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Modified protocol to overcome *in vitro* recalcitrance of premature micro-shoots abscission by silver supplementation in Kinnow mandarin (*Citrus nobilis* L. × *Citrus deliciosa* T.)

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The present study focused on alleviating the issue of premature abscission of micro-shoots emerged from the cultured nodal segments in Kinnow mandarin. To resolve the problem, explants of different maturity stages, culture media, growth regulator combinations and ethylene inhibitors (silver compounds) were evaluated. Hardwood stage was observed to be the most efficient explant maturity stage to boost the vigorous early shoot emergence on MS (Murashige and Skoog) medium. Addition of GA_3 (Gibberellic acid) 10 mg L^{-1} resulted in delayed abscission. Further reduction in abscission rate and healthy shoot growth were observed when subculturing of micro-shoots was done on MS medium supplemented with BAP 2.5 mg L^{-1}, GA_3 10 mg L^{-1} and silver thiosulfate 5 mg L^{-1}. This modified procedure could be effectively utilized for mass multiplication as well as crop improvement in Kinnow mandarin.

Keywords: GA_3, Hardwood, MS medium, Nodal segments

Citrus fruits are rich sources of nutrients. Sweet orange (*Citrus sinensis* Osbeck), mandarin (*C. reticulata* Blanco), lime (*C. aurantifolia* Swingle), lemon (*C. limon* (L) Burm. F), grapefruit (*C. paradisi* Macf.) and pummelo (*C. maxima* (L.) Osbeck) are the widely cultivated and consumed species of the genus. Fruits are loaded with carbohydrate, fibre, vitamins, minerals, antioxidants and phytochemicals beneficial for human health. Besides being consumed in the fresh form, fruits are commercially used for processing into refreshing juice and aroma/flavor compounds, nutraceuticals, secondary metabolites and essential oils. Globally, citrus industry is dominated by sweet orange while, Indian citrus industry is dominated by mandarin (42.25%)[1]. The north western part of India, characterized by arid climate, is the major producing region of Kinnow mandarin.

Kinnow mandarin is known for its higher juice recovery and high productivity. However, excessive number of seeds poses obstacle in processing of fruits into juice because of the high limonin content (9.52 mg/g)[2]. Development of low seeded Kinnow mandarin (<10 seeds fruit) in contrast to 30-35 seeds/fruit in wild type is therefore, of foremost importance. Mutagenesis has been utilized as a breeding tool in developing seedless varieties in orange, mandarin, grapefruit and lemon[3-5]. However, the technique takes longer time to purify the material and get desired mutants. *In vitro* induced mutagenesis could prove to be useful under such condition, for which an efficient regeneration system needs to be standardized. *In vitro* regeneration of citrus has been successfully tried using juvenile as well as mature vegetative explants in sweet orange, mandarin and lemon[6-10]. Although few regeneration protocols using different explants have been reported in Kinnow mandarin[11], the authors experienced difficulty in getting the regeneration success using mature nodal segments as explants. Abscission phenomenon in mature nodal explants during regeneration has been reported in few citrus species such as *C. australasica*, *C. hystrix*, *C. mitis*, *C. paradisi* and *C. sinensis*[9,12,13]. In this backdrop, the current study was aimed to standardize an efficient shoot regeneration system for Kinnow mandarin to produce quality micro-shoots by overcoming the issue of abscission.

Materials and Methods

Plant material

Seven years old budded plants of Kinnow mandarin maintained at a spacing of 6 m x 6 m in the experimental Orchard of Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi,

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India (latitude 28° 38′ 23″ N, longitudes 77° 09′ 27″ E) were used as mother plants. Before collecting the explants, mother plant was sanitized with 100 mg L^{-1} streptomycin and 0.2% each of Bavistin® (Carbendazim) + Ridomil Gold® (Metalaxyl + Mancozeb). Nodal explants were preferred because of their genetic uniformity as the juvenile seedlings may contain zygotic mix. Vegetative shoots at different maturity stages were classified into 4 groups based on their maturity *viz*., E_1: Young tender shoots, E_2: Softwood, E_3: Semi hardwood and E_4: Hardwood. Shoots were detached from the mother plant in the morning hours using sterile surgical blade and were transported to laboratory in clean polythene bags along with ice packs.

Culture medium

The basal media, organic additives, sucrose, plant growth regulators (PGR) and gelling agents of plant tissue culture grade were used for the experiment. The media tried were: M_1: Murashige and Skoog (MS) medium[14], M_2: Murashige and Tuker (MT) medium[15] and M_3: Driver and Kuniyuki walnut (DKW) medium[16]. After adding the required quantity of growth regulators (as detailed in Table 1), additives and 50 g L^{-1} sucrose into basal medium the pH was adjusted between 5.7-5.8 using 1N KOH (Potassium hydroxide) and 1N HCl (Hydrochloric acid) before adding 7 g L^{-1} agar. The media was autoclaved at 121°C (15 psi) for 20 min.

Explant preparation and inoculation

The shoots were washed thoroughly in running water to clean the adhering dust. Using sterile surgical blade, leaves were trimmed off from the shoots by retaining petiole. Thereafter, nodal segments as explant were prepared by retaining 3-4 nodes. Microbial contaminants were removed following sequence of surface sterilization treatments which varied according to stages of shoot maturity. Tender and soft wood nodal segments were immersed in combinations of 0.1% Carbendazim + 0.1% (Metalaxyl + Mancozeb) with 1-2 drops of Tween® 20 (Hi Media, India) for 30 min. in double-distilled water. The nodal segments were then surface sterilized inside a laminar air-flow chamber with agitation in 2.0% sodium hypochlorite (NaOCl) for 15 min. and 70% ethanol (*v/v*) for 30 sec. Semi hardwood and hardwood nodal segments were immersed in 0.5% Carbendazim + 0.5% (Metalaxyl + Mancozeb) + 200 mg L^{-1} 8-Hydroxyquinoline citrate (HQC) along with 1-2 drops of Tween® 20 for 120

Table 1 — Plant growth regulators and silver compounds used for *in vitro* shoot regeneration

PGR treatments						Silver compounds	
Treatment	PGR	Concentration (mg L^{-1})	Treatment	Concentration (mg L^{-1})		Treatment	Concentration (mg L^{-1})
B_0 (control)	-	-		BAP	Kinetin	control	BAP 2.5+ GA_3 10
B_1	BAP	0.5	BK_1	0.5	1.5	AN_1	2.5
B_2		1.0	BK_2	1.0	1.5	AN_2	5.0
B_3		1.5	BK_3	1.5	1.5	AN_3	10.0
B_4		2.0		BAP	GA_3	STS_1	2.5
B_5		2.5	BG_1	0.5	1.5	STS_2	5.0
B_6		5.0	BG_2	1.0	1.5	STS_3	10.0
B_7		10.0	BG_3	1.5	1.5		
K_1	Kinetin	0.5		BAP	NAA		
K_2		1.0	BN_1	0.5	0.5		
K_3		1.5	BN_2	1.0	0.5		
K_4		2.0	BN_3	1.5	0.5		
K_5		2.5	BN_4	2.0	0.5		
K_6		5.0		GA_3	NAA		
K_7		10.0	GN_1	0.5	0.5		
G_1	GA_3	0.5	GN_2	1.0	0.5		
G_2		1.0	GN_3	1.5	0.5		
G_3		1.5	GN_4	2.0	0.5		
G_4		2.0					
G_5		2.5					
G_6		10.0					

AN silver nitrate, *STS* silver thiosulfate

min. followed by sterilization with 4.0% NaOCl for 20 min. in laminar air-flow chamber. Between each step, explants were rinsed thrice with sterile double-distilled water. Finally, nodal segments of 1.5 - 2 cm containing single or double buds were prepared and inoculated onto the culture media as per the treatment. The cultures were maintained under aseptic culture conditions with 25 ± 2°C temperature, 70-80% relative humidity and photoperiod of 16/8 light/dark cycle (2000 lux) using white fluorescent light. Subculturing was done every 12-15 days.

Effect of Silver compounds on culture establishment

Earlier studies showed positive effect of ethylene inhibitors on significant abscission control[9,12,13]. Hence, detailed studies on effect of silver nitrate ($AgNO_3$) and silver thiosulfate (STS) on culture establishment were carried out. MS medium supplemented with BAP 2.5 mg L^{-1} + GA_3 10 mg L^{-1} was taken as control while silver nitrate and silver thiosulfate were added at three different levels of 2.5, 5.0 and 10.0 mg L^{-1}. STS was prepared as per the standard procedure *i.e.* 1:4 molar ratio of freshly prepared 0.1 M silver nitrate stock and 0.1 M sodium thiosulfate stock were mixed together to get STS[17]. The shoots grown in the G_6 medium were subcultured into silver nitrate and silver thiosulfate supplemented media and the growth parameters were recorded. Since silver (Ag) is highly thermolabile, filter sterilized silver compounds were added when autoclaved media cooled down to lukewarm temperature.

Statistical analysis

The experiments were conducted in completely randomized design in three replications and in each replication 25 explants were selected in the study. Standardization of explant and culture media were analyzed in factorial randomized completely block design. Data were analyzed using SAS statistical software (Version 9.3, SAS Institute, NC, USA). Comparison of treatment means were done by Duncan's multiple range test (DMRT) at $P = 0.05$ significant level. The percentage data were transmuted to arc sine prior to ANOVA.

Results

Medium and explant type on shoot emergence

Success of any plant tissue culture system depends on explant type and medium used for culture initiation. In the present study, observations on primary shoot emergence showed that the explant type hardwood (E_4) exhibited earlier bud break (3.72) and shoot emergence (13.69 days) with maximum shoot emergence (38.56%) and leaf unfolding efficiency (26.93%) in contrast to other explant types. Among basal media used, MS (M_1) and MT (M_2) were found to be significantly superior over DKW (M_3) in terms of primary shoot emergence. The interaction between $E_4 \times M_1$ revealed positive results on minimum number of days (3.62) required for sprouting, early induction of shoots (13.45 days) and highest shoot emergence of 39.90% whereas, higher leaf unfolding efficiency (22.01%) was obtained on $E_4 \times M_2$ interaction (Tables 2 & 3). Results inferred that among explant types, hardwood had significant effect on primary shoot emergence (less than fortnight to form) over other maturity stages. Likewise, among the basal media, MS and MT were found to be significantly superior over DKW medium in terms of primary shoot emergence.

Plant growth regulators on shoot regeneration and multiplication

Addition of growth regulators to the basal medium is an inexorable factor for modulating growth of cultured explant. Earlier studies on *in vitro* multiplication of citrus species showed requirements of cytokinins, auxins and gibberellins in culture media[6-10]. Simultaneously, abscission in

Table 2 — Effect of media and explant type on days to bud break and primary shoot emergence in Kinnow mandarin

Explant type	Bud break (days)				Shoot emergence (days)			
	M_1	M_2	M_3	Mean*	M_1	M_2	M_3	Mean*
E_1	19.00±1.00^{b}	19.71±0.92^{b}	22.77±1.53^{a}	20.49^{a}	31.38±0.75^{a}	31.65±0.64^{a}	32.26±0.64^{a}	31.76^{a}
E_2	12.33±0.57^{cd}	11.44±1.04^{de}	14.67±0.97^{c}	12.81^{b}	26.33±0.63^{b}	25.56±0.13^{b}	25.69±0.25^{b}	25.86^{b}
E_3	9.24±0.67^{e}	9.90±0.19^{e}	9.68±0.43^{e}	9.61^{c}	17.67±0.15^{c}	17.63±0.10^{c}	18.83±0.45^{c}	18.04^{c}
E_4	3.62±0.18^{f}	3.78±0.15^{f}	3.76±0.17^{f}	3.72^{d}	13.45±0.54^{d}	13.64±0.22^{d}	14.00±0.53^{d}	13.69^{d}
Mean**	11.05^{b}	11.21^{b}	12.72^{a}		22.21^{b}	22.12^{b}	22.69^{a}	
LSD	Explant (E)		0.77			0.44		
($P \leq 0.05$)	Medium (M)		0.67			0.38		
	E× M		2.36			1.34		

citrus is a complex phenomenon and hormonal signals play a major role in regulation. In general, gibberellins (GAs) and cytokinins are considered to be positive abscission regulators while auxins react the either way. Hence, experiment was conducted to define the optimum level of growth regulators for *in vitro* regeneration of Kinnow mandarin. Among the PGRs tested, G_6 (GA_3 10 mg L^{-1}) recorded early bud break (3.42 days) and shoot emergence within a week (7.63 days) compared to B_0 (control) (13.45 days). This shows the favorable impact of addition of plant growth regulator (GA_3) to the culture medium along with explant competency in Kinnow (Fig. 1). Although highest bud break and shoot emergence was recorded with BAP and GA_3 combinations at lower concentrations (BG_3-92.72% and BG_2-85.68% respectively) but maximum number of micro-shoots (3.90 per explant) were obtained from B_5 (BAP 2.5 mg L^{-1}) while, longer length of micro-shoots (27.23 mm) and more number of leaves were recorded in treatment G_6 (5.96) (Tables 4 and 5).

Table 3 — Effect of media and explant type on shoot emergence and leaf unfolding percentage in Kinnow mandarin

Explant Type	Percent shoot emergence (%)				Leaf unfolding percent			
	M_1	M_2	M_3	Mean*	M_1	M_2	M_3	Mean*
E_1	24.17±0.64^{f} (29.44)	24.30±0.60^{f} (29.53)	25.37±0.17^{f} (30.24)	29.74^{d}	9.50±0.35^{e} (17.95)	9.62±0.03^{e} (18.07)	9.20±0.10^{e} (17.66)	17.89^{d}
E_2	28.93±0.37^{e} (32.54)	29.00±0.33^{e} (32.58)	28.37±0.38^{e} (32.18)	32.43^{c}	11.33±0.14^{d} (19.67)	11.30±0.23^{d} (19.64)	11.00±0.16^{d} (19.37)	19.56^{c}
E_3	35.17±0.45^{c} (36.37)	36.83±0.39^{bc} (37.37)	32.40±0.68^{c} (34.69)	36.14^{b}	15.27±0.05^{c} (23.00)	15.50±0.28^{c} (23.18)	14.77±0.42^{c} (22.60)	22.93^{b}
E_4	39.90±0.26^{a} (39.17)	39.07±0.49^{ab} (38.69)	37.62±0.13^{b} (37.83)	38.56^{a}	20.57±0.25^{ab} (26.97)	21.01±0.17^{a} (27.29)	19.95±0.41^{b} (26.53)	26.93^{a}
Mean**	34.38^{a}	34.54^{a}	33.73^{b}		21.90^{a}	22.04^{a}	21.54^{b}	
LSD ($P \leq 0.05$)	Explant (E)		0.43			0.25		
	Medium (M)		0.38			0.22		
	E× M		1.34			0.75		

Data in the column represent the mean of three replications ± standard deviation and followed by same letter within a column are not significantly different (P < 0.05). Values in the parenthesis are arc sine transformed values. * represent the explant mean irrespective of media. ** represent the medium mean irrespective of explants. E_1: Young tender shoots, E_2: Softwood, E_3: Semi hardwood and E_4: hardwood. M_1: MS medium, M_2: MT medium, M_3: DKW medium.

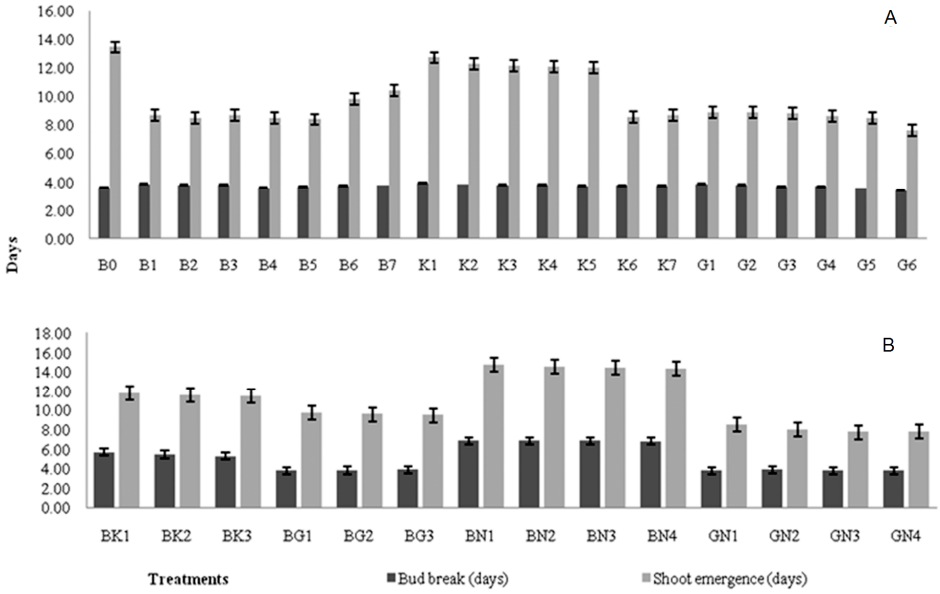

Fig. 1 — Effect of plant growth regulators on days taken for bud break and shoot emergence in Kinnow mandarin. (A) Different concentrations of individual plant growth regulators; and (B) Different combinations of plant growth regulators. Bars represent the mean ± standard error

Table 4 — Effect of plant growth regulators on primary shoot emergence and rate of abscission in nodal segments of Kinnow mandarin

Treatment (mg/L)	Bud break (%)	Shoot regeneration (%)	No. of micro-shoots / explant	Micro-shoot length (mm)	No. of leaves/ micro-shoot	Abscission
B_0 (blank) control	40.98±0.46^{f} (39.80)	39.17±0.26^{n} (39.90)	0.83±0.15^{k}	4.73±0.31^{l}	1.27±0.58^{k}	++
B_1	51.05±0.53^{d} (45.60)	45.25±0.12^{l} (50.43)	1.20±0.26^{j}	9.40±0.26^{ij}	1.87±0.12^{j}	++
B_2	57.30±0.26^{c} (49.20)	50.11±1.10^{jk} (58.87)	1.53±0.12^{hi}	12.30±0.61^{de}	3.53±0.15^{d}	++
B_3	61.33±0.50^{b} (51.55)	63.95±0.82^{d} (80.70)	1.77±0.32^{g}	15.80±0.89^{b}	5.43±0.49^{b}	++
B_4	61.67±1.71^{b} (51.76)	65.20±0.66^{c} (82.40)	2.37±0.25^{de}	11.13±0.25^{fg}	4.50±0.36^{c}	++
B_5	62.93±1.56^{b} (52.50)	67.06±0.40^{b} (84.80)	3.90±0.10^{a}	9.63±0.06^{hi}	3.93±0.12^{d}	++
B_6	67.17±1.62^{a} (55.05)	67.53±1.22^{b} (85.37)	1.73±0.06^{gh}	8.60±0.10^{jk}	3.07±0.15^{e}	++
B_7	67.43±3.71^{a} (55.27)	68.92±1.37^{a} (87.03)	1.71±0.01^{gh}	8.40±0.05^{k}	3.03±0.12^{e}	++
K_1	33.70±0.79^{g} (35.49)	41.57±0.78^{m} (44.03)	1.37±0.06^{ij}	7.77±0.67^{k}	1.93±0.12^{j}	++
K_2	36.77±1.54^{g} (37.32)	49.22±0.41^{k} (57.33)	1.63±0.06^{gh}	9.83±0.15^{hi}	2.37±0.21^{hi}	++
K_3	49.57±0.15^{d} (44.75)	62.16±1.56^{e} (78.17)	1.67±0.12^{gh}	13.80±0.35^{c}	2.83±0.15^{efg}	++
K_4	49.80±0.18^{d} (44.89)	55.59±0.59^{g} (68.07)	2.40±0.10^{de}	13.03±0.90^{cd}	2.43±0.06^{ghi}	++
K_5	50.47±0.52^{d} (45.26)	53.84±0.51^{h} (65.18)	3.17±0.15^{b}	11.70±1.06^{ef}	2.70±0.17^{efgh}	++
K_6	50.50±0.06^{d} (45.29)	53.43±0.16^{hi} (64.50)	2.23±0.15^{ef}	11.08±0.20^{fg}	2.67±0.25^{efgh}	++
K_7	50.57±0.09^{d} (45.32)	53.31±0.12^{hi} (64.30)	2.07±0.06^{f}	11.20±0.10^{fg}	2.87±0.06^{ef}	++
G_1	29.67±0.36^{h} (33.00)	50.38±0.60^{j} (59.33)	1.27±0.12^{j}	10.60±0.95^{gh}	2.10±0.56^{ij}	++
G_2	37.00±0.60^{g} (37.47)	51.12±0.18^{j} (60.60)	1.73±0.06^{gh}	12.70±1.06^{d}	2.47±0.06^{fghi}	++
G_3	45.20±1.90^{e} (42.24)	52.67±0.39^{i} (63.23)	2.37±0.06^{de}	11.40±0.26^{efg}	2.40±0.10^{hi}	++
G_4	49.50±0.52^{d} (44.71)	54.17±0.12^{h} (65.73)	2.84±0.10^{c}	13.10±0.69^{cd}	2.54±0.05^{fgh}	++
G_5	54.67±0.67^{c} (47.68)	57.57±0.38^{f} (71.23)	2.53±0.12^{d}	11.67±0.58^{ef}	2.57±0.08^{fgh}	++
G_6	60.91±1.20^{b} (51.31)	58.37±0.07^{f} (72.50)	1.80±0.10^{g}	27.23±0.67^{a}	5.92±0.03^{a}	+
LSD ($P \leq 0.05$)	2.03	1.10	0.22	0.99	0.42	

Data in columns represent mean of three replications ± standard deviation and the values followed by same letter within a column are not significantly different ($P < 0.05$). Values in the parenthesis are arc sine transformed values. $^{++}$ represent the initiation of abscission less than 30 days after explant inoculation while + represent the delayed abscission of more than 30 days after explant inoculation

Table 5 — Effect of plant growth regulator combinations on primary shoot emergence and rate of abscission in Kinnow mandarin

Treatment	Bud break (%)	Shoot regeneration (%)	No. of micro-shoots/ explant	Micro-shoot length (mm)	No. of leaves/micro-shoot	Abscission
BK_1	85.67±0.22^{f} (67.76)	54.65±0.77^{f} (47.67)	0.90±0.10^{defgh}	6.93±0.15^{g}	2.27±0.15^{b}	++
BK_2	87.13±0.22^{e} (68.98)	54.37±0.58^{f} (47.51)	0.77±0.06^{gh}	7.18±0.18^{g}	2.33±0.06^{ab}	++
BK_3	88.08±0.14^{d} (69.80)	69.02±0.22^{d} (56.18)	1.40±0.26^{ab}	8.47±0.16^{ef}	2.50±0.10^{a}	++
BG_1	91.18±0.08^{c} (72.72)	83.47±0.89^{b} (66.01)	1.00±0.17^{def}	12.57±0.08^{a}	1.27±0.09^{h}	++
BG_2	91.72±0.08^{b} (73.27)	85.68±0.52^{a} (67.77)	1.30±0.10^{bc}	12.73±0.08^{a}	1.50±0.02^{fg}	++
BG_3	92.72±0.12^{a} (74.34)	82.40±0.04^{c} (65.20)	1.37±0.15^{ab}	12.90±0.05^{a}	1.69±0.06^{de}	++
BN_1	73.47±0.46^{k} (58.99)	47.75±0.08i (43.71)	0.93±0.15^{defg}	9.20±0.10^{cd}	1.64±0.01^{ef}	++
BN_2	74.98±0.38^{j} (59.99)	49.43±0.23^{h} (44.68)	0.80±0.10^{fgh}	9.72±0.08^{b}	2.27±0.15^{b}	++
BN_3	76.18±0.45^{i} (60.79)	51.53±0.08^{g} (45.88)	0.83±0.06^{efgh}	9.62±0.08^{bc}	2.38±0.24^{ab}	++
BN_4	76.40±0.20^{i} (60.94)	50.49±0.07^{g} (45.28)	0.72±0.08^{h}	9.68±0.10^{b}	2.08±0.16^{c}	++
GN_1	83.58±0.29^{h} (66.10)	66.65±0.31^{e} (54.72)	0.95±0.04^{defg}	7.30±0.10^{g}	1.85±0.05^{d}	++
GN_2	84.63±0.10^{g} (66.92)	68.67±0.28^{d} (55.96)	1.01±0.08^{de}	8.07±0.65^{f}	1.72±0.03^{de}	++
GN_3	85.74±0.12^{f} (67.81)	69.07±0.24^{d} (56.21)	1.10±0.11^{cd}	9.03±0.60^{d}	1.62±0.03^{ef}	++
GN_4	85.69±0.04^{f} (67.77)	68.75±0.03^{d} (56.01)	1.57±0.06^{a}	8.77±0.24^{de}	1.42±0.03^{gh}	++
LSD ($P \leq 0.05$)	0.35	0.63	0.21	0.45	0.18	

Data in columns represent mean of three replications ± standard deviation and the values followed by same letter within a column are not significantly different ($P< 0.05$). Values in the parenthesis are arc sine transformed values. $^{++}$ represent the initiation of <30 days after explant inoculation

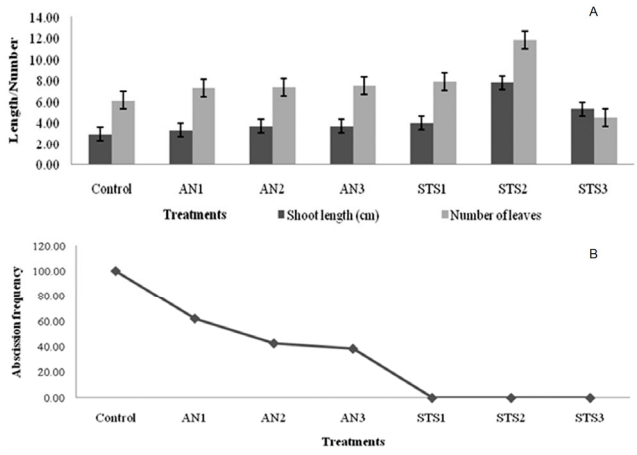

Fig. 2 — Effect of silver compounds on abscission control in Kinnow mandarin. (A) Shoot length (cm) and Number of leaves; and (B) Abscission frequency (%). Bars represent the mean ± standard error

Silver compounds on abscission control and culture establishment

The basic parameters which could prevent synthesis or accumulation of ethylene were evaluated for abscission control. The use of silver compounds for prevention of abscission has been reported recently (Fig. 2). The concentration and type of silver compound which could control abscission have been reported to be species specific. Hence, to determine the suitable composition for Kinnow mandarin an experiment was conducted. The results obtained from the previous experiments showed that BAP 2.5 mg L^{-1} produced multiple shoots and GA_3 10 mg L^{-1} exhibited delayed abscission (Fig. 3A-D). Hence, culture establishment and growth of *in vitro* shoots of Kinnow mandarin were studied by combining BAP 2.5 mg L^{-1} + GA_3 10 mg L^{-1} with

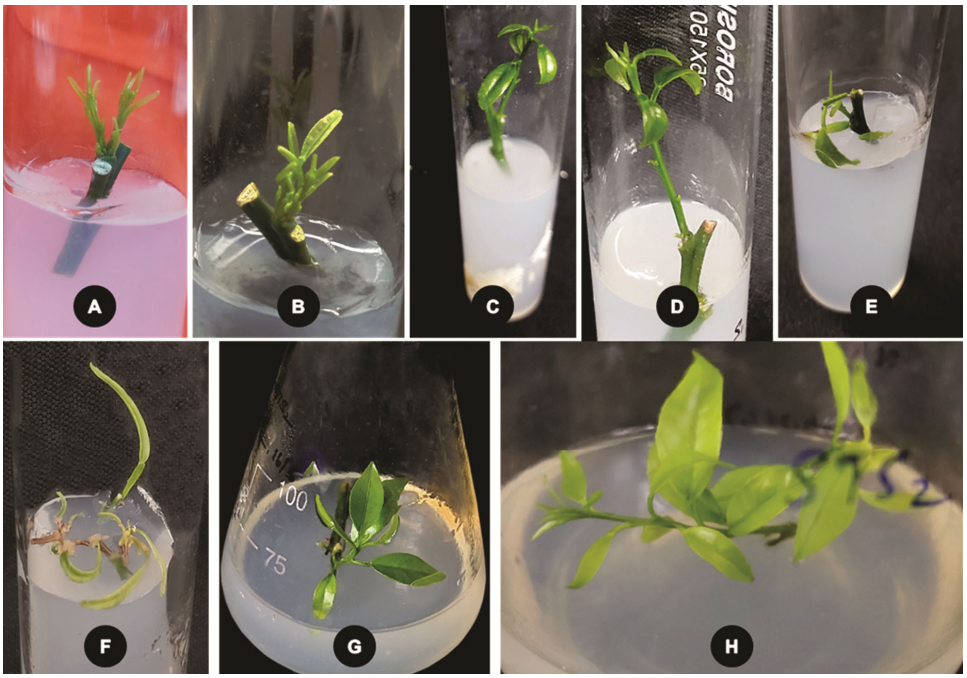

Fig. 3 — *In vitro* shoot regeneration of hardwood explants in Kinnow mandarin. (A-B) Multiple shoot induced by BAP 2.5 mg L^{-1}; (C-D) Primary shoot growth on GA_3 10 mg L^{-1}; (E-F) Premature abscission of primary shoots; (G-H) Abscission free enhanced shoot growth on MS + BAP 2.5 mg L^{-1}+ GA_3 10 mg L^{-1} + Silver thiosulfate (5 mg L^{-1}) supplemented medium

three different levels of two silver compounds. Result inferred that among the combinations tested BAP 2.5 mg L^{-1} + GA_3 10 mg L^{-1} + STS 5.0 mg L^{-1} generated longest microshoots (7.81 cm) with more number of leaves (11.82) and the same combination maintained abscission free cultures upon subculture (Fig. 2, Fig. 3E-H).

Discussion

In the present study significant shoot emergence was achieved in matured hardwood nodal explants of Kinnow mandarin compared to other growth stages. This favorable effect of hardwood explants might be due to the presence of pre-existing meristem and endogenous reserves for competency and are similar with the earlier findings[18]. In citrus use of matured dormant bud stick cultured *in vitro* is the source of scion for shoot tip grafting.

The data on per cent shoot emergence and leaf unfolding efficiency of hardwood was notably higher on MS and MT media over DKW media (Table 3). Lower response of primary shoot emergence on DKW medium may be due to low availability of macro nutrients[19]. These observations point out the requirement of nutrients from MS basal medium along with endogenous substances of hardwood explant to support primary shoot growth in Kinnow mandarin. Favorable effect of MS media on *in vitro* nodal explant culture of citrus have been reported in lime, Kinnow, calamondin, grapefruit, sweet orange and Australian finger lime[9,13,11,20]. In the present study, premature abscission (shoot and leaves detachment) was noticed in newly emerged shoots invariably on all the media and explant tested, which was not reported in any of the earlier studies in Kinnow mandarin. Though culture establishment was comparatively better with hardwood explants of August month, coincidence of monsoon rain in north Indian region induced latent expression of microbial contaminants. Advantage of multiple flushing in Kinnow was utilized and same experiment with hardwood explants of February dormant shoots showed reduced contamination. Hence for further experiments, February hardwood explants were chosen.

From these results, it can be implicated that among the growth regulators BAP and GA_3 have a positive effect on primary shoot regeneration of Kinnow nodal explants either alone or in combination, but response vary based on their concentrations and combinations. Cytokinin BAP showed superior performance over kinetin on shoot multiplication. Interestingly BAP supported primary growth by producing more micro-shoots which is in accordance with the findings in

Kinnow and lemon[11,21]. In addition BAP and GA_3 combinations showed positive effect on per cent bud break and shoot regeneration efficiency at lower concentrations which confirms the finding of earlier studies on lemon and citrus rootstocks[22,23]. However, BAP induced substantial multiple shoots but severe abscission was noticed invariably on all the concentrations and combinations used. Addition of GA_3 at higher concentration (10 mg L^{-1}) improved primary shoot regeneration by inducing early bud break and shoot emergence along with elongated green thick shoots having more number of normal leaves. This signifies the favorable function of GA_3 on cell division and organogenesis with its role in cell elongation by interaction with endogenous growth substances. Observation on abscission rate in the present study showed delayed abscission in G_6 treatment (4 weeks after culture initiation) compared to other combination. Also, the leaf drop did not take place, but shoots got detached at the apical region while in other treatments severe abscission was observed at all the abscission zones within one or two week after the shoot initiation.

Major cause of abscission in citrus tissue culture has been reported due to the accumulation of gaseous ethylene inside the closed culture vessels. Addition of ethylene biosynthesis inhibitors/modulators in the culture medium to prevent abscission has been attempted in certain citrus species recently to prevent pre mature abscission[9,12,13,17]. Among the various ethylene biosynthesis inhibitors/modulators $AgNO_3$ and STS have been studied frequently in *Citrus* species with encouraging positive results. Although, $AgNO_3$ and STS had positive influence on abscission frequency in Kinnow mandarin STS was comparatively superior to $AgNO_3$. The mode of action of silver ions on ethylene inhibition was not understood fully but involvement of silver ions on the blockage of ethylene receptors have been hypothesized[12]. Similarly, in the present study 5.0 mg L^{-1} STS was found to control abscission along with enhanced growth, while further increase in STS concentration controlled abscission but reduced the shoot growth. This emphasizes the need of optimum concentration of silver ion and ability of STS on enhanced supply of Ag^+ ion through thiosulfate. This result corroborates with the findings in finger lime and lemon[12,17]. However, it contradicts the findings in grapefruit and *C. hystrix* where $AgNO_3$ has been reported to control leaf abscission[9,12]. This shows the species specificity for requirement of particular silver compound.

Conclusion

From the results it can be concluded that premature abscission is the major problem associated with *in vitro* regeneration of mature vegetative explants in Kinnow mandarin. However, the present study emphasizes the role of medium, explants and Gibberellin on primary shoot emergence. While, subculturing of primary shoots on BAP 2.5 mg L^{-1} + GA_3 10 mg L^{-1} + STS 5.0 mg L^{-1} medium enhanced the shoot growth and reduced the premature abscission. This protocol fulfilled the research objective of obtaining premature abscission free shoots for further experiments. The protocol could also be effectively utilized for mass multiplication and crop improvement *via In-planta* transformation in Kinnow mandarin.

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Conflict of interest

All authors declare no conflict of interest.

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