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Effect of Growth Regulators on Multiple Shoot Induction from Shoot Tip of Nagpur Mandarin (*Citrus reticulata* Blanco)

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Abstract

Nagpur mandarin (*Citrus reticulata* Blanco) orange is a prominent group within the citrus family, highly valued for its fruit with sweet taste, ease of peeling and rich nutritional profile. It is a widely cultivated member of the *citrus* genus. However, the conventional propagation of Nagpur mandarin is limited due to its susceptibility to various diseases such as Citrus canker, Huang long bing (HLB or Citrus greening), and Citrus triste zavirus (CTV). These bud-transmittable diseases and a low success rate hinder its propagation. Therefore, the present investigation was undertaken to develop an *in vitro* regeneration protocol for the rapid multiplication of mandarin (*C. reticulata*). The shoot tip of *in vitro* plantlets derived from seeds were used as explants in this study. The explants were cultured on full-strength MS media supplemented with different concentrations and combinations of plant growth regulators, *viz.*, BAP, Kinetin and GA3. The combination of BAP 0.5 mg/L + GA3 1.0 mg/L produced the maximum number of shoots (8.6) at 90 days after shoot initiation, maximum shoot length was recorded in medium containing Kinetin 1.0 /1.5 mg/L + GA3 2.0 mg/L and maximum leaves (30) was observed in medium containing BAP 0.5 + GA3 1.0mg/L.

Keywords : Nagpur mandarin, Growth regulator, *In-vitro* regeneration, Shoot tip

Nagpur Mandarin is a well-known citrus variety primarily cultivated in the Nagpur region of Maharashtra, India. The fruits are small to medium-sized with a slightly flattened shape and the peel is thin and easy to remove. This mandarin is prized for its distinctive taste, juiciness and vibrant colour, making it one of the most popular and highly sought-after citrus varieties in the country (Bopanna *et al.,* 2016). It is a commercially significant cultivar, accounting for 41 per cent of India's total citrus production, with an average yield of 6.0 tons per hectare. The Vidarbha region of Maharashtra, along with neighbouring parts of Madhya Pradesh and Rajasthan (Notably the Jhalawar district), shares similar agro-climatic conditions, fostering the successful and growing cultivation of this variety (Karwa, 2003).

Nagpur mandarin is traditionally propagated through seeds and budding, but *in vitro* propagation offers a

faster way to produce uniform, true-to-type plants. Tissue culture techniques allow for the rapid multiplication of thousands of genetically identical elite plants in a compact space, with year-round availability. Nagpur mandarin is prone to various diseases, including Phytophthora rot (Fungal), greening (Bacterial) and viral infections such as psorosis and exocortis. However, tissue culture propagation generates large number of disease-free plants. Propagation through nucellar tissue from fertilized or unfertilized ovules is labour-intensive and ovules at the optimal developmental stage are only available for a short time each year. Additionally, the presence of juvenile traits and delayed fruiting pose challenges in producing true-to-type, virus-free plants (Bodade *et al*., 2017).

Our research paper aims to establish a standardized micro-propagation protocol for Nagpur mandarin, with a specific focus on using shoot tips as explants. It also explores the potential of individual and combined growth regulators to revolutionize Nagpur mandarin cultivation.

Material and Methods

The present research was conducted at the Plant Tissue Culture Laboratory, Department of Horticulture, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bengaluru, during the years 2022 to 2024.

Shoot tips collected from seed-derived plantlets were used as explants for this experiment. Upon seed swelling, the germinating nucellar and zygotic embryos were identified following the procedure standardized by Tisserat (1985). The seeds were collected from healthy and ripe fresh fruits procured from KR market, Bengaluru. The fruit was cut horizontally without causing any damage to the seeds. The seeds were then removed from the fruits and soaked in water for 2 to 3 hours in tap water. The extracted seeds were washed thoroughly to remove the sticky pulp from the surface to enable easy handling without any slip. Surface sterilization was carried under the aseptic condition just before inoculation. The concentrations and durations of the chemicals used in the experiment were as follows: Bavistin 0.20 (%) for 15 minutes, sodium hypochlorite (5%) with two drops of Tween 20 for 15 minutes, mercuric chloride (HgCl) 0.10 (%) for 3 minutes and alcohol 70 (%) for 1 minute (Ranganath *et al*., 2023).

The seeds were transferred to bottle containing 0.2 per cent of Bavistin (Carbendazim 50% WP) and continuously shaken for 15 minutes and rinsed with sterile water for 3 times (5 minutes each). The seeds were transferred to bottle containing 5 per cent sodium hypochlorite along with Tween 20, a wetting agent and were continuously shaken for 15 minutes followed by three sterile water wash (5 minutes each). Further the seed coat was removed using forceps and scalpel followed by treatment with 0.1 per cent Mercuric chloride ($HgCl_2$) for 3 minutes. The seeds were then again washed with sterile water for three times (5 minutes each). After washing the seeds were transferred to 70 per cent alcohol for a period of 1 minute after which they were placed on the basal medium. Removal of the seed coat was necessary as it facilitated easier germination when compared to the intact seed coat, because seeds cultured with the seed coat took longer to germinate or did not germinate at all.

The seeds were allowed to germinate and grow into seedlings *in vitro* before collecting shoot tips. The excised shoot tips were then placed on medium consisting of MS salts supplemented with 3 per cent (w/v) sucrose solidified with 0.6 per cent agar having different concentrations of growth regulators *viz*., cytokinin with concentration of 0.5, 1.0, 1.5 and 2.0 mgL^{-1} BAP, Kinetin with concentration of 0.5, 1.0, 1.5 and 2.0 mgL^{-1} and Gibberellin GA_3 concentration of 0.5, 1.0, 1.5 and 2.0 mgL^{-1} were used for shoot proliferation.

Results and Discussion

Effect of BAP, Kinetin and GA_3 on Shoot Regeneration from Shoot Tip Explant

The effect of BAP, Kinetin and GA_3 on shoot regeneration was studied by culturing the shoot

tips on MS medium supplemented with BAP, Kinetin and GA_3. Medium with BAP at 1 mg/L^{-1} produced a higher number of shoots compared to BAP at 0.5 mg/L^{-1} (3) and BAP at 1 mg/L^{-1} (2.3), as shown in Table 1 and Table 2. Cytokinins, particularly BAP, promoted maximum shoot formation at lower concentrations, indicating the inhibitory effects of higher hormone concentrations on shoot production (Plate 1 & 2). These data are comparable to those reported by Costa *et al.* (2004), who reported variations in shoot production at 1 mg/L BAP in Foster grapefruit. Haripyaree *et al*. (2011) also demonstrated the efficacy of BAP in their micropropagation studies on *Citrus megaloxycarpa* Lush. Their study showed that MS medium fortified with varying amounts of BAP, Kin and NAA induced the peak shoot count (6.0) at 0.25 mg/L BAP.

It was observed that among all the treatments, GA at 2 mg/L resulted in the highest shoot length. This finding is in consistent with a report on lemon by Perez-Tornero *et al*., 2009. Conversely, the lowest shoot length was recorded when BAP was used at a higher concentration of 2 mg/L. GA specifically targets the internodes, the segments of the stem between nodes, promoting their elongation, which leads to an overall increase in shoot length (Hedden and Phillips, 2000).

Reports show that using kinetin alone resulted in the lowest percentage of shoot induction and multiple shoot formation in Meyer lemons (*Citrus meyeri*), consistent with the findings of this study. The interaction between exogenous and endogenous plant growth regulators is often species-specific, as various species, genotypes and explant sources can significantly influence the response of plant cells and tissues (Haradzi *et al.,* 2021). Further research on rough lemon and Cleopatra mandarin (Mwaniki *et al.,* 2019), Pomelo (*Citrus grandis* L.) (Sharma *et al.,* 2009) and *Citrus macroptera* (Begum *et al*., 2004) also supports the conclusion that BAP at 1 mg/L is optimal for shoot regeneration.

Table 1

Influence of Cytokinin (BAP, Kinetin) and GA_3 on number of shoots after 30, 60 and 90 days from shoot initiation

	Treatment	Number of Shoots (at 30 days)	Number of Shoots (at 60 days)	Number of Shoots (at 90 days)
T_1	Basal medium (Control)	1.33	2.33	2.33
T_2	BAP 0.5	1.66	1.33	3.00
T_3	BAP 1.0	1.00	2.33	2.33
T_4	BAP 1.5	1.33	1.66	1.66
T_5	BAP 2.0	1.00	1.33	1.33
T_6	Kinetin 0.5	1.00	1.00	1.00
T_7	Kinetin 1.0	1.00	1.00	1.00
T_8	Kinetin 1.5	1.00	1.00	1.00
T_9	Kinetin 2.0	1.00	1.33	1.33
T_{10}	GA_3 0.5	1.00	1.33	1.66
T_{11}	GA_3 1.0	1.00	1.00	1.00
T_{12}	GA_3 1.5	1.00	1.00	1.00
T_{13}	GA_3 2.0	1.00	2.33	2.33
	F-test	1.72	1.98	*
	S.Em±	0.16	0.38	0.40
	CD (1%)	NS	NS	1.58

TABLE 2

Influence of Cytokinin (BAP, Kinetin and GA_3) on shoot length at 30, 60 and 90 days from shoot initiation

	Treatment	Length of Shoots (at 30 days)	Length of Shoots (at 60 days)	Length of Shoots (at 90 days)
T_1	Basal medium (Control)	0.63	1.26	1.66
T_2	BAP 0.5	0.56	1.13	1.26
T_3	BAP 1.0	0.46	0.70	0.80
T_4	BAP 1.5	0.80	0.93	0.96
T_5	BAP 2.0	0.40	0.46	0.6
T_6	Kinetin 0.5	0.56	0.76	0.96
T_7	Kinetin 1.0	0.43	0.56	0.96
T_8	Kinetin 1.5	0.60	0.80	0.96
T_9	Kinetin 2.0	0.60	0.70	0.83
T_{10}	GA3 0.5	0.40	0.8	0.90
T_{11}	GA3 1.0	0.76	1.26	1.43
T_{12}	GA3 1.5	0.50	0.66	1.13
T_{13}	GA3 2.0	1.10	2.23	2.36
	F-test	1.36	*	4.20
	S.Em±	0.16	0.19	0.22
	CD (1%)	NS	0.75	0.88

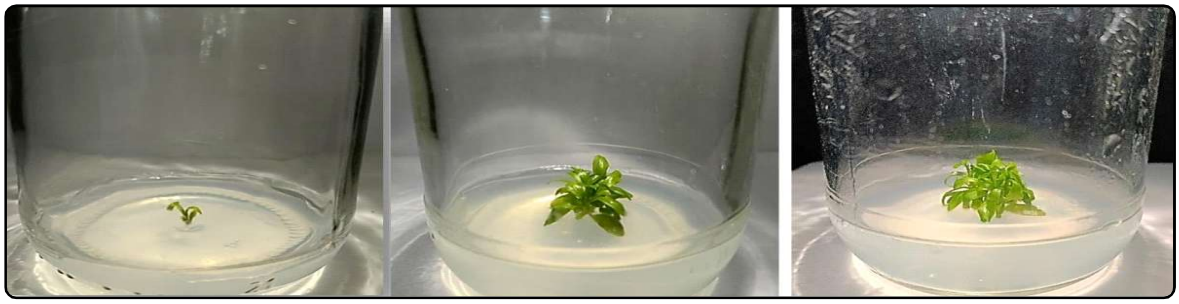

Plate 1 : Influence of BAP, Kinetin and GA_3 on shoot induction from shoot tip. (a) Shoot formation in BAP 0.5 mg/L^{-1} 30 Days (b) Shoot formation in BAP 0.5 mg/L^{-1} 60 Days (c). Shoot formation in BAP 0.5 mg/L^{-1} 90 Days

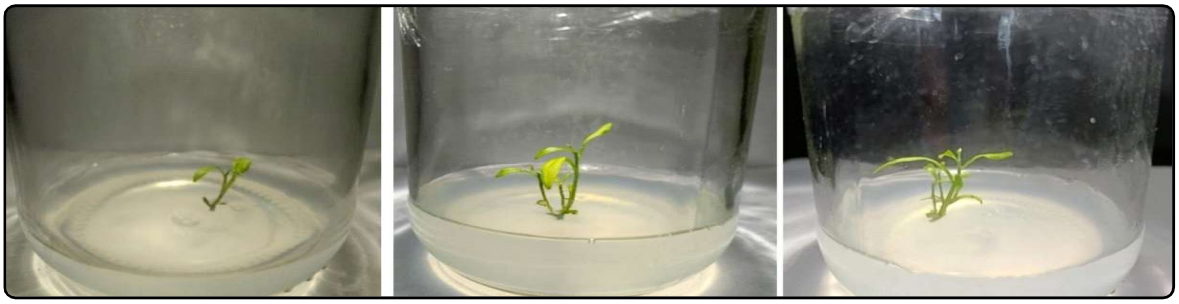

Plate 2 : Influence of BAP, Kinetin and GA_3 on shoot length from shoot tip. (a) Shoot length in GA_3 2.0 mg/L^{-1} 30 Days (b) Shoot length in GA_3 2.0 mg/L^{-1} 60 Days. (c). Shoot length in GA_3 2.0 mg/L^{-1} 90 Days

Combined Effect of BAP and Kinetin on Shoot Regeneration from Shoot Tip

The effects of different BAP and Kinetin concentrations on the number of shoots after initiation are shown in (Table 3 and Plate 3). Maximum number of shoots (5.3) at 90 days post-initiation was achieved with 1.0 mg/L BAP and 1.5 mg/L Kinetin. In Nagpur mandarin, when explants were cultured on MS medium supplemented with BAP (8.88 µL) and Kinetin (2.32 µL), maximum number of shoots regenerated (Singh *et al*., 1994). Similarly, highest number of shoots per explant was recorded in *Citrus reticulata* with 1.5 mg/L Kinetin and 0.5 mg/L BAP (Mukhtar *et al*., 2005). Multiple shoot formation was highest in media containing 1.0 mg/L BAP and 0.5 mg/L Kinetin from shoot tip explants of *C. megaloxycarpa* (Haripyaree *et al*., 2011).

The combination of 0.5 mg/L BAP with 1.5 mg/L Kinetin resulted in the longer shoots1.76 cm (Table 4 and Plate 4). Interestingly, the number of shoots per

TABLE 3

Influence of BAP and Kin on number of shoots produced at 30, 60 and 90 days from shoot initiation

	Treatment	Number of Shoots (at 30 days)	Number of Shoots (at 60 days)	Number of Shoots (at 90 days)
T_1	Basal medium (Control)	1.33	2.33	2.33
T_2	BAP 0.5 + Kinetin 0.5	1.00	2.66	3.00
T_3	BAP 0.5 + Kinetin 1.0	1.00	1.66	2.33
T_4	BAP 0.5 + Kinetin 1.5	1.33	2.66	3.00
T_5	BAP 0.5 + Kinetin 2.0	1.33	2.66	3.33
T_6	BAP 1.0 + Kinetin 0.5	1.66	3.33	4.33
T_7	BAP 1.0 + Kinetin 1.0	3.00	4.66	5.00
T_8	BAP 1.0 + Kinetin 1.5	2.66	4.66	5.33
T_9	BAP 1.0 + Kinetin 2.0	2.00	3.33	4.00
T_{10}	BAP 1.5 + Kinetin 0.5	2.00	3.33	3.33
T_{11}	BAP 1.5 + Kinetin 1.0	2.00	2.66	2.66
T_{12}	BAP 1.5 + Kinetin 1.5	2.66	4.00	4.00
T_{13}	BAP 1.5 + Kinetin 2.0	2.00	3.33	3.33
	F-test	4.08	1.20	0.84
	S.Em±	0.32	0.80	1.03
	CD (1%)	1.25	NS	NS

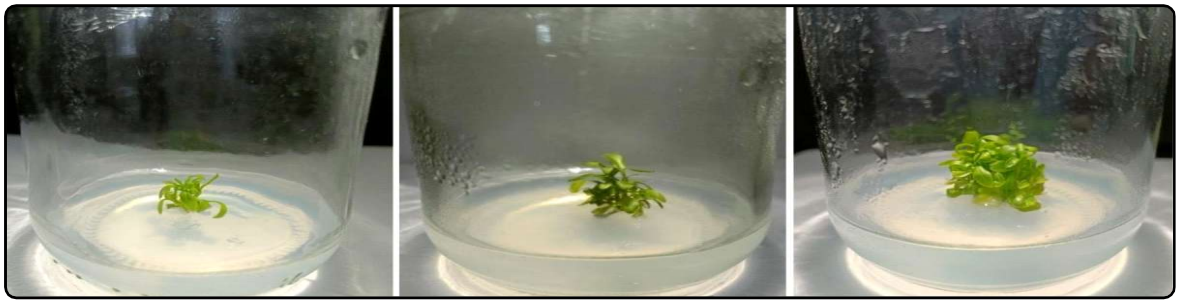

Plate 3 : Influence of BAP and Kinetin on shoot induction from shoot tip. (a) Shoot formation in BAP 1 mg/L^{-1} + Kinetin 1.5 mg/L^{-1} 30 Days; (b) Shoot formation in BAP 1 mg/L^{-1}+Kinetin 1.5 mg/L^{-1}60 Days; (c) Shoot formation in BAP 1 mg/L^{-1}+ Kinetin 1.5 mg/L^{-1} 90 Days

TABLE 4

Influence of BAP and Kinetin on length of shoots produced at 30, 60 and 90 days after shoot initiation

	Treatment	Length of Shoots (at 30 days)	Length of Shoots (at 60 days)	Length of Shoots (at 90 days)
T_1	Basal medium (Control)	0.63	1.26	1.66
T_2	BAP 0.5 + Kinetin 0.5	0.53	1.00	1.10
T_3	BAP 0.5 + Kinetin 1.0	0.50	0.73	0.86
T_4	BAP 0.5 + Kinetin 1.5	0.70	1.43	1.76
T_5	BAP 0.5 + Kinetin 2.0	0.53	1.20	1.40
T_6	BAP 1.0 + Kinetin 0.5	0.66	0.93	1.00
T_7	BAP 1.0 + Kinetin 1.0	1.03	1.20	1.40
T_8	BAP 1.0 + Kinetin 1.5	0.70	1.10	1.46
T_9	BAP 1.0 + Kinetin 2.0	0.56	0.83	1.06
T_{10}	BAP 1.5 + Kinetin 0.5	0.46	0.66	0.73
T_{11}	BAP 1.5 + Kinetin 1.0	0.60	0.70	0.73
T_{12}	BAP 1.5 + Kinetin 1.5	0.50	0.96	1.03
T_{13}	BAP 1.5 + Kinetin 2.0	0.50	0.93	1.06
	F-test	1.45	1.34	*
	S.Em±	0.12	0.20	0.22
	CD (1%)	NS	NS	0.86

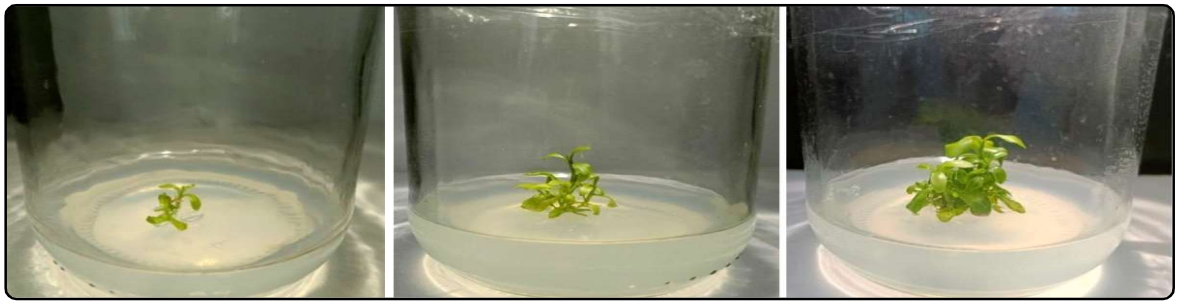

Plate 4 : Influence of BAP and Kinetin on shoot length from shoot tip. (a) Shoot length in BAP 0.5 mg/L^{-1} + Kinetin 1.5 mg/L^{-1} 30 Days (b) Shoot length in BAP 0.5 mg/L^{-1} + Kinetin 1.5 mg/L^{-1} 60Days (c) Shoot length in BAP 0.5 mg/L^{-1} + Kinetin 1.5 mg/L^{-1} 90 Days

explant was inversely proportional to shoot length with 3 number of shoots. In *Cleopatra mandarin*, the combination of 0.5 mg/L BAP and 2.0 mg/L Kinetin produced the longest shoots when nodal segments were used as explants (Kumar *et al*., 2014).

Effect of BAP and GA_3 on Different Regeneration Parameters when Shoot Tip are used as Explants

Among the various combinations tested, highest number of shoots was recorded with 0.5 mg/L BAP and 1.0 mg/L GA_3 (Table 5 and Plate 5). Lower concentrations of BAP combined with higher concentrations of GA_3 were optimal for producing a greater number of shoots. The shoot formation is influenced by the concentrations of BAP and GA_3, with the best results observed in lemon (*Citrus limon*) using 2 mg/L BAP and 1-2 mg/L GA_3 as reported by (Perez-Tornero *et al*., 2010). Similarly, the exogenous addition of 4.44 µM BAP with 1.54 µM GA_3 significantly enhanced shoot multiplication rates compared to the control in *Citrus sinensis* (Pandey *et al*., 2016). In *Citrus carrizo*, the number of shoots

TABLE 5

Influence of BAP and GA_3 on number of shoots produced at 30, 60 and 90 days from shoot initiation

	Treatment	Number of Shoots (at 30 days)	Number of Shoots (at 60 days)	Number of Shoots (at 90 days)
T_1	Basal medium (Control)	1.33	2.33	2.33
T_2	BAP 0.5 + GA_3 0.5	1.66	2.66	3.33
T_3	BAP 0.5 + GA_3 1.0	2.00	6.00	8.66
T_4	BAP 0.5 + GA_3 1.5	1.66	3.33	4.00
T_5	BAP 0.5 + GA_3 2.0	1.66	2.66	2.66
T_6	BAP 1.0 + GA_3 0.5	1.00	1.33	1.66
T_7	BAP 1.0 + GA_3 1.0	2.33	2.66	2.66
T_8	BAP 1.0 + GA_3 1.5	2.33	4.33	4.33
T_9	BAP 1.0 + GA_3 2.0	1.33	2.00	4.00
T_{10}	BAP 1.5 + GA_3 0.5	1.66	4.33	5.00
T_{11}	BAP 1.5 + GA_3 1.0	3.00	5.00	6.00
T_{12}	BAP 1.5 + GA_3 1.5	1.66	2.66	4.33
T_{13}	BAP 1.5 + GA_3 2.0	2.00	2.00	2.66
	F-test	0.52	0.65	0.84
	S.Em±	0.71	1.66	2.00
	CD (1%)	NS	NS	NS

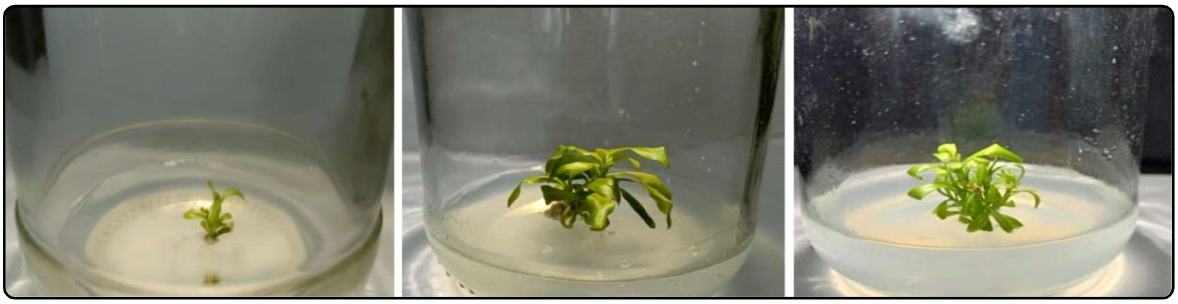

Plate 5 : Influence of BAP and GA_3 on shoot induction from shoot tip. (a) Shoot formation in BAP 0.5 mg/L^{-1} + GA_3 1.0 mg/L^{-1} 30 Days (b) Shoot formation in BAP 0.5 mg/L^{-1} + GA_3 1.0 mg/L^{-1} 60 Days (c) Shoot formation in BAP 0.5 mg/L^{-1} + GA_3 1.0 mg/L^{-1}90 Days

Plate 6 : Influence of BAP and GA_3 on shoot length from shoot tip. (a) Shoot length in BAP 0.5 mg/L^{-1} + GA_3 1.0 mg/L^{-1} 30 Day (b) Shoot length in BAP 0.5 mg/L^{-1} + GA_3 1.0 mg/L^{-1} 60 Day (c) Shoot length in BAP 0.5 mg/L^{-1} + GA_3 1.0 mg/L^{-1} 90 Days

TABLE 6

Influence of BAP and GA_3 on Shoot length produced at 30, 60 and 90 days from shoot initiation

	Treatment	Length of shoot (at 30 days)	Length of shoot (at 60 days)	Length of shoot (at 90 days)
T_1	Basal medium (Control)	0.63	1.26	1.66
T_2	BAP 0.5 + GA_3 0.5	0.73	1.23	1.40
T_3	BAP 0.5 + GA_3 1.0	1.26	2.40	2.53
T_4	BAP 0.5 + GA_3 1.5	1.20	1.50	1.70
T_5	BAP 0.5 + GA_3 2.0	0.73	1.26	1.46
T_6	BAP 1.0 + GA_3 0.5	0.56	1.23	1.30
T_7	BAP 1.0 + GA_3 1.0	0.56	1.03	1.10
T_8	BAP 1.0 + GA_3 1.5	0.93	1.40	2.16
T_9	BAP 1.0 + GA_3 2.0	0.76	1.20	1.60
T_{10}	BAP 1.5 + GA_3 0.5	0.43	0.86	1.16
T_{11}	BAP 1.5 + GA_3 1.0	0.86	1.33	1.40
T_{12}	BAP 1.5 + GA_3 1.5	0.70	1.20	1.26
T_{13}	BAP 1.5 + GA_3 2.0	0.60	0.70	1.13
	F-test	0.72	0.69	0.81
	S.Em±	0.28	0.47	0.46
	CD (1%)	NS	NS	NS

was also dependent on BAP and GA_3 concentrations, with the best results obtained using 1 mg/L BAP + 1.0 mg/L GA_3 and shoot length increased with higher concentrations of GA_3 (Kanwar *et al*., 2013).

The effect of BAP and GA_3 combinations on shoot length differed significantly across the concentrations used. The maximum shoot length (2.16 cm) was observed in a medium containing 0.5 mg/L BAP and 1.0 mg/L GA_3 (Table 6 and Plate 6). GA_3 promoted the multiplication of adventitious shoots and subsequently stimulated their elongation.

The present study demonstrated that MS medium containing combination of 0.5 mg/L BAP and 1.0 mg/L GA_3 produced the maximum number of shoots making it an ideal combination for regenerating a higher number of shoots for shoots for further root induction and rapid multiplication of plants.

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