Original Research Article

Direct Plant Regeneration From Nodal Explants OfGrapefruit (*Citrus paradisi*Macfad.)

ABSTRACT

Aims: The present study was undertaken to develop a protocol for *in vitro* regeneration and multiplication of disease-free quality planting material of Grapefruit (*Citrus paradisi*Macfad.).
Study design: Completely Randomized Design (CRD)
Place and Duration of Study: The study was conducted at the Plant Tissue Culture Laboratory, Department of Horticulture, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bengaluru, from 2020 to 21.
Methodology: Nodal segments from the young shoots of field growing plants were used as explants to conduct the experiment. The explants were sterilized and placed on MS medium supplemented with different concentrations and combinations of growth regulators, namely BAP, Kinetin and Gibberellic acid.
Results: Among the different combinations of growth regulators, the combination of BAP 1.0 mgL^{-1} and GA_3 3.0 mgL^{-1} produced maximum number of shoots (7) from nodal segments
Conclusion: The use of growth regulators such as BAP and $GA_3$is reliable for shoot regeneration even when the field explants are used.

Keywords: In-Vitro, BAP, Kinetin, ~~Gibberelic~~Gibberellic acid, IBA

1. INTRODUCTION

A new member of the genus citrus is Grapefruit (*Citrus paradisi*Macfad), which has an interesting history, its one-of-a-kind origin is shrouded in mystery. Hughes in 1750[1]described a tree growing as the "forbidden fruit" in Barbados, although Browne (1756) [2] reported the same and associated it to pummelo (*Citrus maxima*), which was called "smaller shaddock". Later, Macfadyen (1830) [3] classified the “forbidden fruit” reported by both Browne and Hughes as *Citrus paradisi*, also known as “Barbados grapefruit”. Chemotaxonomy [4] and molecular data [5] suggest that accidental hybridization produced an interspecific hybrid of pummelo (*Citrus grandis*) and sweet orange (*Citrus sinensis*), which is now classified as grapefruit.
The*in vitro* regeneration of grapefruit plants offers the benefit of producing disease-free planting material and a source for transformation experiments. The traditional method of propagation is either through budding or grafting which have posed the problem of graft transmissible diseases. However, crop improvement is hindered by the biological characteristics of woody plants, such as nucellar polyembryony, high heterozygosity, long juvenile period, and auto incompatibility.

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In vitro regeneration is one of the most important steps in plant transformation experiments, and it enables crop improvement. Several studies have been conducted to establish a protocol for the *invitro* regeneration of grapefruit using explants such as seeds [6] and leaves [7], nodal segments [8], internodal segments [9], epicotyl [10] and roots.
This study aimed to develop a protocol for *in vitro* regeneration of grapefruit (*Citrus paradisi*Macfad). Young nodal segments from field-grown trees were used as explants in the experiments.

2. MATERIAL AND METHODS

The study was conducted at the Plant Tissue Culture Laboratory, Department of Horticulture, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bengaluru, from 2020 to 21. The media used in the in vitro study were full-strength solid MS (Murashige and Skoog, 1962) [11] media for both shoot and root initiation studies.
The field explants (nodal segments) were subjected to running tap water for 20 minutes and further sterilization was carried out under aseptic conditions. The explants were treated with 0.5 per cent bavistin followed by three sterile water washes (5 minutes each). The explants were treated with 0.3 per of mercuric chloride ($HgCl_2$) for 4 minutes, washed with sterile distilled water three times (5 minutes each), and transferred to 70 per cent alcohol for 1 minute. The explants were then cut to a length of 1 cm, comprising one node each, and transferred to a solution containing 0.1 per cent streptomycin for half an hour. Then the explants were immediately placed on the medium.
The explants were placed on medium consisting of MS salts supplemented with 3 per cent (w/v) sucrose, and the media were solidified by 0.6 per cent agar with different concentrations of growth regulators, viz., cytokinin with concentrations of 0.5, 1.0, 1.5, and 2.0 mg L-1 BAP (6 Benzyl amino purine), KIN (Kinetin) with concentrations of 0.5, 1.0, 2.0, and 3.0 GA_3 with concentrations of 0.5, 1.0, 2.0, and 3.0. IBA (Indole-butyric acid) and IAA (indole-acetic acid) with concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 mgL^{-1} for root initiation.
The culture bottles were maintained in a growth room at a temperature of 24 ± 2^{0}C. A light intensity of 2000 lx was provided using white fluorescence tubes for eight hours of light and 16 h of dark. The chamber was fumigated with potassium dichromate and formaldehyde weekly.
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3. RESULTS AND DISCUSSION

3.1. Effect of BAP and Kinetin on different regeneration parameters

In the first experiment, we observed the effects of BAP and Kinetin on shoot regeneration. The nodes were cultured separately on MS medium supplemented with BAP and Kinetin.
When nodes were cultured on the media, BAP 1 mgL^{-1} showed early shoot initiation (39.4 days), and the number of shoots was higher in BAP 0.5 mgL^{-1} (2.6) and BAP 1 mgL^{-1} (2.4). Shoot length (1.08 cm) was also the highest at BAP 1 mgL^{-1} (Table 1 and Figure.1). The variation in the days taken for shoot initiation at higher concentrations of BAP might be due to the increased level of BAP, which shows a significant effect on shoot initiation, and the low concentration may be insufficient to promote or accelerate shoot growth. The number of days to initiation also depends on the combination of species and media.
After the initial development of buds, the shoots did not regenerate for 30 to 60 days, but bud proliferation continued. None of the buds regenerated into shoots; some buds were arrested at the bud proliferation phase, along with callus formation, and did not form shoots. These observations were also reported previously in sweet orange (*Citrus sinensis*)[12] [13]. They suggested that the small size of regenerated buds may be the reason for this problem. Similar observations were recorded in nodal explants of *Citrus indica*[14].
It was noticed that, among all the treatments the concentration of BAP at 1 mgL^{-1} showed increased shoot length. There was no production of shoots at 30 and 60 days, but at 90 days, the

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Or
The first material taken from the field were propagated until there was sufficient number of explants for the experiments? (if so, please provide the information needed)

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Comment [ӨT7]: Please, mention how long does the experiment lasted and how often the culture medium was renewed during the experiment.

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maximum shoot length noticed was 1.08 cm after shoot initiation, and the lowest was recorded when BAP was used at a higher concentration of 2 mgL^{-1}. Reports state that,when kinetin was used alone, it exhibited the lowest percentage of shoot induction and number of multiple shoots in Meyer lemons (*Citrus meyeri*), which supports the results of the present study. The synergistic and inhibitory interactions of exogenous and endogenous plant growth regulators are specific, as different species, genotypes, and explant sources largely influence the responses of plant cells and tissues [16]. Many other research findings in rough lemon and cleopatra [17],Pumello (*Citrus grandis* L.) [18] and*Citrus macroptera* [19]also suggest that 1 mgL^{-1} is superior for shoot regeneration

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Table 1: Influence of BAP and Kinetin on number of days taken for shoot initiation, number of shoots and shoot length using nodal segment as explant

Comment [ΘT13]: Please provide the results from the statistical analysis.

Treatments	Days taken for shoot initiation	Number of Shoots (at 90 days)	Shoot Length (at 90 days)
T_1 - Basal medium (Control)	50.0	0.0	0.00
T_2 -BAP 0.5	42.2	2.6	0.34
T_3 -BAP 1.0	39.4	2.4	1.08
T_4 -BAP 1.5	41.2	1.4	0.98
T_5 -BAP 2.0	49.6	1.0	0.24
T_6 -KINETIN 0.5	46.8	1.8	0.54
T_7 -KINETIN 1.0	45.8	2.2	1.00
T_8 -KINETIN 2.0	46.2	1.2	0.70
T_9 -KINETIN 3.0	46.4	1.0	0.50
F-test	*	*	*
S.Em±	0.722	0.277	0.277
CD (1%)	2.055	0.789	0.789

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Figure. 1: Shoot formation in BAP 1.0 mgL^{-1}

3.2. Effect of BAP and Kinetin combination on different regeneration parameters when nodal segments were used as explants

Comment [ΘT14]: Experiment and treatments should be mentioned in the Material and Methods section.

The effects of BAP and Kinetin levels on the number of days required for shoot initiation are presented in Table 2. The number of days taken for shoot initiation was significantly different between treatments. The earliest shoot initiation was observed at 35.20 days in the media containing BAP 1.5 mgL^{-1} and Kinetin 1 mgL^{-1}. The maximum duration (79.80 days) for shoot initiation was seen in the media with BAP 2.0 mgL^{-1} and Kinetin 3.0 mgL^{-1} (Figure 2 A). Media with BAP 2.0 mgL^{-1} and different combinations of kinetin did not show any response. The explants in such media remained green, but failed to regenerate. This may be due to the supraoptimal concentration of BAP, which was detrimental for shoot proliferation, and also due to the endogenic levels of cytokinin in different species. *In vitro* propagation of *Citrus reticulata* Blanco and *Citrus limon*Burm showed that, the mean minimum number of days required for regeneration was directly dependent on the species and medium combination in which the explants were placed[20].

Although there was proliferation of buds on the explants, none of the buds formed regenerated into shoots. This may be due to the recalcitrant nature of the explants and the small size of the buds. The maximum number of shoots (5.0) at 90 days after shoot initiation was obtained in BAP 1.0 mgL^{-1} with Kinetin 1.0 mgL^{-1} (Figure 2 B). In Nagpur mandarin when explants were inoculated with MS medium supplemented with BAP (8.88 µl) and (2.32 µl) kinetinmaximum shoots (9.11)had regenerated [21]. Similarly, highest number of shoots per explant was found in 1.5 mgL^{-1} Kinetin with 0.5 mgL^{-1} BAP in *Citrus reticulata*[22]. Highest number of multiple shoot formation per explant was observed (4.4 ± 0.4 shoots)in media containing 1.0 mgL^{-1} BAP and 0.50 mgL^{-1} Kinetin from shoots tip explants of *C.megaloxycarpa*[23].

The combination of BAP 2.0 mgL^{-1} with Kinetin 0.5 mgL^{-1} showed highest shoot length of 1.54 cm (Figure 2 C). The number of shoots produced per explant was inversely proportional to shoot length. The combination of BAP 0.5 mgL^{-1}+ Kinetin 2.0 mgL^{-1}showed the highest shoot length when nodal segments were used as explantsfor Cleopatra mandarin [24].

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Table 2: Influence of BAP and Kinetin combinations on number of days taken for shoot initiation, number of shoots and shoot length using nodal segment as explant

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Treatments	**Days taken for shoot initiation**	**Number of Shoots (at 90 days)**	**Shoot Length (at 90 days)**
T1 - Basal medium (Control)	40	0.0	0.00
T2 -BAP 0.5 + Kinetin 0.5	42.6	2.0	1.14
T3 -BAP 0.5 + Kinetin 1.0	35.8	4.2	0.00
T4 -BAP 0.5 + Kinetin 2.0	43.6	2.6	1.12
T5 -BAP 0.5 + Kinetin 3.0	00.0	0.0	0.46
T6 -BAP 1.0 + Kinetin 0.5	36.2	2.2	0.94
T7 -BAP 1.0 + Kinetin 1.0	37.6	5.0	0.44
T8 -BAP 1.0 + Kinetin 2.0	43.0	4.0	0.76
T9 -BAP 1.0 + Kinetin 3.0	00.0	0.0	0.00

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T10 -BAP 1.5 + Kinetin 0.5	00.0	0.0	0.00
T11 -BAP 1.5 + Kinetin 1.0	35.2	4.0	1.18
T12 -BAP 1.5 + Kinetin 2.0	41.0	2.2	1.80
T13 -BAP 1.5 + Kinetin 3.0	37.2	3.2	1.08
T14 -BAP 2.0 + Kinetin 0.5	45.8	4.2	1.54
T15 - BAP 2.0 + Kinetin 1.0	00.0	0.0	0.00
T16 - BAP 2.0 + Kinetin 2.0	00.0	0.0	0.00
T17 - BAP 2.0 + Kinetin 3.0	46.4	3.0	1.10
F-test	*	*	*
S.Em±	1.19	0.417	0.134
CD (1%)	3.36	1.18	0.378

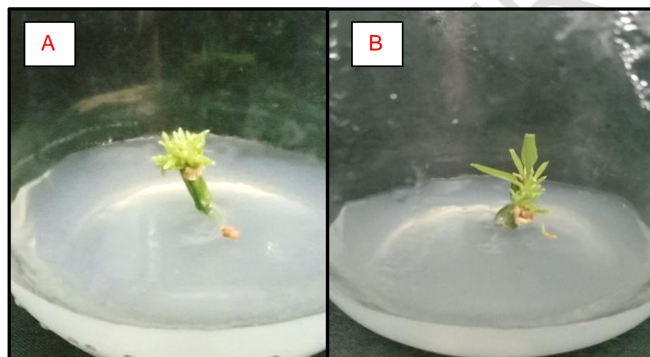

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Figure 2: Influence of BAP and Kinetin on shoot induction from nodal segments. **A**. Bud proliferation in BAP 0.5 mgL^{-1} + Kinetin 2.0 mgL^{-1} **B.** Multiple shoot formation at BAP 1.0 $mgL^{-1}$with Kinetin 1.0 mgL^{-1}**C.** Shoots formed in BAP 1.5 mgL^{-1} with Kinetin 2 mgL^{-1}

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3.3. Effect of BAP and GA_3 on different regeneration parameters when nodal segments are used as explants

The time taken for shoot initiation from nodal segments when BAP and GA_3 were used is shown in Table 3. Significant differences were observed among the treatment combinations. Among the different combinations, the culture media which had BAP 2.0 mgL^{-1} and GA_3 3.0 mgL^{-1} showed early shoot initiation (31.8 days) compared to all the other combinations and control (Figure 3A). Lower concentrations required a longer period for shoot initiation. Bud proliferation occurred before complete shoot formation. All the buds that proliferated did not regenerate into shoots. The highest number of shoots (7) was recorded for BAP 1.0 mgL^{-1} and GA_3 3.0 mgL^{-1} (Figure 3B). The results showed that lower concentrations of BAP and higher concentrations of GA_3 were suitable for producing a greater number of shoots. The number of shoots formed was dependent on the BAP and GA_3 concentrations and the best results with 2 mgL^{-1} BAP and 1 or 2 mgL^{-1} GA_3 were reported in lemon (*Citrus limon*)[25]. Exogenous addition of 4.44 μM BAP in combination with 1.54 μM GA_3 enhanced shoot multiplication rate significantly (17.73±1.69 shoots/explant) in comparison to control (0.00±0.00 shoots/explant) in *Citrus sinensis* (L.) Osbeck [26]. Also, the number of shoots was dependent on the BAP and GA3 concentrations, and the best result was 1 mgL^{-1} BAP + 1.0 mgL^{-1} GA3, and the shoot length was greater with increasing concentrations of GA3 in carrizo (*Citrus carrizo*)[27].
The influence of BAP with GA3 combinations differed significantly among the different concentrations used on the shoot length at 90 days after shoot initiation (Table 3). The maximum length of shoots (1.7 cm) was observed in a medium containing BAP 1.0 mg/L and GA3 2.0 mg/L (Figure. 3C).The treatment with GA3 had the effect of first promoting the multiplication of adventitious shoots and then stimulating their elongation.

Table 3: Influence of BAP and GA_3 combination on number of days taken for shoot initiation, number of shoots and shoot length using nodal segment as explant

Comment [ΘT17]: Pleaseprovidestatisticalanalys is in the table and mention the experiment in the Material and Methods section.

Treatments	Days taken for shoot initiation	Number of Shoots (at 90 days)	Shoot Length (at 90 days)

T_1 - Basal medium (Control)	40.0	0.0	0.00
T_2 -BAP 0.5 +$GA_3$0.5	42.8	3.6	1.08
T_3 -BAP 0.5 + GA_3 1.0	37.4	3.0	0.30
T_4 -BAP 0.5 + GA_3 2.0	38.8	3.4	1.16
T_5 -BAP 0.5 + GA_3 3.0	40.0	5.4	1.00
T_6 -BAP 1.0 + GA_3 0.5	34.4	5.0	1.60
T_7 -BAP 1.0 + GA_3 1.0	44.2	3.8	1.00
T_8 -BAP 1.0 + GA_3 2.0	37.4	4.8	1.70
T_9 -BAP 1.0 + GA_3 3.0	61.0	7.0	0.64
T_{10} -BAP 1.5 + GA_3 0.5	38.2	1.4	0.60
T_{11} -BAP 1.5 + GA_3 1.0	52.8	3.0	0.22
T_{12} -BAP 1.5 + GA_3 2.0	60.4	2.4	0.80
T_{13} -BAP 1.5 + GA_3 3.0	59.0	3.2	0.80
T_{14} -BAP 2.0 + GA_3 0.5	60.4	3.4	0.64
T_{15} - BAP 2.0 + GA_3 1.0	61.4	3.0	0.68
T_{16} - BAP 2.0 + GA_3 2.0	39.4	3.4	0.42
T_{17} - BAP 2.0 + GA_3 3.0	31.8	2.8	0.70
F-test	*	*	*
S.Em±	1.36	0.459	0.125
CD (1%)	3.86	1.298	0.355

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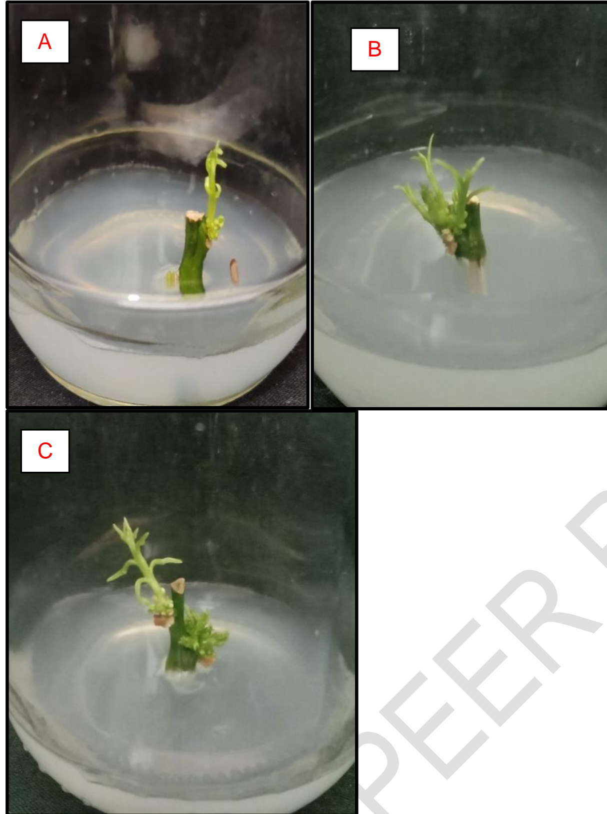

Figure 3: Influence of BAP and GA_3 on shoot induction from nodal segments. A. Shoot formation in BAP 1.0 mgL^{-1} + GA_3 2.0 mgL^{-1} ,B. Multiple shoot formation at BAP 1.0 mgL^{-1} + GA_3 3.0 mgL^{-1}, C. Shoots formed in BAP 1.0 mgL^{-1} and GA_3 0.5 mgL^{-1}

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3.4. Effect of IBA and IAA alone on root regeneration parameters

For root regeneration, the shoots that were regenerated in the trials were placed on medium enriched with IBA and IAA at varying concentrations from 0.5 to 2.5 mg/L. The effect of different concentrations of IAA and IBA on the number of days taken for root initiation did not show any significant difference among treatments (Table 4). The media supplemented with IAA 0.5 mgL^{-1} showed a better response for the production of roots (1.6). Significant differences were observed in root length after 45 days, and IBA 1 mgL^{-1} showed its supremacy over all the other auxins used in the experiment. It was found that, IBA 1 mgL^{-1} showed a maximum root length of 8.6 cm at 45 days. IBA was better for rooting, as this might be due to the fact that IAA is photo-oxidised rapidly than IBA in tissue culture media. So, IAA degrades soon after initial root induction in the rooting medium. IBA, even at a lower concentration, remained active in the medium for a longer period of time, which positively affected the root length. Some of the reports with similar findings where 1 mgL^{-1} of IBA was used and reported a maximum number of roots, 3.19 per shoot on MS + 1 mgL^{-1}l NAA + 1 mgL^{-1} IBA and a minimum of 0.75 on MS + 0.5 mgL^{-1} NAA + 0.5 mgL^{-1} IBA [28]. Also, higher shoot length at 3 mgL^{-1} IBA were also reported [29].

Table 4: Influence of IBA and IAA alone on days for root initiation

Treatment	Days for root initiation	Number of roots at 45 Days	Root length (cm) at 45 Days
T_1 - Basal medium (Control)	42.4	0.2	1.0
T_2 – IBA 0.5	12.4	1.0	5.70
T_3 – IBA 1.0	22.4	1.2	8.60
T_4 – IBA 1.5	31.4	1.4	5.96
T_5 - IBA 2.0	12.8	1.0	6.10
T_6 – IBA 2.5	27.0	1.2	6.84
T_7 – IAA 0.5	11.2	1.6	4.32
T_8 – IAA 1.0	19.2	1.2	4.22
T_9 – IAA 1.5	20.0	1.4	6.06
T_{10} – IAA 2.0	23.0	1.0	5.02
T_{11} – IAA 2.5	36.6	1.4	5.10
F-test	NS	*	*
S.Em±	NS	0.191	2.29
CD (1%)	NS	0.545	0.80

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4. CONCLUSION

The present study reveals that, the combination of bap 1.0 mgl^{-1} and ga_3 3.0 mgl^{-1} produced maximum number of shoots (7) in nodal segments, which could be used for producing a greater number of planting material. Iba 1 mgl^{-1} produced the longest roots of 8.6 cm at 45 days after root initiation and could be recommended for in vitro rooting of grapefruit plants.

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Comment [ΘT19]: 19/29 references have been publishes over a decade ago, while only 3/29 are published within the last 5 years. Please use more recent bibliography when possible.

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