In vitro shoot organogenesis in sweet orange (Citrus sinensis L.) cv. Mosambi and the effect of ethylene adsorbents on micro-shoot quality

Reena Prusty
Indian Agricultural Research Institute
OM PRAKASH AWASTHI (awasthiciah@yahoo.com)
IARI: Indian Agricultural Research Institute https://orcid.org/0000-0002-9222-8605
Sajnjay Kumar Singh
Indian Agricultural Research Institute
Kanika Kumar
ICAR - National Institute for Plant Biotechnology

Research Article

Keywords: Ethylene adsorbents, micro-shoot quality, basal medium, leaf abscission, shoot organogenesis

Posted Date: July 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1730471/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on April 6th, 2023. See the published version at https://doi.org/10.1007/s11240-023-02499-2.

Abstract

An improved organogenesis protocol for multiplication of sweet orange cv. Mosambi has been standardized by the use of different PGRs, basal media and ethylene adsorbents. Incorporation of both cytokinins, BAP (8.8µM) and kinetin (6.97µM) resulted in best shoot organogenesis with the highest response (81.40%), No. of micro-shoots/explant (2.06), mean micro-shoot length (1.30 cm) and No. of leaves/micro-shoot (3.55) owing to synergistic effect of these factors. However, the regenerated micro-shoots failed to establish due to 100% leaf abscission on micro-shoot. To retard the effect of ethylene accumulation on the regenerated micro-shoots, two types of ethylene adsorbents carrying silver ions, namely, $AgNO_3$ and $Ag_2S_2O_3$ and two gelling agents (agar-agar and Phytagel™) were tested at different concentrations. Addition of $AgNO_3$ (5.88µM) to the medium containing Phytagel™ along with cytokinin (BAP 8.8µM and kinetin 6.97µM) led to significant reduction in shoot abscission rate (4.20), while 17.66µM $AgNO_3$ supplementation improved No. of micro-shoots/explants (2.19) and micro-shoot length (3.36 cm) whereas $Ag_2S_2O_3$ at 20µM enhanced the total chlorophyll content (3.47 mg g^{-1} FW) three times as compared to control. Similarly, among the tested basal media, MS basal medium induced best response on shoot organogenesis. Rooting of micro-shoots was highest (81.12%) with the supplementation of NAA (5.37µM), which also affected No. of roots/explant (4.52) and mean root length (5.26 cm). The supplementation of ethylene adsorbents during in vitro micro-shoot multiplication significantly improved their quality, which provides ideal rooting for development of complete plantlets in sweet orange cv. Mosambi.

Key Message

Ethylene accumulation in glass containers inhibited micro-shoot establishment of Citrus sinensis **L. Use of silver ions enhanced culture establishment and quality of the micro-shoot.**

Introduction

Citrus is one of the important fruit crops of the world and rightly, renowned as the “world fruit” for its high socio-economic and nutritional attributes. Among different citrus groups, sweet orange (*Citrus sinensis* L.) tops the world production with 48.8 million metric tones (FAS, 2021). In India, amongst the cultivated sweet orange cultivars, ‘Mosambi’ has gained momentum in terms of area and production due to its desirable attributes like high sweetness, TSS, vitamin C, minerals, total polyphenols and antioxidant properties.

During the recent past, the demand of planting material having desirable traits such tolerance to various biotic (virus free planting material) and abiotic stresses (drought, salinity, thermal stress etc.) and particularly the low seeded varieties are now in great demand. To achieve the said objective within a shorter span of time, plant tissue culture emerged as a boon in the field of plant biotechnology (George et al. 2008). Production of propagules under controlled environmental condition offers an additional advantage for round the year production and supply of planting material as compared to the conventional propagation (Marutani-Hert et al. 2011). Standardization of in vitro regeneration protocol is

a basic requisite for an experiment to proceed. Furthermore, the successful standardization of an efficient plant regeneration protocol is genotype-dependent and specific for selected explants which depend on the media constituents, including growth regulators and their concentration, explants preparation methods, different surface sterilization treatments, their duration and environmental condition of the culture room.

Among the different components that make a micro-environment congenial for the success of culture establishment, ethylene, a gaseous hormone has a throwback effect. During the various steps of micro-propagation, ethylene produced by different stress treatments (high, low light intensity, wounding) (Feher et al. 2003) affects the physiological activities of the explant (Lemos and Blake 1996). The effect of ethylene on response to cultured plant cell are diverse depending on plant species, cultivar, explants and the different culture conditions followed (Hu et al. 2006). In in vitro conditions, the positive impact of ethylene on callus induction, organogenesis, culture growth in suspension, root formation, embryogenesis and secondary metabolite production have been reported. In contrast, ethylene accumulation was found to inhibit regeneration of several plant species with effects like inhibition of shoot formation, leaf elongation, hypertrophy, leaf epinasty, senescence and diminution of foliar area (Kumar et al. 1998). The leaf abscission in micro-shoot is the major limiting factor for the successful establishment of plantlets through nodal segments. Our initial observation while working on sweet orange cv. Mosambi showed similar throwback effect which might be a consequence of ethylene accumulation. To overcome ethylene effect, various researchers have attempted its regulation with the use of different adsorbents and inhibitors. Ethylene production in the plants is inhibited by weak antagonists like CO_2 and powerful antagonists like silver compounds. General properties of silver ion carrying compound silver nitrate ($AgNO_3$) with good water solubility, specificity, and stability make it ideal for a variety of applications in plant growth regulation and morphogenesis under in vivo and in vitro. Silver thiosulfate ($Ag_2S_2O_3$) is more effective in tissues than $AgNO_3$ because of its faster mobility and less phytotoxicity. The present investigation was therefore aimed to study the counteract effect of ethylene accumulation by using ethylene adsorbent carrying Ag^+ like $AgNO_3$ and $Ag_2S_2O_3$ at different concentrations. Interaction effect of the silver compounds was also investigated to establish a reliable protocol for in vitro control of micro-shoot necrosis/leaf fall in sweet orange cv. Mosambi.

Material And Methods

Plant material and explant preparation

The experiment was carried out at Central Tissue Culture Laboratory, ICAR-National Institute of Plant Biotechnology, ICAR-IARI, New Delhi during 2020 to 2022. The explants (nodal stem segments, 10-15 cm) were collected from the mother plant of sweet orange cv. Mosambi, maintained at the Experimental Fruit Orchard of Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi. The collected explants were transported to laboratory in an icebox and immediately washed under running tap water followed by washing in distilled water with the addition of 1-2 drops of Tween-20™ (surfactants) to remove the contaminants. The washed nodal segments were trimmed from both the ends and leaves were excised

keeping a short petiole intact. The nodal stem segments were treated with 0.1% carbendazim (Bavsitin BASF, India) + 0.1% metalaxyl + mancozeb (Ridomil Gold®, Syngenta, India) suspension and kept on a horizontal shaker (60 rpm) for 1 hour. The nodal segments were then shifted to the laminar and subjected to surface sterilization with freshly prepared sterile 0.1% mercuric chloride solution for 10 min. Thereafter, a quick 70% ethanol dip (10 s) was followed by three rinsing with sterile double-distilled water for 5 min. The surface sterilized explants were trimmed with a sterile scalpel and cut to 3-5 cm segments having one or two nodes and then used for in vitro culture into the test-tubes (150x25 mm) containing different medium treatment combinations.

Effect of BAP and kinetin on culture establishment/shoot organogenesis

This set of first experiment comprised of two cytokinins, i.e., BAP (6-benzylaminopurine) (4.44, 6.66, 8.88 and 11.1µM) and kinetin (N^6-furfuryladenine) (4.65, 6.97, 9.3 and 11.62µM) each at four different concentrations. The combinations were supplemented with basal MS medium (Murashige and Skoog 1962) (Hi-Media, Mumbai PT021).The nodal explants were then transferred onto the glass test tubes of 150×25mm containing 15 ml of MS medium supplemented with different levels of growth regulators. After 4 weeks of culture, data regarding the response of nodal segments to different growth regulators and their combinations (shoot organogenesis (%), days taken for organogenesis, No. of micro-shoots/explant, mean micro-shoot length (cm), mean No. of leaves per micro-shoot, productivity and shoot forming index) were recorded using the following formula:

$$\text{Shoot organogenesis}(\%) = \frac{\text{No.of explants produced micro-shoot}}{\text{Total No.of explants inoculated}}$$

$$\text{Productivity} = \text{No. of micro} - \text{shoot/explant} \times \text{mean micro} - \text{shoot length}$$

$$\text{Shoot forming index}(\text{SFI}) = \frac{\text{shoot organogenesis}\ (\%) \times \text{No. of micro} - \text{shoot/explant}}{100}$$

Effect of basal medium on shoot organogenesis

In the second experiment, 5 different basal media, i.e., MS (Murashige and Skoog 1962), MT (Murashige and Tucker 1969), DKW (Driver and Kuniyuki 1984), WPM (Lloyd and Mc Cown 1980) and B5 (Gamborg et al. 1968) were fortified with BAP 11.1µM and kinetin 9.3µM, 5% sucrose, 200 mg L^{-1} activated charcoal (Qualigens, Mumbai) and 0.7% agar-agar (Hi media PCT0412) to study the comeback effect of different medium on nodal explant proliferation, shoot organogenesis and the data were recorded after 4 weeks of culture.

Effect of ethylene adsorbents and gelling agents on micro-shoot quality

This experiment aimed to improve the micro-shoot quality and prevention of shoot fall with the use of two factors, (i) ethylene adsorbents (ii) gelling agents. Different ethylene adsorbents ($AgNO_3$- 5.88, 17.66

and 29.43μM) and ($Ag_2S_2O_3$- 20, 40 and 60 μM) were supplemented at different concentrations to MS medium and without any ethylene adsorbent solidified with agar-agar and Phytagel™ (Sigma-Aldrich, St. Louis, USA) as control. These ethylene adsorbents were added in the MS medium supplemented (BAP 11.1μM and kinetin 9.3μM) individually with agar-agar (0.7%) and Phytagel™ (0.25%). For preparing 10mM STS ($Ag_2S_2O_3$), 100mM stock solution of Ag_2NO_3 was added to 100mM $Ag_2S_2O_3$ (1:4 M) (Navarro-García et al. 2016a). The stock solutions of ethylene adsorbents were added using sterile micro-filter (0.22 μm; Sartorius, USA). The gelling agents were used as a factor as they affect the physical properties (e.g., water potential) of the media. The micro-shoots obtained from the experiment were used for sub-cultures (4 week) for multiplication. The data regarding the experiment were recorded on the elicited micro-shoot one month after the initiation of culture. Total leaf chlorophyll contents were estimated using DMSO (Dimethyl sulfoxide, AR grade) as per the method suggested by Hiscox and Israelstom (1979). The absorbance was read at 645 and 663 nm wavelengths and total chlorophyll were calculated by using the following formula and the value was expressed as mg g^{-1} of fresh weight of leaves.

$$\text{Total chlorophyll} = \frac{(20.7 \times \text{OD645}) + (8.02 \times \text{OD663}) \times \text{volume} \times \text{dilution}}{100 \times \text{Weight of the sample}}$$

Micro-shoot/leaf abscission control rate was rated as 1 = all leaves dropped, 2 = ≥ 70% of leaves dropped, 3 = 30–70% of leaves dropped, 4 = ≤ 30% of leaves dropped, 5 = no leaves dropped (Marutani-Hert et al. 2011).

Rooting of micro-shoots and acclimatization of plantlets

In this experiment, rooting of micro-shoots was initiated using two different auxins; NAA (2.68, 5.37, 10.74 μM) and IBA (2.46, 4.92, 9.84 μM) in MS medium with and without auxin and activated charcoal (control). Rhizogenesis data was recorded two months after root initiation. After the initiation of roots, micro-shoots were transferred from the rooting media to auxin-free basal media for the elongation of roots.

After 5 weeks, the rooted plantlets were washed with sterile double-distilled water to remove the agar and transferred to the glass jars filled with autoclaved potting medium (coco peat: vermiculite and perlite in 1:1:1) moistened with half-strength liquid MS medium basal salts solution and covered with polythene bags (one week) to maintain the relative humidity. Initially, spraying of water was carried out using 0.1% carbendazim (Bavsitin BASF, India) to prevent fungal infection and thereafter with half-strength sterilized liquid MS medium to prevent mortality of the in vitro raised plantlets.

Media preparation and culture maintenance

All the media used in the experiments were supplied with 5% sucrose (w/v) except for rooting the sucrose was supplemented at 3% (w/v). The pH of the culture media in all the four experiments was adjusted to 5.7±0.5 with the addition of 1N NaOH before the addition of gelling media and after that, the medium was sterilized at 121°C for 20 min. Each treatment comprised of 20-25 test tubes. All the cultures were

maintained in the controlled culture room (24±2°C). The culture maintenance room was programmed to maintain a 16/ 8 h light/ dark cycle using cool white fluorescent lights (54µmol/m^2/s).

Experimental design and statistical analysis

The statistical analysis of the first two and fourth experiment comprising of different treatment combination and four replications were analyzed in a completely randomized design (CRD) using statistical analysis system software, SAS package (9.3 SAS Institute, Inc. USA), followed by a t-test (LSD). The third experiment with two factors, i.e., ethylene adsorbents and gelling agents was analyzed in two factors CRD. P-values ≤0.05 were considered significant. Regression analysis was done to analyze trends and relationships between leaf abscission rates with other growth parameters using ethylene adsorbents. The treatment means were computed by Pearson's simple correlation.

Results

The salient finding of the experiments for mass multiplication of micro-shoot and improvement of micro-shoot quality are represented as below

Effect of PGRs on culture establishment/shoot organogenesis

Nodal explants inoculated on solid MS medium supplied with different PGRs at different concentrations and their combinations were symbolized as T_1-T_{24}. The optimal regeneration in-terms of direct shoot organogenesis (%), days taken for organogenesis, mean No. of micro-shoot/explant, mean micro-shoot length, mean No. of leaves/explant were documented (Table 1). PGR treatments significantly influenced the morphological characters. The highest shoot organogenesis (81.40%) was noted in treatment combination T_{18} (8.8µM BAP+6.97µM kinetin) followed by T_2 (6.6µM BAP) (66.68%). The lowest shoot organogenesis (26.45%) was recorded in treatment T_5 (11.62µM kin), which did not differ statistically with treatment T_8. Among different treatments followed, kinetin at all concentrations showed the poor response with regard to shoot organogenesis.

The minimum time taken for shoot organogenesis (5.47 days) was registered in the treatment T_{17} (8.8µM BAP+4.65µM kinetin) and it maintained statistical parity with T_{18} (8.8µM BAP+6.97µM kinetin) i.e., 5.52 days. The maximum time taken for the shoots to sprout (8.07 days) was recorded in treatment T_{12} (4.4µM BAP+11.62µM kinetin) followed by treatment 11.62µM kin (7.67 days).

The highest number of micro-shoot/explant (2.06) and mean micro-shoot length (1.30 cm) were recorded in T_{18} (8.8µM BAP+6.97µM kinetin), whereas the number of micro-shoot was recorded lowest (1.17) in T_5 (4.65µM kinetin), and the lowest mean micro-shoot length (0.54 cm) was observed in T_{15} (6.6µM BAP+9.3µM kinetin). The maximum number of leaves (3.55) were recorded in T_{18} (8.8µM BAP+6.97µM kinetin) and T_{13} (6.6µM BAP+4.65µM kinetin), with similar values in treatment T_9 (4.4µM BAP+4.65µM

kinetin), while the minimum number of leaves/micro-shoot (1.13) were recorded in T_{20} (8.8µM BAP +11.62µM kinetin).

Shoot productivity and shoot forming index response among different treatments have been depicted (Figure 1). The response showed significant response among different treatments on shoot productivity and shoot forming index. The highest shoot productivity (2.70) and shoot forming index (1.68) were registered in T_{18} (8.8µM BAP+6.97µM kinetin), while the lowest productivity (0.66) was registered in treatment T_{15} (6.6µM BAP+9.3µM kinetin) and minimum shoot forming index (0.32) was noted in T_5 (4.65µM kinetin).

However, these micro-shoots failed to maintain their quality due to the 100% leaf abscission thereby affecting shoot proliferation and rooting. As evident, the combinations of BAP and kinetin treatments were found to be superior in comparison with their individual levels.

Effect of different basal media on shoot organogenesis

A comparative analysis among MS, MT, DKW, WPM and B5 with supplementation of T_{18}-8.8µM BAP+6.97µM kinetin (proved best for shoot organogenesis from the first experiment) was conducted to identify the most effective basal medium for shoot organogenesis, micro-shoot growth and proliferation (Table 2). The highest shoot organogenesis (68.97%), and number of micro-shoot/explant (2.04) were documented for MS in the shortest time (7.6 days). The minimum shoot organogenesis (44.78%) and number of micro-shoots (1.49) were recorded on WPM medium compared to other basal media. The longest (1.60 cm) and the shortest (1.41 cm) micro-shoots were recorded on DKW and B5 medium respectively. The results clearly revealed the superiority of MS basal medium in shoot organogenesis in Mosambi.

Effect of ethylene adsorbents

The results on the effect of ethylene adsorbent and gelling agent on shoot organogenesis, foliar abscission and micro-shoot quality are shown in Table 3. Supplementation of ethylene adsorbents in the form of $AgNO_3$ and $Ag_2S_2O_3$ exhibited significant effect on micro-shoot development, control of micro-shoot/leaf abscission and quality of the micro-shoots. Ethylene absorbents and their interaction with gelling agent significantly affected the different parameters, though gelling agent individually had non-significant effect on different parameters except micro-shoot/leaf abscission rate. Among gelling agents, Phytagel™ was most effective for minimizing micro-shoot/leaf abscission (3.51), number of micro-shoot (1.82), and total leaf chlorophyll content (2.61mg g^{-1} FW), except micro-shoot length (2.84 cm) which was documented highest on agar-agar.

Among the tested ethylene adsorbents, $AgNO_3$ was noted superior to $Ag_2S_2O_3$ in inducing increased number and length of the regenerated micro-shoots. In contrast, $Ag_2S_2O_3$ controlled leaf abscission and improved the quality of micro-shoots with higher total leaf chlorophyll content. The highest number of

micro-shoots (2.14) was noted for treatment 17.66µM $AgNO_3$ followed by 5.88µM $AgNO_3$ (1.85), which were significantly different compared to other treatments. The mean micro-shoot length was recorded maximum (3.20 cm) for the treatment 17.66µM $AgNO_3$ followed by 29.43µM $AgNO_3$ (3.04 cm). The control rate of micro-shoot/leaf abscission (3.96) and leaf chlorophyll content (3.40 mg g^{-1} FW) were recorded highest with 20µM $Ag_2S_2O_3$ followed by 40µM (3.93, 3.20mg g^{-1} FW, respectively) and was significantly superior over other treatments. Contrary to this, the lowest micro-shoot number (1.40), micro-shoot length (1.43 cm), leaf chlorophyll content (1.17 mg g^{-1} FW) and highest micro-shoot/leaf abscission rate (1.09) were recorded in control.

Interaction among ethylene adsorbents and gelling agent levels were statistically similar except the highest and the lowest values which differed than others (Table 4). The highest No. of micro-shoot/explant (2.19) was recorded in the treatment combination 17.66µM $AgNO_3$+Phytagel™, whereas micro-shoot length (3.36 cm) was recorded highest in 17.66µM $AgNO_3$+agar-agar. The lowest micro-shoot/leaf abscission control rate (4.20) was recorded for the combination 5.88µM $AgNO_3$+Phytagel™, while the highest total leaf chlorophyll (3.47 mg g^{-1} FW) was noted for 20µM $Ag_2S_2O_3$+Phytagel™. Agar-agar alone (control) induced lowest number of micro-shoot (1.31), total leaf chlorophyll (1.11 mg g^{-1} FW) with maximum micro-shoot/ leaf abscission (1.05), while Phytagel™ alone recorded the shortest micro-shoots (1.34 cm). The increase in frequency of sub-culture enhanced the regeneration of higher number of micro-shoots from the base of the primary micro-shoot (Figure 3). The result on multiplication in number of micro-shoot varied significantly among each other. The number of micro-shoots increased to 3.06 in first subculture which was further goes on increasing (7.67) with the increase in number of subcultures i.e., fifth subcultures.

Effect of auxins on rooting

Micro-shoots obtained from the fifth sub-culture were used for in vitro rooting trials. The micro-shoots cultured on half-strength MS medium supplemented with different auxins did not give root initiation even after 6 weeks of culture onto rooting media (Data not presented). The observations on rhizogenesis of micro-shoot in full-strength MS medium supplemented with different auxins, however, had significant effects. The highest rooting (64.26%) was observed on MS medium supplemented with 5.37µM NAA followed by 10.74µM NAA (58.22%), while in control (without auxin supplementation) the rooting were not observed. The highest No. of roots/ micro-shoot (4.52) and mean root length (5.26 cm) were documented for 5.37µM NAA followed by 9.84µM IBA for number of micro-shoot (3.82) and 4.92µM IBA for micro-shoot length (4.70 cm), respectively.

Discussion

Bud break and shoot organogenesis were successfully induced with the application of plant growth regulators in the MS medium. Treatments with different BAP levels provided satisfactory results but the treatments supplemented with kinetin were observed to be less effective as compared to other

treatments. With the increase in concentration up to an optimum level (BAP 8.8µM and kinetin 6.97µM), the response on shoot organogenesis, shoot number and shoot length were enhanced but further increase in the concentration led to decline in shoot/leaf number and also shoot length. The effect of BAP in increment in shoot organogenesis may be attributed to the increase in nucleic acid and protein content, thereby leading to enhanced enzymatic activity within the cell and the ultimate increase in cell division, micro-shoot multiplication, micro-shoot length and number of leaves. The findings of the study are in consonance with those reported by Rattanpal et al (2011) in *C. jambhiri* and Al-Bahrany (2002) in *C. lemon* in which they stated that the presence of kinetin and NAA without supplementation of BAP did not show any shoot elongation, thus clearly explaining the role of BAP in inducing and stimulating cell division. Similar variable response of BAP was also reported in different lemon genotypes at varying concentration by Navarro Garcia et al. (2016b). They also reported deterioration in quality at higher concentration of BAP, which was also observed in the present study. Our results on number of micro-shoot/explants supports the earlier result of Tallon et al. 2013 who reported that maximum number of buds (2.5-3.0 buds/explant) with BAP concentration varying from 1.0-3.0 mg L^{-1}. The supremacy of BAP over kinetin might be due to rapid transport and uptake by the explants thereby activating the quiescent cell or meristem to function more efficiently.

Effect of basal media

Most of the reports on micro-propagation focuses on the role of the PGR combinations but have ignored the importance of different basal medium compositions and also the concentration of macro/micro salts. Although all the reported regeneration protocol on citrus was based on the compositions of MS and MT media, Kotsias and Roussos (2001) proposed the superior response of DKW for micro-propagation of lemon. In our study, full-strength MS medium followed by DKW was found superior as compared to the other media evaluated. The variable response of different basal medium treatments is basically attributed to the difference in the nutrient levels, i.e., micro-nutrients, vitamins and amino acids.

The highest shoot organogenesis on MS followed by B5could be attributed to the high ammonia content (20.6 mM) which must have increased synthesis of nucleic acid and protein resulting in expression of genes associated with optimal regeneration. Besides the ammonia content, the vitamins i.e., thiamine, pyridoxine and nicotinic acid among different medium, MS (0.00037, 0.0029, 0.00406mM), MT (0.037, 0.059, 004mM), WPM (0.0037, 0.0029, 0.004mM), B5 (0.037, 0.0059, 0.00812mM), must have contributed significantly towards organogenesis improvement. In the present observation, the lowest shoot organogenesis was documented in WPM, which is well in line with that of Navarro Garcia et al (2016b), who reported MS as the best and WPM as the least effective among the used MS, DKW and WPM. Similarly, the superiority of MS medium compared to WPM for morphogenesis and growth stimulation was earlier reported by Oliveira et al. (2010). In contrast to the effectiveness of WPM over MS basal medium was reported for in vitro organogenesis of Alemow and sour orange (Tallon et al. 2013), which signifies that the different tissue requires different nutrients, vitamin and minerals for different responses which could not be supplied alone in MS medium.

Effect of ethylene adsorbents

Among the ethylene adsorbents tested, none of the combinations recorded complete inhibition of micro-shoot/leaf abscission but $Ag_2S_2O_3$ (STS) was comparatively more effective than $AgNO_3$, as it improved the quality of micro-shoots and increased the chlorophyll content. Incorporation of $Ag_2S_2O_3$ into the medium must have counteracted the ill effects of ethylene by restoring the chlorophyllase gene resulting in improved chlorophyll content and leaf colour (Veen and Vandejein 1978). Similar were the observations of with Mahmoud et al. (2020) who also reported better efficacy of $Ag_2S_2O_3$ against $AgNO_3$ in control of leaf abscission in Australian finger lime. Lemos and Blake (1996) reported 100% leaves abscission within 4 days after culture initiation in *Annona squamosa* L. Although there is dearth of literature on leaf abscission pattern in citrus, the present observation on the effect of silver compounds showed delayed abscission which took place 8 days after culture initiation and the plants died within 10 days.

The better efficacy of $AgNO_3$ in improving the number of micro-shoot in comparison to $Ag_2S_2O_3$ suggests the inhibitory effect of $AgNo_3$ on ethylene action which might have improved shoot organogenesis. The beneficial effect of $AgNO_3$ on improved regeneration of plants has also been reported in different crop species like *Punica granatum* (Naik and Chand 2003), *Manihot esculenta* (Zhang et al. 2001), *Coffea canephora* (Sridevi et al. 2010) and *Hevea brasiliensis* (Sirisom and Te-chato 2012). The decreasing response on number of shoots and length with high concentration of $AgNO_3$ might be due to the toxicity of $AgNO_3$, which increased the ethylene concentration, thereby showing a detrimental effect on shoot organogenesis. Similar inhibitory effect of $AgNO_3$ has been recorded in *A. annua,* in with high concentration i.e., above 6 mg L^{-1} leads to toxicity in the plants (Lei et al. 2014). In the present study, Phytagel™ was found more effective in controlling leaf abscission than agar-agar. However, the results were statistically at par among gelling agents and varied significantly between adsorbents. Mahmoud et al (2020) reported no significant effect of the gelling agent on the control of leaf abscission.

The increment in subcultures had a major effect on the multiplication of micro-shoot and also varied significantly. The number of micro-shoots increased four-fold more than the primary subcultures, because the plantlets limit the growth after a certain period due to the depletion of media constituents thereby requiring further subculture. Again the proliferation and multiplication in number of micro-shoots was due to transfer of the plantlets to the fresh medium containing ample nutrients.

Effect of auxin on rooting

The MS medium without any supplementation of auxins recorded the lowest rooting percentage, number of roots and root length, which signifies the importance of auxin in root initiation. In the present study the observed effectiveness of NAA over IBA might be due to the greater stability and persistence of NAA than IBA. Our results contradict the general belief that IBA is a more potent auxin than NAA for rooting as it is transported faster than IBA in the cell (Epstein and Ludwig-Muller 1993). Similar result of NAA on rooting was also reported by Rashad et al (2005). Hence, it is convincing to say that 5.4µM NAA is the optimum

concentration for induction of roots for micro-shoot derived from nodal segments (Nwe et al. 2014). Further, the depressing effect on rhizogenesis with increasing auxin concentration is in consonance with Al-Bahrany 2002 in *Citrus aurantifolia* (Christm.) Swing.

Correlation and regression analysis

The figure 5 depicts the correlation and regression analysis among the micro-shoot/leaf abscission control rate with No. of micro-shoot/explant, mean micro-shoot length and total chlorophyll content by use of ethylene adsorbents. The correlation study indicated a statistically strong significant correlation among the parameters undertaken. A significant positive correlation was observed between leaf abscission control rate with number of micro-shoot/explant (0.775), mean micro-shoot length (0.964) and total chlorophyll content (0.809).

The regression analysis between leaf abscission rate and other organogenesis parameters portrayed the highest R^2 value for the micro-shoot length (0.930) followed by chlorophyll content (0.654) and the lowest R^2 value was recorded for number of micro-shoot/explant (0.601). As evident in the regression equation, all were documented with positive equations. The value of the coefficient of x-variables for number of micro-shoot (0.162), micro-shoot length (0.560) and total chlorophyll content (0.183) were noted which signifies the value of y changes with an increase in 0.162, 0.560 and 0.183 of x-variable in number of micro-shoot, mean micro-shoot length and chlorophyll content, respectively.

Conclusion

The present study concludes the effectiveness of different PGRs, basal media, and ethylene absorbents ($AgNO_3$ and $Ag_2S_2O_3$) in the standardization of a protocol for mass multiplication of sweet orange and at the same time improving the quality of the regenerated plants. Inoculation of nodal segments in the medium containing the BAP and kinetin resulted in patronizing the effect on shoot organogenesis than the individual effects. Among the tested basal medium, MS medium comeback with effect of maximizing shoot organogenesis as compared to the other mediums. Both the ethylene absorbents contributed in preventing micro-shoot abscission at similar level but $Ag_2S_2O_3$ has a superior effect than $AgNO_3$ in controlling micro-shoot abscission and enhancing chlorophyll content of the leaves whereas $AgNO_3$ proved best for increasing number of micro-shoot/explants and micro-shoot length. In the context of rooting, NAA proved best in increasing the rooting efficiency of micro-shoots. The standardization of this protocol adds value to the existing protocol and to carry out the experiment in developing the plantlets controlling abscission. These results can further be tested for different genotypes to manage the leaf abscission and also establishment of a reliable protocol.

Abbreviations

BAP 6-benzylaminopurine; NAA 1-Napthalene acetic acid; IBA Indole-3-butyric acid; SFI Shoot forming index STS Silver thiosulfate; DMSO Dimethyl sulfoxide; AC Activated Charcoal; CRD Completely

randomized design; LSD Least significant difference; MS Murashige and Skoog medium; MT Murashige and Tucker medium DKW Driver and Kuniyuki medium; WPM Lloyd and Mc Cown medium; B5 Gamborg B5 medium

Declarations

Acknowledgments

The main author sincerely acknowledges the ICAR-Indian Agricultural Research Institute, New Delhi for providing the facility for carrying out the research and Department of Science and Technology, Ministry of Science and Technology (DST) Government of India for the grand of DST-INSPIRE fellowship.

Authors Contribution statement

Research was work conceptualized by Dr. O. P. Awasthi. Data collection, statistical analysis and the original draft was prepared by the main author. The draft was corrected by Dr. S. K. Singh and Dr. Kanika.

Authors have no conflict of interest. The research was funded by DST, Govt of India.

Data Availability Statement

All data generated or analysed during this study are included in this published article.

References

1. Al-Bahrany AM (2002) Effect of phytohormones on in vitro shoot multiplication and rooting of lime *Citrus aurantifolia* (Christm.) Swing. Sci Hortic 95(4):285-295. https://doi.org/10.1016/S0304-4238(01)00349-1
2. Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut rootstock. HortSci 19:507-509
3. Epstein E, Ludwig-Muller J (1993) Indole 3-butyric acid in plants: Occurance, synthesis, metabolism and transport. Physiol Plant 88:382-289. http://doi.org/10.1111/j.1399-3054.1993.tb05513.x
4. Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult 74:201-228. https://doi.org/10.1023/A:1024033216561
5. Gamborg OL, Miller R, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exper Cell Res 50:151-158. https://doi.org/10.1016/0014-4827(68)90403-5
6. George EF, Hall MA, Klerk GJD (2008) Plant propagation by tissue culture. 3rd edn, Springer, Dordrecht, The Netherlands
7. Hiscox JD, Israelstom GF (1979) A method for the extraction of chlorophyll from leaf tissues without maceration. Can J Bot 57:1332-1334. https://doi.org/10.1139/b79-163
8. https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf

9. Hu WW, Gong H, Pua, EC (2006) Modulation of SAMDC expression in *Arabidopsis thaliana* alters in vitro shoot organogenesis. Physiol Plant 128(4):740-750. https://doi.org/10.1111/j.1399-3054.2006.00799.x
10. Kotsias D, Roussos PA (2001) An investigation on the effect of different plant growth regulating compounds in in vitro shoot tip and node culture of lemon seedlings. SciHortic 89:115-128. https://doi.org/10.1016/S0304-4238(00)00227-2
11. Kumar PP, Lakshmanan P, Thorpe TA (1998) Regulation of morphogenesis in plant tissue culture by ethylene. In Vitro Cell Dev Biol Plant 34(2): 94-103. https://doi.org/10.1007/BF02822771
12. Lei C, Wang H, Liu B, Ye H (2014) Effects of silver nitrate on shoot regeneration of *Artemisia annua* L. Plant Biotechnol 13-1109. https://doi.org/10.5511/plantbiotechnology.13.1109a
13. Lemos EEP, Blake J (1996) Control of leaf abscission in nodal cultures of *Annona squamosa* L. J Hortic Sci 71(5): 721-728. https://doi.org/10.1080/14620316.1996.11515452
14. Lloyd G and Mc Cown (1980) Commercially feasible micropropagation of mountain laurel *Kalmia latifolia*, by use of shoot tip culture. Proc Int Comb Plant Prop Soc 30:421-427.
15. Mahmoud LM, Grosser JW, Dutt M (2020). Silver compounds regulate leaf drop and improve in vitro regeneration from mature tissues of Australian finger lime (*Citrus australasica*). Plant Cell Tissue Organ Cult 141(3):455-464. https://doi.org/10.1007/s11240-020-01803-8
16. Marutani-Hert M, Evens TJ, McCollum GT, Niedz, RP (2011) Bud emergence and shoot growth from mature citrus nodal stem segments. Plant Cell Tissue Organ Cult 106(1):81-91. https://doi.org/10.1007/s11240-010-9896-0
17. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
18. Murashige T, Tucker DPH (1969) Growth factor requirements of citrus tissue culture. Proc 1st Int Citrus Symp 3: 1155
19. Naik SK, Chand NK (2003) Silver nitrate and amino-ethoxy-vinylglycine promote in vitro adventitious shoot regeneration of pomegranate (*Punica granatum* L.). J Plant Physiol 160:423–430. https://doi.org/10.1078/0176-1617-00949
20. Navarro-García N, Martínez-Romero D, Pérez-Tornero O (2016a) Assessment of the impact of ethylene and ethylene modulators in *Citrus limon* organogenesis. Plant Cell Tissue Organ Cult 127:405-415. https://doi.org/10.1007/s11240-016-1062-x
21. Navarro-García N, Morte A, Pérez-Tornero O (2016b). In vitro adventitious organogenesis and histological characterization from mature nodal explants of *Citrus limon*. In Vitro Cell Dev Biol Plant 52(2):161-173. https://doi.org/10.1007/s11627-015-9743-4
22. Nwe YY, Myint KT, Mochizuki Y, Vazirzanjani M, Okayasu K, Suzuki S, Ogiwara I (2014) In vitro regeneration through direct shoot organogenesis in Honey Orange (*Citrus tangerina).* Plant Biotechnol J 31(4):341-344. https://doi.org/10.5511/plantbiotechnology.14.0929a
23. Oliveira MLP, Costa MGC, Silva CV, Otoni WC (2010) Growth regulators, culture media and antibiotics in the in vitro shoot regeneration from mature tissue of citrus cultivars. Pesqui Agropecu Bras

45:654-660. https://doi.org/10.1590/S0100-204X2010000700004

24. Rattanpal HS, Kaur G, Gupta M (2011) In vitro plant regeneration in rough lemon (*Citrus jambhiri* Lush) by direct organogenesis. Afr J Biotechnol 10(63): 13724-13728. https://doi.org/10.5897/AJB09.1264
25. Rashad M, Khan MM, Ranzan R, Adnan S, Khan FA (2005) In vitro regeneration and somatic embryogenesis in *Citrus aurantifolia* and *Citrus sinensis*. Int J AgricBiol 7:518-520.
26. Sirisom Y, Te-chato S (2012) The effect of peptone and silver nitrate on in vitro shoot formation in *Heveabrasiliensis*Muell Arg. J Agric Technol 8:1509-1516. http://www.ijat-aatsea.com/pdf/v8_n4_12_July/31_IJAT_2012_8_4__Yupaporn Sirisom-Plant Science-accepted.pdf
27. Sridevi V, Giridhar P, Simmi PS, Ravishankar GA (2010) Direct shoot organogenesis on hypocotyl explants with collar region from in vitro seedlings of *Coffea canephora Pierre* ex. Frohner cv. CxR and *Agrobacterium tumefaciens*-mediated transformation. Plant Cell Tissue Organ Cult 101: 339-347. https://doi.org/10.1007/s11240-010-9694-8
28. Tallón CI, Porras I, Pérez-Tornero O (2013) High efficiency in vitro organogenesis from mature tissue explants of *Citrus macrophylla* and *Citrus aurantium*. In Vitro Cell Dev Biol Plant 49:145-155. https://doi.org/10.1007/s11627-012-9476-6
29. Veen H, vandegeijn, SC (1978) Mobility and ionic form of silver as related to longevity of cut carnations. Planta 140(1):93-96. https://doi.org/10.1007/BF00389386
30. Zhang P, Phansiri S, Puonti-Kaerlas J (2001) Improvement of cassava shoot organogenesis by the use of silver nitrate in vitro. Plant Cell Tissue Organ Cult 67: 47-54. https://doi.org/10.1023/A:1011654128198

Tables

Table 1 Effect of different concentrations of cytokinin (BAP and Kinetin) on culture establishment/ shoot morphogenesis of inoculated nodal explants

Treatment	Shoot organogenesis (%)	Days taken for organogenesis	No. of micro-shoots/ explant	Mean micro-shoot length (cm)	Mean No. of leaves/ micro-shoot
BAP (µM)					
4.4 (T_1)	48.20^{hg} (43.97)*	6.42^{fg}	1.20^{lk}	0.87^{likj}	2.91^{d}
6.6 (T_2)	66.68^{b} (54.76)	5.97^{h}	1.25^{kij}	0.96^{hg}	3.12^{c}
8.8 (T_3)	59.76^{c} (50.63)	5.77^{i}	1.42^{e}	1.24^{ba}	3.28^{b}
11.1 (T_4)	55.10^{d} (47.93)	6.32^{g}	1.26^{hij}	0.84^{lkmj}	2.30^{hgi}
Kinetin (µM)					
4.65 (T_5)	27.80^{l} (31.82)	6.90^{e}	1.17^{l}	0.87^{likj}	2.39^{g}
6.9 (T_6)	42.53^{i} (40.70)	6.80^{e}	1.22^{kij}	1.10^{ecd}	2.62^{f}
9.3 (T_7)	32.66^{k} (34.85)	7.15^{d}	1.24^{kij}	1.02^{efg}	3.25^{b}
11.62 (T_8)	26.45^{l} (30.95)	7.67^{b}	1.26^{hkij}	0.84^{lkmj}	2.37^{hg}
BAP + Kinetin (µM)					
4.4+4.65 (T_9)	43.22^{i} (41.10)	7.21^{dc}	1.81^{a}	1.19^{bcd}	3.51^{a}
4.4+6.97 (T_{10})	55.41^{d} (48.11)	5.72^{i}	1.54^{d}	0.70^{n}	3.28^{b}
4.4+9.3 (T_{11})	46.27^{h} (42.86)	7.66^{b}	1.64^{c}	0.82^{lm}	2.31^{hgi}

4.4+11.62(T_{12})	33.19^{kj} (35.18)	8.07^{a}	1.70^{b}	0.91^{likhj}	2.54^{f}
6.6+4.65 (T_{13})	56.05^{d} (48.48)	6.25^{g}	1.63^{c}	0.76^{nm}	3.55^{a}
6.6+6.97 (T_{14})	43.57^{i} (41.31)	7.40^{dc}	1.27h^{i}	0.87^{likj}	1.93^{j}
6.6+9.3 (T_{15})	54.75^{d} (47.73)	7.18^{d}	1.20^{lk}	0.54^{o}	2.28^{hi}
6.6+11.62 (T_{16})	34.97^{j} (36.25)	7.22^{dc}	1.36^{fg}	0.88^{likj}	1.73^{k}
8.8+4.65 (T_{17})	64.82^{b} (53.63)	5.47^{j}	1.71^{b}	1.16^{bc}	2.79^{e}
8.8+6.97 (T_{18})	81.40^{a} (64.46)	5.52^{j}	2.06^{a}	1.30^{a}	3.55^{a}
8.8+9.3 (T_{19})	52.66^{e} (46.53)	7.62^{b}	1.63^{c}	1.01^{fg}	1.41^{l}
8.8+11.62 (T_{20})	59.56^{c} (50.51)	7.40^{c}	1.31^{hg}	0.94^{ihg}	1.13^{m}
11.1+4.65 (T_{21})	50.44^{f} (45.26)	6.72^{e}	1.20^{lkj}	1.08^{efd}	2.64^{e}
11.1+6.97 (T_{22})	51.38^{fe} (45.79)	5.97^{h}	1.41^{fe}	0.96^{hg}	2.30^{hgi}
11.1+9.3 (T_{23})	49.55^{fg} (44.75)	6.32^{g}	1.22^{lkij}	0.92^{ihj}	2.26^{i}
11.1+11.62(T_{24})	47.62^{hg} (43.63)	6.52^{f}	1.23^{lkij}	0.91^{ikhj}	2.26^{i}
Mean	0.71	0.01	0.002	0.003	0.005

LSD (P≤0.05)	1.19	0.17	0.06	0.08	0.10

*Arc Sine √% transformed data. Mean values in a column are separated by different alphabetical letter(s) in superscript are significant as per Tukey's test

Table 2 Effect of different media on shoot organogenesis on the nodal segment in sweet orange cv. Mosambi

Treatment	Shoot organogenesis (%)	Days to organogenesis	No. of micro-shoots/explant	Mean micro-shoot length (cm)
MS	87.05[a] (68.97)	7.6[c]	2.04[a]	1.44[b]
MT	71.26[c] (57.58)	8.22[b]	1.72[b]	1.47[b]
DKW	63.49[d] (52.82)	8.15[b]	1.76[b]	1.60[a]
WPM	49.62[e] (44.78)	8.73[a]	1.49[c]	1.47[b]
B5	82.82[b] (65.54)	8.90[a]	1.97[a]	1.41[b]
Mean	1.62	0.03	0.012	0.003
LSD (P≤0.05)	1.96	0.24	0.17	0.10

*Arc Sine √% transformed data; Mean values in a column are separated by different alphabetical letter(s) in superscript are significant as per Tukey's test. Each medium was supplemented with 8.8µM BAP + 6.97 µM kinetin; MS = Murashige and Skoog (1962); MT = Murashige and Tucker (1969); DKW= Driver and Kuniyuki (1984); WPM = Lloyd and Mc Cown (1980); B5 =Gamborge et al (1968).

Table 3 Effect of different ethylene adsorbents and gelling agents on micro-shoot multiplication, micro-shoot quality and micro-shoot/leaf abscission control rate in sweet orange cv. Mosambi

Treatment	Mean No. of micro-shoots/explant	Mean micro-shoot length (cm)	Micro-shoot/leaf abscission control rate (%)	Total chlorophyll (mg g^{-1} FW)
Gelling agent				
Agar-agar	1.76^{a}	2.84^{a}	3.36^{b}	2.52^{a}
Phytagel™	1.82^{a}	2.71^{a}	3.51^{a}	2.61^{a}
Mean	0.05	0.07	0.06	0.03
LSD(P≤0.05)	0.12	0.14	0.13	0.09
Ethylene adsorbents				
Control	1.40^{c}	1.43^{c}	1.09^{c}	1.17^{f}
$AgNO_3$(μM)				
5.88	1.85^{b}	3.01^{ba}	3.91^{a}	2.30^{e}
17.66	2.14^{a}	3.20^{a}	3.80^{ba}	2.48^{d}
29.43	1.76^{b}	3.04^{ba}	3.78^{ba}	2.46^{ed}
STS (μM)				
20	1.82^{b}	3.03^{ba}	3.96^{a}	3.40^{a}
40	1.73^{b}	2.86^{b}	3.93^{a}	3.20^{b}
60	1.84^{b}	2.88^{b}	3.59^{b}	2.94^{c}
Mean	0.05	0.07	0.06	0.03
LSD (P≤0.05)	0.23	0.27	0.24	0.17

Data presented in parenthesis are Arc Sine √% transformed values; Mean values in a column are separated by different alphabetical letter(s) in superscript are significant as per Tukey's test

Table 4 Individual Effect of different ethylene absorbents and gelling agents on micro-shoot multiplication, micro-shoot quality and micro-shoot/leaf abscission control rate in sweet orange cv. Mosambi

Treatment	Mean No. of micro-shoots/explant		Mean micro-shoot length (cm)	
	Agar-agar	Phytagel™	Agar-agar	Phytagel™
Control	1.31^{c}	1.49^{bc}	1.52^{c}	1.34^{c}
$AgNO_3$ (µM)				
5.88	1.85^{bac}	1.84^{bac}	3.06^{ba}	2.98^{ba}
17.66	2.10^{a}	2.19^{a}	3.36^{a}	3.04^{ba}
29.43	1.73^{bac}	1.79^{bac}	3.06^{ba}	3.02^{ba}
STS (µM)				
20	1.71^{bac}	1.93^{ba}	3.07^{ba}	2.99^{ba}
40	1.81^{bac}	1.65^{bac}	3.04^{ba}	2.69^{b}
60	1.83^{bac}	1.86^{bac}	2.80^{ba}	2.96^{ba}
Mean	0.05		0.07	
LSD (P≤0.05)	0.56		0.67	

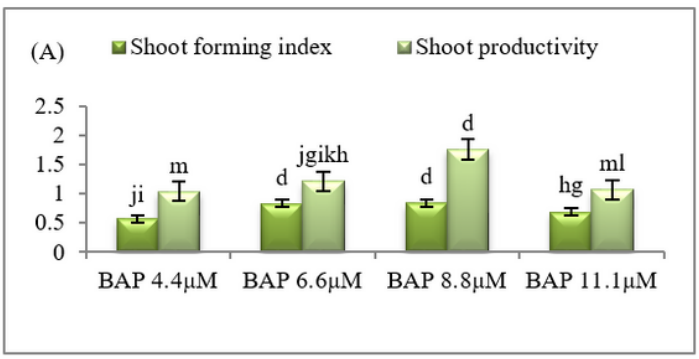
Data presented in parenthesis are Arc Sin √% transformed values; Mean values in a column are separated by different alphabetical letter(s) in superscript are significant as per Tukey's test

Table 5 Effect of auxins supplemented with or without activated charcoal on in vitro rooting in sweet orange cv. Mosambi micro-shoots

Treatment	Rooting (%)	Mean No. of roots/ micro-shoot	Mean root length (cm)
Control (Full MS + 30g L^{-1} Sucrose)	0.00^{f}	0.00^{f}	0.00^{e}
Full MS + 2.68µM NAA + 200mg L^{-1} AC + 30g L^{-1}sucrose	62.83^{d} (52.44)*	3.37^{d}	4.22^{c}
Full MS + 5.37 µM NAA + 200mg L^{-1} AC + 30g L^{-1} Sucrose	81.12^{a} (64.26)	4.52^{a}	5.26^{a}
Full MS + 10.74 µM NAA + 200mg L^{-1}AC + 30g L^{-1} Sucrose	72.26^{b} (58.22)	3.70^{cb}	4.66^{b}
Full MS + 2.46 µM IBA + 200mg L^{-1} AC + 30g L^{-1} Sucrose	57.12^{e} (49.09)	2.70^{e}	3.22^{d}
Full MS + 4.92 µM IBA + 200mg L^{-1} AC + 30g L^{-1} Sucrose	65.38^{dc} (53.96)	3.42^{cd}	4.70^{b}
Full MS + 9.84µM IBA + 200mg L^{-1} AC + 30g L^{-1} Sucrose	66.08^{c} (54.39)	3.82^{b}	4.39^{cb}
Mean	1.31	0.04	0.05
LSD (P≤0.05)	1.70	0.28	0.32

*Arc Sine √% transformed data. Mean values in a column are separated by different alphabetical letter(s) in superscript are significant as per Tukey's test

Figures



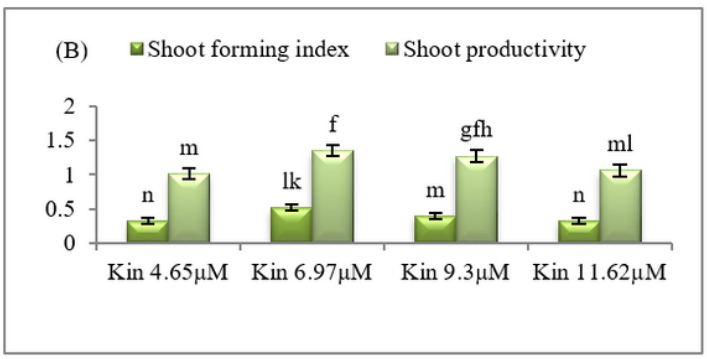


Figure 1

Effect of different concentrations of (A) BAP and (B) Kinetin on shoot forming index and shoot productivity. Bars represented as mean± standard error. Means followed by the same letter were not significantly different as α=0.05 using student's t-test. BAP-(N6-Benzyl aminopurine), Kin- (6-furfuryl amino purine).

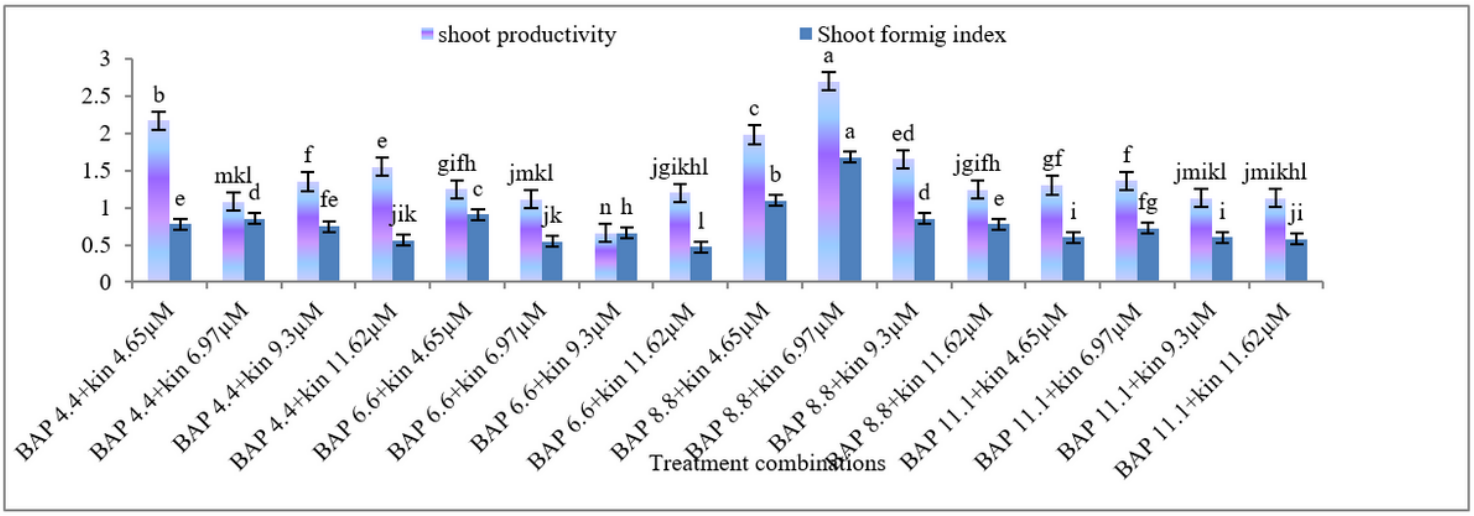


Figure 2

Interaction effect of BAP and kinetin in different concentrations on shoot forming index and shoot productivity. Bars represented as mean± standard error. Means followed by the same letter were not significantly different as α=0.05 using student's t-test. BAP- N6-Benzyl amino purine, Kin-6-furfuryl amino purine.

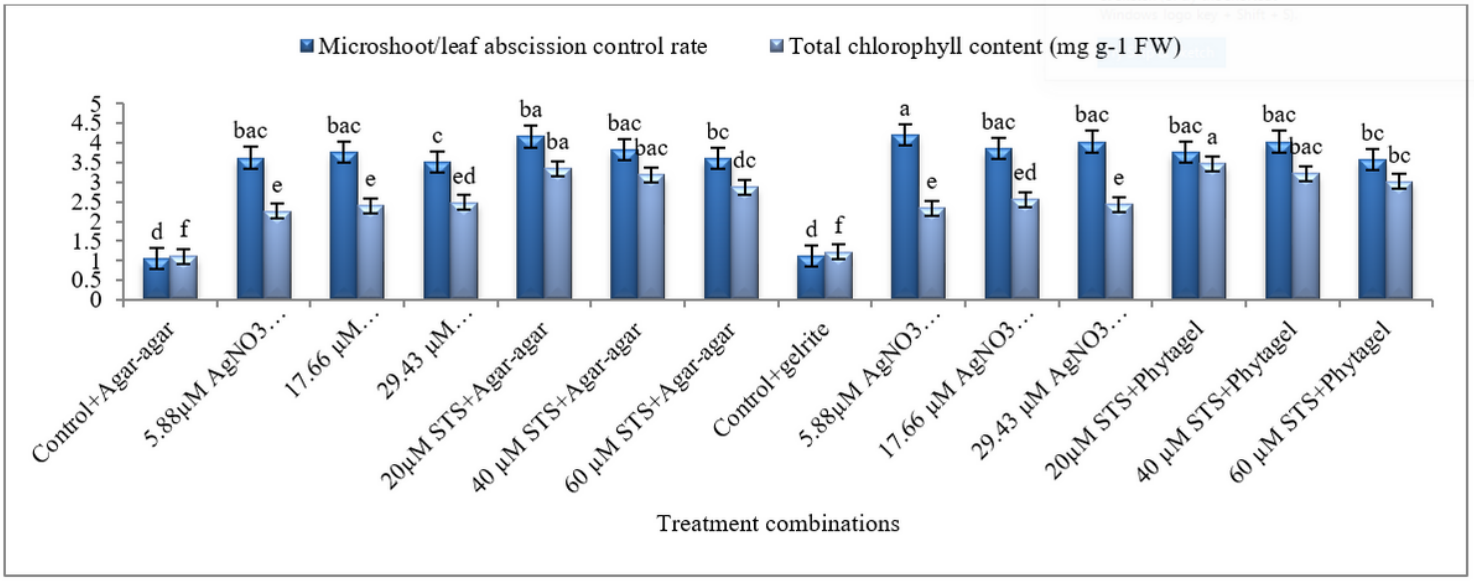


Figure 3

Interaction effect of ethylene adsorbents and gelling agents on micro-shoot/leaf abscission control rate and total chlorophyll content. Bars represented as mean± standard error. Means followed by the same letter were not significantly different as α=0.05 using student's t-test.

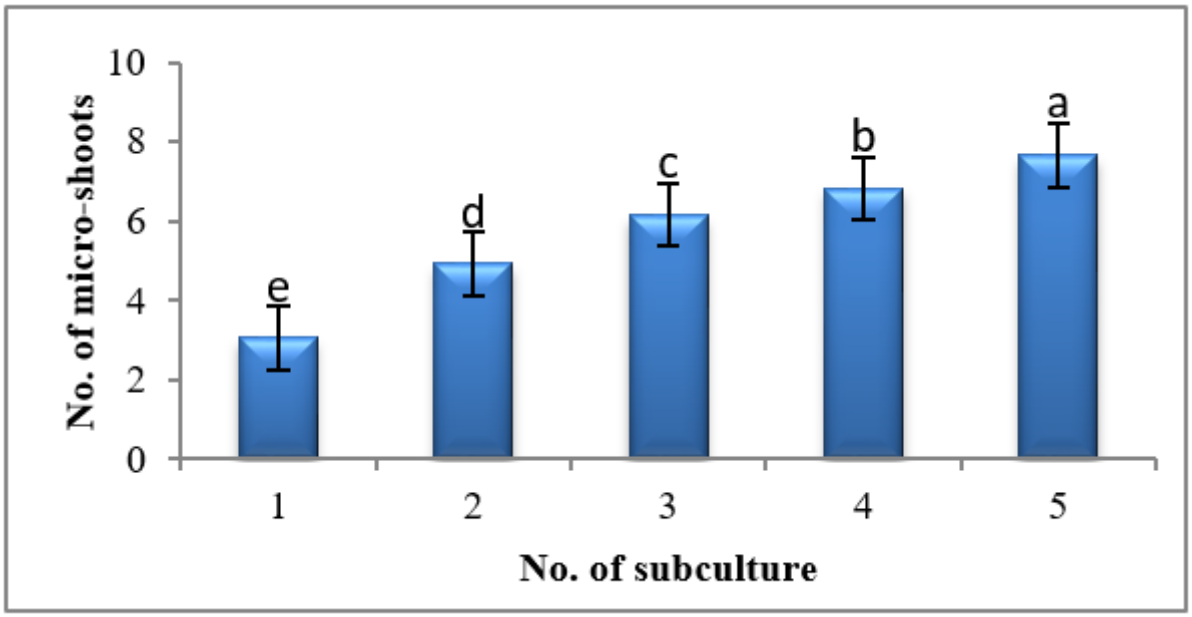


Figure 4

Increase in number of micro-shoot with increment in subculture. Bars represented as mean± standard error. Means followed by the same letter were not significantly different as α=0.05 using student's t-test.

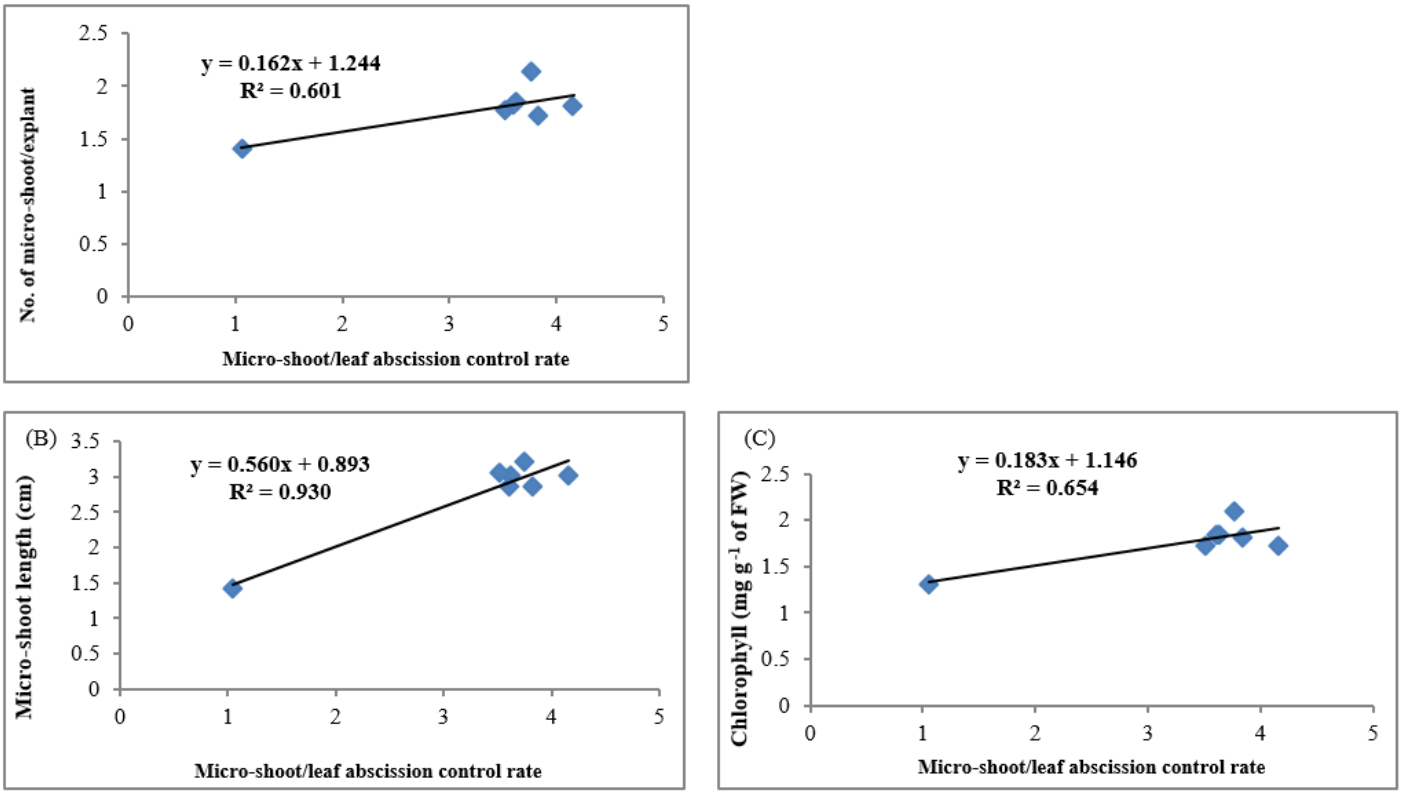


Figure 5

Regression analysis of micro-shoot/leaf abscission with relation to the shoot organogenesis parameter in sweet orange cv. Mosambi (A) No. of micro-shoot/explants (B) Micro-shoot length (C) Chlorophyll content. X-axis corresponds to micro-shoot/leaf abscission while Y-axis to various shoot organogenesis and leaf total chlorophyll content by use of ethylene adsorbents

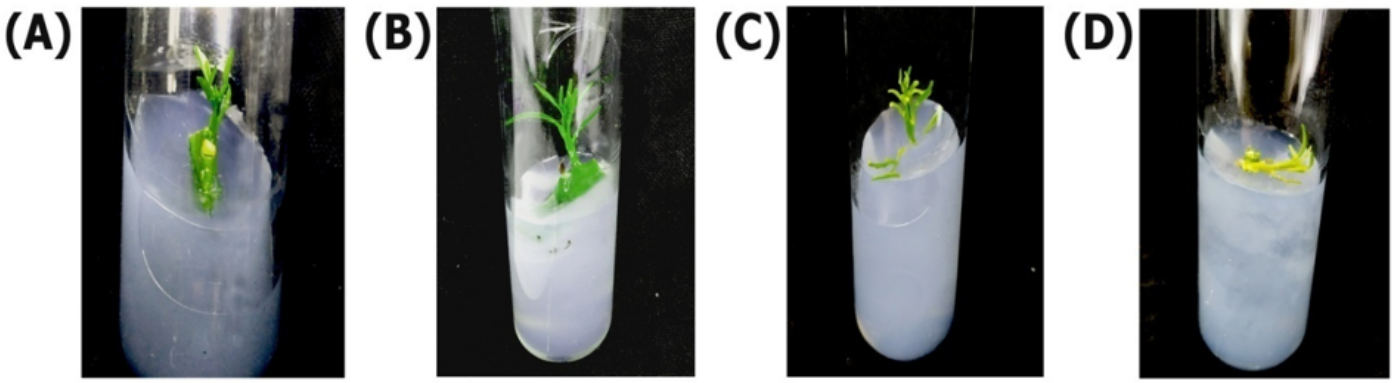


Figure 6

Shoot organogenesis in effect of BAP+Kinetin. (A) On the length of micro-shoot and No. of leaves (B) On the No. of micro-shoot/explant (C) Abscission of leaves (D) Chlorosis of leaves with complete abscission of micro-shoot

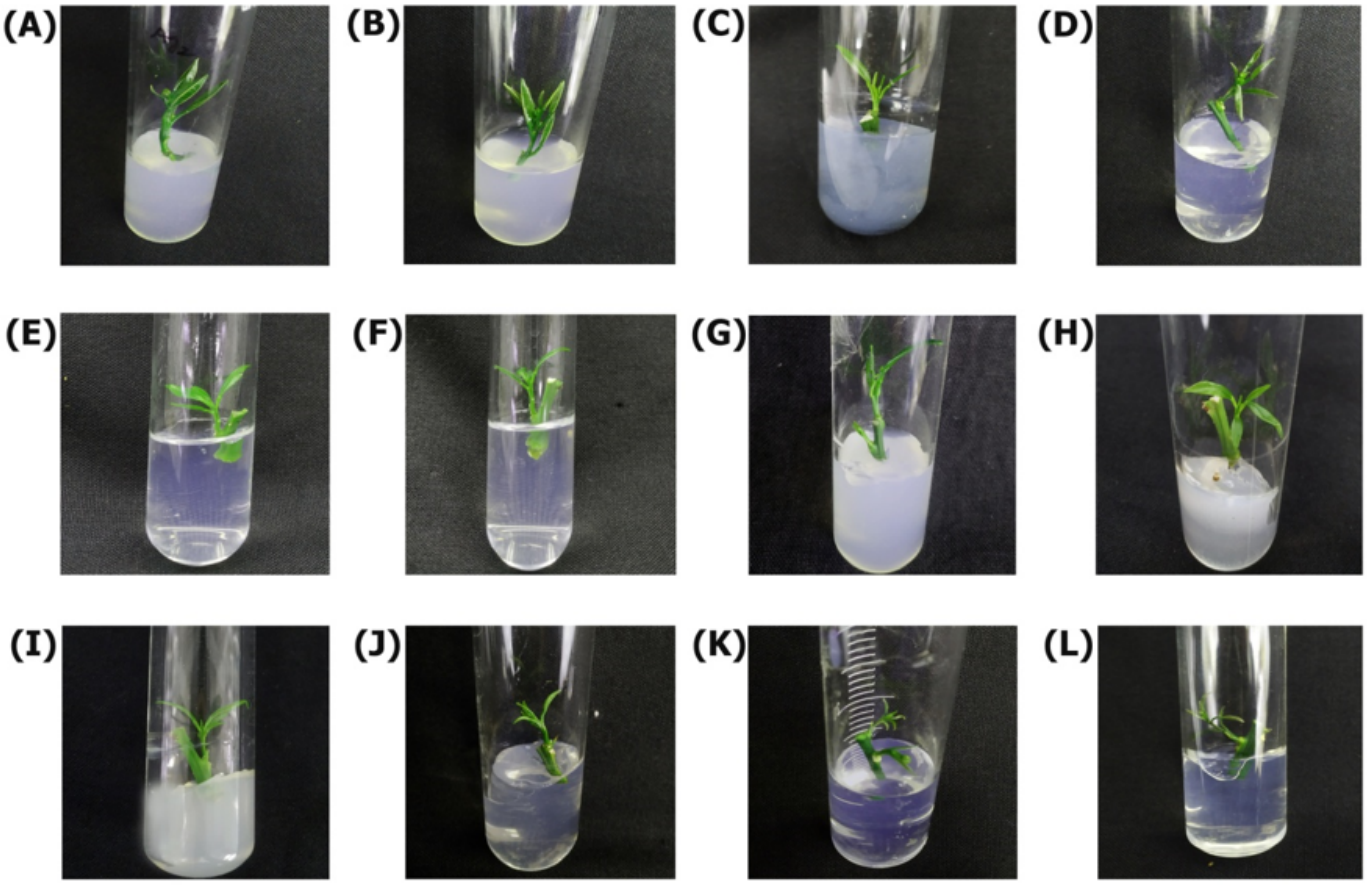


Figure 7

Response of ethylene adsorbents on shoot-organogenesis and micro-shoot quality improvement (A) 5.88µM $AgNO_3$-agar agar (B) 17.66 88µM $AgNO_3$-agar agar (C) 29.43µM $AgNO_3$-agar agar (D) (A) 5.88µM $AgNO_3$-Phytagel™ (E) 17.66 88µM $AgNO_3$- Phytagel™ (F) 29.43µM $AgNO_3$- Phytagel™ (G) 20µM $Ag_2S_2O_3$-agar agar (H) 40µM $Ag_2S_2O_3$-agar agar (I) 60µM $Ag_2S_2O_3$-agar agar (J) 20µM $Ag_2S_2O_3$- Phytagel™ (K) 40µM $Ag_2S_2O_3$- Phytagel™ (L) 60µM $Ag_2S_2O_3$- Phytagel™

Figure 8

(A) Multiplication of micro-shoot (B) Rooting of micro-shoot (C) Hardening of plantlets in potting media