µm to 1 mm of two citrus species namely *Citrus aurantiifolia* and *C. sinensis* in addition to using *in vitro* 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 meristemst of length 500µm derived from scion of rootstock species *Citrus limonia* or *C. limon* resulted in 57.14% success in *C. aurantiifolia* 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. aurantiifolia* (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 micrografting 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 polyethylene, 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 CaCl2.2H2O 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-benzylaminopurine) 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% CaCl2 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 °C) 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 semwild 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 \textit{in vitro} growth conditions. Cultures of proliferating shoots derived from nodal stem explants of mature trees of \textit{C. aurantifolia}, \textit{C. sinensis}, \textit{C. jambhiri}, \textit{Citrus karna} and \textit{Citrus limonia} or \textit{C. limon} had been maintained as normal growth cultures without any decline 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 \textit{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]. \textit{In vitro} conservation strategies include several techniques involving short, medium and long term preservation of \textit{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 \textit{in vitro} collections including some general process such as culture initiation, 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 \textit{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 germplasm 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 \textit{C. indica}, \textit{C. assamensis}, \textit{C. latipes}, \textit{C. ichagensis}, \textit{C. macroptera}, \textit{C. aurantium}, \textit{C. reticulata}, \textit{C. megaloxycarpa}, \textit{C. jambhiri}, \textit{C. aurantifolia}, \textit{C. grandis}, \textit{C. limonand} and \textit{C. karna}, hence continuous efforts have been made by several researchers in terms of \textit{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 \textit{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 \textit{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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