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Research Article

IN VITRO MICRO PROPAGATION OF SWEET ORANGE

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Abstract: Present investigation was conducted to standardize a protocol for in-vitro propagation of citrus spp. *i.e.* sweet orange for commercial purpose. The shoot tip explant was found better for callus induction of these plants than the nodal segment and epicotyls. Maximum callus formation (40.0% and 23.3% 22.2%) of shoot tip explants was obtained respectively in treatment MS basal media + 0.8mg/l Kinetin, 1.5mg/l NAA, and 2.5 mg/l 2, 4-D. Furthermore, the maximum number of shoots per explant was obtained through the callus in MS basal media + BA 1mg/l. Maximum rooting of shoots (1.11%) was noted sweet orange for the ½ MS media supplemented with 0.2 mg L-1 NAA plus 0.1 mg L-1 BA. Although the callus development and bud proliferation were recorded in all explants however, shoot and root formation did not occur. The potting media composing of soil, sand and FYM in the ratio of 1:1:1 by volume was better with maximum survival rate of hardened plants six weeks after transferring to the pots under greenhouse. In this way we can use this standardized protocol for regeneration of different rootstock for purity and uniformity purpose in seedling.

Keywords: *In-vitro multiplication, Citrus, Sweet orange, Micropopogation*

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Introduction

The citrus has been recognized as one of the most economically important group of plants in the world. Improvement of citrus by conventional method is hampered by polyembryony, sexual incompatibility and male or female sterility [1]. Citrus propagation by conventional means is restricted to particular season and availability of plant material.

It doesn't guarantee trueness of cultivars and mass production of certified Citrus plants throughout the year. Plant tissue culture has emerged as a powerful tool for propagation and improvement of many woody plant species including Citrus. Citrus also stands among difficult to root crops and micropropagation offers rapid propagation of such crops in limited space and time under controlled conditions throughout the year. *In vitro* culture further eliminates diseases [2], provides scope for the development of new cultivars through somaclonal variation [3] and somatic hybridization [4-7], that have improved Citrus rootstock resistance against nematode infestation and other pests as well. Reports on Citrus mircopropagation revealed maximum callus induction percentage in Kinnow (86.8%) on Murashige and Tucker's medium supplemented with 0.01mg/L BA, NAA and 500mg/L malt extract [8]. Different concentrations of growth regulators 10mg/L benzyladenine (BA), 0.1mg/L NAA and 500mg/L malt extract caused maximum initiation of shoot buds from Citrus stem explants grown *in vitro* [9].

The best rooting (100%) in the minimum time (15 days) occurred in the half strength MS medium supplemented with growth hormones (1.0mg/L NAA). The present research work was planned to estimate the effect of growth regulators on the enhancement of growth and development. Further, to induce multiple shoots in Citrus cultivars for mass propagation of certified disease-free plant material. Kinnow has replaced the traditional cultivars of sweet orange due to its outstanding adaptation to agroecology of different region in India, which led to profuse vegetative growth and heavy yield with good fruit quality. Because of these the requirement of disease-free planting material required for plantation purpose.

Source of explant, photoperiodic factors, cut modes, hormonal concentrations and additives may affect *in vitro* citrus shoot regeneration. Epicotyl segments excised from seedlings germinated in the dark for 3 - 6 weeks [10] and then transferred to a 16 h photoperiod that varied from 1 to 3 weeks [11,12] improved the transformation efficiency. For hormones, the effect of auxin on shoot regeneration was rarely concerned, though the main hormone effect on bud formation was due to the addition of BAP [13].

Almeida *et al.* (2003) [14] recorded maximum number of shoots when epicotyl segments were cultured on regeneration EME medium supplemented with 25 g/l additional sucrose and 1 and 2 mg/l BAP for sweet orange and rangpur lime, respectively. Among cut modes, transversal cut, the most popular cut mode [15] is simple to manipulate but produces the fewest adventitious buds. Longitudinal cut, a newly developed but infrequently used cut mode produced the most adventitious buds [16].

Materials and Methods

Plant material and explants preparation

Seeds were extracted from ripe fruits sweet orange. Fruits were collected from a citrus germplasm collection of NRCC, Nagpur. Seed integuments were removed and disinfestation was done with 1% Mercuric Chloride for 5 min. Three washes in distilled and sterilized water were done before the seeds were introduced in culture jars containing 50 ml of MS medium [17], supplemented with 30 g l-1 sucrose. The seeds were maintained at 29 ± 2°C in the dark for three weeks, followed by one, two or three weeks under a 16 h photoperiod.

Explants selected for study

The shoot tip, nodal segment and epicotyls were selected for the study from the sweet orange plant germinated in controlled condition as shown in [Fig-1].

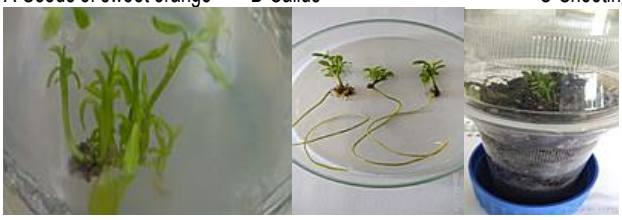
Media and culture conditions

Three different media were used; MS (Murashige and Skoog, 1962) medium supplemented with 500 mg l-1 malt extract and 25 g l-1 sucrose (N1), MS supplemented with 500 mg l-1 malt extract and 40 g l-1 sucrose (N2), and MS supplemented with 500 mg l-1 malt extract, 50 g l-1 sucrose, and 3 mg l-1 BAP (N3). After adjusting the pH to 5.8 ± 0.1 using 1 M NaOH, 1% Difco Bacto agar was added to the media. The media were sterilized by autoclaving at 121°C for 15 min, and 25 ml medium was stored in 100-ml flasks and sealed with Parafilm (American Can, USA). The shoot tip, nodal segment and epicotyls were regularly subcultured on the same fresh medium every 21 days. callus obtained from explants were isolated and cultured in test tubes containing 15 ml solid MS medium with agar (10 g l-1) supplemented with 45 g l-1 sucrose; this medium was with different concentration of hormone. The culture tubes and flasks containing the explants were incubated in a culture room at 25 ± 1°C under 16-h day length with an illumination of 100 µmol m-2 s-1 white 18 W fluorescent lamps. Plantlets were stored for a year under the above-mentioned conditions. The green and healthy callus was transferred on the shooting media in combination with kinetin, NAA, 2, 4-D. After 2-3 weeks regenerated shoots were transferred on the rooting media containing ½ MS with IBA and BAP with different concentration.

A-shoot tip B-Nodal segment C-epicotyl

Fig-1 Explants used in study

A-Seeds of sweet orange B-Callus C-Shooting



D-Multiple shooting E-Rooted shoot F-hardened plant

G-seedlings micropropagated plants

Fig-2 Steps followed in regeneration of Sweet orange

Treatments used

Indirect organogenesis

Two concentrations of BA (2 and 4 mg L-1) with 0.1 mg L-1 NAA were tested in a basal medium for callus induction. In addition, 1 or 2 mg L-1 BA with 0.1 mg L-1 NAA was tested for shoot induction from callus.

Rooting of shoots

The rooting medium contained half strength MS medium supplemented with 0.2 mg L-1 NAA plus 0.1 mg L-1 BA.

Acclimatization

The rooted plantlets were transplanted into 15 cm in diameter pots containing a mixture of sand: peat moss: FYM (1:1:1), placed in a growth room under controlled conditions (temperature 29±2°C,16/8 h photoperiod and light intensity 1500 Lux). The process of acclimatization continued for 8 months, and the rate of survival was 100 % as shown in [Fig-2].

Results

Among the explants selected epicotyls show best results than the other on different concentration of growth media for indirect organogenesis. Under dark/light condition, the buds differentiated from the callus formed at the cut end. Bud formation increased when BA concentration was enhanced. Meanwhile, the number of quiescent shoots regenerated increased. When combined with 0.2 mg/l IbA, the additive effect appeared at 2.0 mg/l of BA. The mean number of buds reached a maximum of 9.8 per explant, among which about five buds could elongate to shoots. Therefore, MBI medium was chosen as the optimal medium for use during micropopogation of sweet orange epicotyls explants

Discussion

In some species, somatic embryos were obtained from special explants cultured on hormone-free media but epicotyls show better regeneration in the medium supplemented with high sucrose and BAP. Regarding root and shoot induction *in vitro* exists in case of Citrus cultivars too. Supplement of both BA (1mg/L) and NAA (10mg/L) in the basal media showed multiple shoot and root formation in sweet orange. BA as a cytokinin was found inhibitory at higher levels for shoot induction in all cultivars leading to the fact that hormone sensitivity was similar for the cultivars studied while NAA when used for root formation did not show any inhibitory response. Such studies might be a promising step towards mass production of sanitized plant material of Citrus.

Application of research: In the present investigation efforts have been made to standardize the protocol for *in vitro* multiplication of sweet orange. It may be possible to use same standardized protocol to propagate some Citrus rootstocks *viz*. Rough lemon, Cleopatra mandarin and other desired rootstocks available *in vitro* if they are desirable enough to justify the labor and expense that would be involved.

Research Category: Micro-propagation

Abbreviations: BAP: Benzyl amino purine, IBA: Indoline butyric acid, MS: Murashige and skoog, NAA: 1-Naphthaleneacetic acid.

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Study area / Sample Collection: NRCC, Nagpur

Cultivar / Variety / Breed name: Citrus

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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