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Review of Micropropagation Methods in *Citrus indica*: Enhancing Cultivation Practices

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ABSTRACT:

Citrus indica, an endangered *citrus* species native to north-eastern India, holds significant genetic and medicinal value, making it a vital resource for *citrus* breeding programs and conservation efforts. Traditional cultivation methods face challenges such as low seed viability, disease susceptibility, and habitat destruction. Micropropagation offers a promising solution for the large-scale production of disease-free and genetically uniform plants, enhancing cultivation and conservation practices for *C. indica*. This review explores the various micropropagation techniques employed for *C. indica*, including explant selection, culture media composition, and optimal environmental conditions. The stages of micropropagation, from initiation to acclimatization, are detailed, highlighting their critical role in successful plantlet establishment. Additionally, the advantages of micropropagation, such as mass production, disease-free plants, and genetic uniformity, are discussed alongside the limitations, including high initial costs, technical expertise requirements, contamination risks, and somaclonal variation. Future challenges and prospects, such as protocol optimization, genetic fidelity assessment, cryopreservation, genetic transformation, and collaborative efforts, are addressed to underline the need for continued research and innovation. By overcoming these challenges, micropropagation can significantly enhance the cultivation practices of *C. indica* and contribute to the conservation of this valuable species.

Keywords: Citrus, Micropropagation, somaclonal, cultivation, genetic

Introduction:

In nature, plant propagation can be either asexual (by multiplication of vegetative parts) or sexual (through the generation of seeds) (Hartmann and Kester1959). Sexually propagated plants demonstrate a high degree of heterogeneity since their seeds progeny are not true-to-type, as they originate from inbred lines (Fehr 1991). Asexual reproduction, on the other hand, produces plants that are genetically identical to the parent plants and thus permits perpetuation of the unique characters of the cultivars-propagation or multiplication of

heterozygous genotypes is of necessity by vegetative propagation as it involves only mitotic cell divisions. Progeny obtained through vegetative propagation (or sexual reproduction) of a single plant constitutes a clone. By definition, members of a single clone share the same genotype (Bhojwani and Razdan 1996). Traditionally, vegetative reproduction is achieved by cutting, budding, grafting, etc. Tissue culture also enables rapid clonal propagation plants, commonly referred to as micro-propagation (George et al 2008). This process is the primary plant biotechnology utilized by industries in India for the commercial production of mainly ornamental plants and fruit trees such as banana, lemon, etc. Ornamental plants are predominantly produced for export, often under contracts with foreign companies, while fruits and plantation crops like cardemon are primarily grown for the domestic purpose/market (Govil and Gupta 1997).

1.1 Micropropagation:

The *in vitro* propagation of plants through methods such as cutting vegetative parts, layering, grafting, and budding can often be challenging, expensive and even sometimes unsuccessful. Tissue culture methods offer an alternative approach to vegetative propagation, commonly known as **micropropagation** (Bhojwani and Razdan 1996)**.** Micropropagation allows for the rapid production of a large number of plants from a single individual within a short period. The use of tissue culture for micropropagation was initiated by G. Morel in 1960, who identified it as the only commercially viable methods for orchid propagation. Since then, micropropagation has been applied to various crop species, and established protocols are now available, which can be adopted by growers trained in aseptic manipulation and plant husbandry (Hartmann and Kester1959).

The **Advantage** of micropropagation over sexual reproduction include:

- **Bypassing the juvenile phase:** Micropropagation allows for the direct propagation of woody perennials or certain cultivars from adult material, avoiding the undesirable juvenile phase associated with seed- raised plants.
- **Establishing gene banks:** Micropropagation facilitates the multiplication of variants among clonally propagated plants, enabling the creation of gene banks.
- **Reduction in labor cost**: The technique is less labor- intensive compared to traditional propagation methods.
- **Avoidance of field infection:** Micropropagation occurs in a controlled environment, reducing the risk of field- related infections.
- **Environmental protection:** Plants propagated *in vitro* are shielded from environmental hazards like hail and frost,
- **Availability of propagation material**: Material for micropropagation is readily accessible.
- **High Multiplication Rate:** The chief advantage of micropropagation is its extremely high multiplication rate, making it ideal for rapidly propagating rare genotypes, and plants with low natural multiplication rates.
- **Use of small explants:** Micropropagation allows for the use of very small explants, which is not possible with conventional techniques.

- **Disease- free plants:** During micropropagation, fungi and bacteria are usually eliminated mainly due to the rejection of contaminated cultures. Therefore, the plants obtained are clean, while conventional methods propagate the disease as well.
- **Pathogen-free maintenance**: Plants can be maintained *in vitro* in a pathogen-free state, facilitating easier export due to the absence of quarantine problem, and simplified packing because of smaller plant size.
- **Propagation of dioecious species**: In dioecious species (those with separate male and female plant), micropropagation can be used to propagate the more desirable sex.
- **Rapid cloning of elite trees:** For forest trees, mature elite trees can be quickly identified and cloned using micropropagation.
- **Year round propagation:** Micropropagation can be carried out throughout the year, independent of seasonal variations.
- **Enhanced growth in ornamentals:** In many ornamentals plants, tissue culture produces plants with better growth, more flowers, and less plant loss (Hartmann and Kester1959; Thorpe 2012; George and Sherrington 1984).

Limitations:

- **High production costs**: The High production costs associated with micropropagation have limited its application primarily to more valuable ornamental crops and some fruit trees (Thorpe 2012).
- **Lack of Suitable techniques**: Micropropagation techniques are not yet available for many valuable species such as mango, coconut, etc. Further research is needed to resolve the challenges, particularly with woody plant species (Bhojwani and Razdan 1996).
- **Risk of Somaclonal variation:** Somaclonal variation can occur during *in vitro* culture, especially when a callus phase is involved, potentially leading to genetic instability (Jain et al 1998).
- **Vitrification issues:** Vitrification (hyperhydracity) may be a problem in some species, affecting the success of micropropagation (Ziv 1991).

1.2 Techniques in micropropagation:

In vitro micropropagation is a complicated process that involves multiple stages. Murashige and Skoog (1962) proposed four distinct stages for the commercial production of clones, with Stage I-III occurring under *in vitro* conditions, and Stage IV taking place in a greenhouse environment. Later, Debergh and Maene (1981) introduced an additional Stage 0 to enhance various micropropagating systems.

Stage 0: Selection and maintenance of stock plants for culture initiation.

Stage I: Initiation and establishment of aseptic culture which includes explant isolation, surface sterilization, washing, and establishment on appropriate culture media.

Stage II: Multiplication of shoots or rapid formation of somatic embryos using a defined culture medium.

Stage III: Germination of somatic embryos and/or rooting of regenerated shoots *in vitro.*

Stage IV: Transfer of plantlets to sterilized soil for hardening in a greenhouse environment.

Fig1.2.1: Major steps of *in vitro* micropropagation

1.2.1 Stage 0: Preparations of stock plants:

Stock plants intended for culture initiation are grown under carefully monitored glasshouse conditions for at least 3 months. They are maintained at low humidity and watered via irrigation tubes or capillary sand beds/mats (Meena et al, 2022).

1.2.2 Stage I: Initiation of Aseptic culture:

As defined by Murashige (1974), this stage involves preparing explants from stock plants and establishing them on a suitable culture medium. Shoot tips and axillary buds are commonly used for commercial micropropagation (Chhetri et al 2021).

1.2.3 Stage II: Shoot multiplication:

This stage focuses on the proliferation of regenerated shoots using a defined culture medium. Approaches include:

- Multiplication of meristems from apical and auxiliary shoots.
- Induction and multiplication of adventitious meristems through organogenesis or somatic embryogenesis.
- Multiplication of call, with subsequent organogenesis or somatic embryogenesis in serial subcultures (Singh 2002).

1.2.4 Stage III: Rooting and preparation for soil:

Proliferated shoots are transferred to rooting media or directly to soil as micro cutting. Factors to consider include:

- Dividing and rooting shoots individually.
- Hardening shoots to resist moisture stress and disease
- Preparing plants for autotrophic development and breaking dormancy (Holder and Johan, 2021).

1.2.5 Stage IV: Acclimatization:

- Plantlets from Stage III are transferred from the lab to a greenhouse environment. Unrooted Stage from stage II can also be acclimatized in soil or compost under controlled light, temperature and humidity conditions (Hazarika, 2023).

1.3 Micro propagation in *Citrus* plants

Citrus, a vital horticultural crop known for its fruit and juice, comprises about 30 species in India. Nine species are widely distributed across country, while 17 are native to the North-Eastern states, a biodiversity hotspot. Several *Citrus* species, including *C. indica, C. macroptera, C. latipes*, are considered endangered or nearly so by the IUCN (Sharma et al 2016).

Conventional *Citrus* propagation is limited by seasonal constraints and the availability of plant material, which may not ensure cultivars trueness or mass production of certified plants year- round (Grosser and Gmitter 1990). Micropropagation, a tissue culture technique, has emerged as a powerful tool for *Citrus* propagation, allowing rapid multiplication of difficult-to-root crops in a controlled environment throughout the year. This method not only eliminates diseases but also enables the development of new cultivars with improved pest resistance through somaclonal variation and somatic hybridization (Tomaz et al 2001).

Vegetative propagation is preferred for *Citrus* to ensure true-to-type plants and uniform quality. Micropropagation offers significant advantages over conventional methods, including faster production of

genetically identical, physiologically uniform and developmentally normal plants at a reduced in a reduced lowered cost. The application of micropropagation in *Citrus* is expanding globally, aiming to match or surpass the performance of conventionally raised plants (Gmitter et al 2007).

REVIEW OF LITERATURE

Micropropagation, the propagation of selective genotype using *in vitro* culture techniques is originated from the pioneering research of Gottlieb Haberlandt (2003) who first attempted to culture isolated plant cells. As a concept, micropropagation was first presented to the scientific community in 1960 by G.M.Morel producing virus –free Cymbidiums. The necessary tools that made micropropagation a possibility, such as the media and an understanding of plant growth regulator, have been available only since the late 1950s. And it was not until the early 1960s that a generalized culture medium was established .The actual establishment of commercial micropropagation as an industry became a reality during the 1970s and 1980s.

The classical findings of Skoog and Miller (1957) continued to be guiding principal on *in vitro* organogenesis. *In vitro* organogenesis has been achieved in over 1000 plants through empirical selection of the explant, the medium composition and control of the physical environment.

***Citrus indica* Tanaka-**

Scientific classification

Kingdom-Plantae

Division-Magnoliophyta

Class-Magnoliopsida

Sub-class-Rosidae

Order-Sapindales

Family-Rutaceae

Genus-Citrus

Species-indica

The genus *Citrus* includes some of the principal fruit crops of the world such as the *citrons (C.medica L.), lemon (C.limon (L.)Osbeck), limes (C.aurantifolia (Christm.)* Swingle, *mandarins (C. reticulata Blanco),* sour oranges *(C.aurantium L.),* sweet oranges *(C. aurantium (L.) Osbeck),* grapefruits *(C. aurantium L., C .paradise Macf.) and pummeles (C. maxima (Burm.)Merr.).*

There are two widely used classification systems in *Citrus*: Swingle, 1943; Swingle and Reece, 1967 and Tanaka, 1976. The Swingle system included 16 species under two subgenera-*Citrus* and *Papeda*, while the Tanaka system recognized 162 species under the subgenera Archicitrus and Metacitrus. Advanced studies based on biochemical and morphological characterization ,suggest that there are only three basic species , i.e. citron (*C.medica L.)* , mandarian (*C.reticulata Blanco*) ,and pummelo (*C.maxima (burm.)Merr.)* within the subgenus *Citrus* and that the other edible *citrus*, e.g. lemon, lime, sour, orange, grapefruit, etc. are apomictictically perpetuated biotypes with probable hybrid origin (Scora, 1975; Barrett and Rhodes, 1976) Mabberley (1998, 2004) treated *Citrus* in a broader sense by merging three of its closely allied genera-

Fortunelle Swingle, Eremocitrus Swingle and Microcitrus Swingle within it .*Citrus* is thus believed to have its primary centre of origin in North-east India ,China ,Malaysia and Australia (Swingle and Reece,1967; Scora, 1975.;Gmitter and Hu, 1990; Mabberley,2004).*Citrus* fruits trees are now commercially grown in more than 100 countries in the tropical regions of the world ,many of them located far away from their actual centers of origin.

India has a distinct position in the *Citrus* belt of the world due to remarkable diversity in *citrus* genetic resources, both in cultivation and wild. Apart from the most commonly cultivated species /cultivars /hybrids of *citrons*, lemons. limes, mandarins, sour oranges, pummelos and grapefruits, three wild species and one variety , viz *Citrus indica* Tanaka (Indian wild orange), *C.latipes* (Swingle) Tanaka (Khasi Papeda), *C.ichangensis* Swingle (Ichang *Papeda*) (a synonym of *C. cavaleriei* H. Leveille ex *Cavalerie*; cf. and *C. hystrix* DC (*C. macroptera* Montrouz. *var*. annamensis Tanaka- Melanesian Papeda) were reported to occur in the subtropical forests of North-east India and the foot hills of the East Himalayas (Nair and Kumar 2016; Sharma and Tripathi 2006).

Reasons for micropropagation of *C. indica*:

Citrus indica, one of the primitive wild species of *Citrus*, is endemic to the Tura ranges in Garo hills of Meghalaya in North-East India. A *Citrus* gene Sanctuary for preserving the indigenous germplasms of *Citrus*, particularly of *C. indica* has been established in the Nokrek Biosphere Reserver (NBR) in the Garo Hills along the Turs ridge in Meghalaya (Singh, 1981). The Indian wild orange is popularly known among the Garo tribes as 'Memang-narang' (i.e. orange for ghosts or departed human spirits; in Garo language 'memang' means ghost and 'narang' means orange). The plant is thus revered with religious sentiments and grown in the backyards of several Garo halmets for medicinal uses (Malik et al. 2006). The Garo settlements are located along the forest fringes and they do not practice an organized farming or cultivation system for *C. indica.* The Garos usually nurture the wild plants of *C. indica* that are found growing naturally in and around their dwellings.

The indigenous genetic resources of *Citrus* have great utility in citriculture and *citrus* industry. Unfortunately, like in many other crop plants, the genetic base of indigenous and wild species of Indian *Citrus* including *C. indica* is being eroded due to habitat destruction, introduction of new exotic cultivars/varieties, and lack of appropriate conservation and management strategies.

Despite its medicinal and genetic resource values, no detailed study has ever been carried out to evaluate the extent and pattern of genetic diversity found within *C. indica* in its native distributional range.

Micropropagation of sweet orange *Citrus sinensis* Osbeck for the development of nucellar seedling

Das A. *et al* (2000) standardized the protocol for micropropagation of elite plants of sweet orange (*Citrus sinensis*) through nucellar embryo culture. Nucellar embryos and a zygotic embryo could be excised from a single mature seed and successfully generated as healthy plants in basal MS medium. MS medium supplemented with NAA (1 mg/L) or 2,4D (1mg/L) promoted callus development in both nuclear and zygotic embryos.

***In vitro* micropropagation of *Citrus aurantifolia* (lime):**

Al-Khayri and Al-Bahrany 2001 describes a micropropagation technique for lime, *Citrus aurantifolia.* Swing. using nodal explants of manure trees. Nodes were cultured on Murashige and Skoog medium containing indole-3-butyric acid (IBA) at 0.05 and 1 mg/L combined with 6-benzylaminopurine (BAP) at 0, 0.25, 0.5, 1 and 2 mg/L in combination with 6-furfurylaminopurine (kinetin) at 0, 0.5 and 1 mg/L. Best results for multiple shoot formation, 8 shoots per node, were obtained with 1mg/L BAP and 0.5 mg/L kinetin. The concentration of IBA has little effect on shoot multiplication. Shoot elongation appeared to favor 0.25mg/L BAP combined with 1mg/L kinetin .Shoot elongation and leaf size were inhibited in response to high levels of BAP. Transfer of shoots to a rooting medium induced the highest percentage of rooting, 56%, on 1mg/L IAA .Plantlets survived in soil and exhibited normal growth in a greenhouse.

***In vitro* root induction of regenerated shoot of *Citrus jambhiri* Lush.**

Ali S. and Mirza B .(2006) performed in rooting in 25 150 mm culture tubes containing 25 mL of MS medium containing 3% sucrose and solidified with 0.8% agar ,having 0.5 mg/L,NAA or 1 mg/L, 2,4-D. Twenty five regenerated shoots were cultured for rooting and each experiment was conducted three times. Visual observations were taken every three days and the effect on different shoots was qualified on the basis of percentage of shoots showing response for rooting.

Micropropagation of *Citrus halimii*-an endangered species of South –East Asia:

Normah M. N *et al* (1997) described a successfully system of direct organogenesis for the wild citrus tree, *Citrus halimii* Stone which used in *in vitro* seedling explants cultured on Murashige and Skoog medium supplemented with 0.4-11.1 M 6-n benzyladenine . Hypocotyl was the best explants for multiple shoots regeneration. Maximum number of shoots was obtained on medium with 2.2-11.1M 6-n benzyladenine. Rooting of regenerated shoots was best on Murashige and Skoog medium supplemented with 2.7 M - naphthalenacetic acid.

In vitro* shoot induction *Citrus cultivars:

Mukhtar et al (2005) explored *Citrus* cultivars for multiple shoot induction and root regeneration in different media. The multiple root and shoot induction was found directly proportionate to the increase in the levels of benzylaminopurine (BA) and naphthalene acetic acid (NAA) in the modified Murashige and Skoog medium. The study might be promising towards *in vitro* propagation of *Citrus* plant material.

Acclimatization of micropropagated *C. limon:*

J.S.Rathore *et al* (2007) micropropagated several hundreds of shoots. These were efficiently rooted *in vitro* or under greenhouse conditions. About 95%of the rooted plantlets were hardened and transferred to polybags containing garden soil, sand and manure. The plantlets hardened in the greenhouse were shifted to the nursery and then placed out for field evaluation. After 4 years, clone plantlets have flowered and produced fruits under field conditions.

Challenges and Future Prospects

While significant progress has been made in the micropropagation of *Citrus* species, several challenges remain. Contamination, somaclonal variation, and the need for species-specific protocol optimization are major hurdles. Future research should focus on developing efficient, reproducible protocols and exploring the potential of molecular markers for genetic fidelity assessment. Moreover, integrating biotechnological approaches such as genetic transformation and cryopreservation can further enhance the conservation and sustainable utilization of *Citrus* species. Collaborative efforts between research institutions, government agencies, and local communities are essential for the successful conservation and commercialization of this valuable species.

1. **Protocol Optimization**

Further research is needed to optimize protocols specific to *C. indica* to improve efficiency and reproducibility (Singh 2002).

2. **Genetic Fidelity Assessment**

The use of molecular markers can help in assessing genetic fidelity and ensuring the true-to-type nature of the propagated plants (Halder & Jha, 2021).

3. **Cryopreservation**

Integrating cryopreservation techniques can aid in the long-term storage of *C. indica* germplasm, providing a backup against the loss of genetic material.

4. **Genetic Transformation**

Advanced biotechnological approaches, such as genetic transformation, can be explored to introduce desirable traits and enhance disease resistance in *C. indica*.

5. **Collaboration and Knowledge Sharing**

Collaboration between research institutions, government agencies, and local communities is essential to overcome challenges and ensure the successful conservation and commercialization of *C. indica* (Hazarika, 2023).

Conclusion

Micropropagation offers a viable solution for the large-scale production and conservation of *Citrus indica*. Optimizing explant selection, culture media, and environmental conditions are crucial for successful micropropagation. Addressing the limitations and future challenges through continued research and innovation will enable the full potential of micropropagation to be harnessed, enhancing the cultivation practices of *Citus* species.

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