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Efficient micropropagation of *Vanilla planifolia* Andr. under influence of thidiazuron, zeatin and coconut milk

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Vanilla planifolia multiplication through tissue culture was worked out with new leads in elucidation of synergistic activity of zeatin, synthetic cytokinin thidiazuron (TDZ) and coconut milk (CM) as well as N^6-benzyladenine(BA) . Multiple shoots were developed from axillary bud explants using Murashige and Skoog (MS) medium supplemented with zeatin, BA+zeatin , TDZ and TDZ+ coconut milk (CM). The nature of explant and the method of explant inoculation onto the medium influence not only multiple shoot production, but also bulbous shoot buds (BS) formation. Zeatin supported the growth of mostly single shoots, whereas formation of BS was induced in zeatin and BA combination and also in media supplemented with TDZ+10% CM. Subsequent transfer of these BS onto shoot proliferation medium supplemented with BA (8.87 μM) or α-naphthalene acetic acid (NAA) (2.69 μ*M)* resulted in multiple shoot proliferation 17 ± 2.5 shoots and 30 ± 2.1 shoots respectively per explant. The multiple shoots so obtained were transferred to Nitsch medium (N69) containing BA (2.22 μ*M*) and gibberellic acid (GA_3) (0.029 μ*M*) and also onto simultaneous shoot multiplication and root forming medium for further growth. For the first time the influence of zeatin, TDZ and coconut milk on shoot multiplication was studied. This protocol is effective in producing micropropagated vanilla plants with successful hardening and field transfer.

Keywords: axillary bud, bulbous shoot buds, explant, gibberilic acid, plantlets, rooting

Introduction

Vanilla planifolia Andr. (Vanilla) is a tropical orchid grown for its pods (beans), which yield vanillin[1,2]. Vanilla is the second-most expensive spice (after saffron), which is mostly subjected to competition from imperfect substitutes (low-cost artificial flavouring). Vanillin is mainly used in flavouring ice creams, soft drinks, candies, baked goods, condiments and oleoresins. The world production of vanilla beans is estimated to be about 3500 tonnes per annum and nearly 3000 tonnes are used in USA alone. Madagascar and Indonesia each accounted for 40% of the vanilla trade. Synthetic vanillin constitutes one-hundredth the price of the natural vanilla extracts. Despite the strong competition from synthetic vanilla, the increased health awareness and preference for natural products have strengthened the demand for vanilla beans during the past decade[3]. The conventional method of vanilla propagation is inefficient[4,5], hence tissue culture methods have been adopted for propagation[6,7].

In vitro multiplication of *V. planifolia* has been reported through the culture of callus masses[6,8], protocorms, root tips[9] and axillary bud explants[10,11]. For efficient shoot multiplication of vanilla, the present study was taken up with a view to get high multiplication rate. This report deals with efficient *in vitro* shoot multiplication of *V. planifolia* from axillary bud cultures under the influence of thidiazuron (TDZ) and zeatin along with coconut milk(CM).

Materials and Methods

Plant Material and Explant Preparation

Shoot tips and nodal explants containing dormant axillary buds excised from 1 yr old field grown vines of *Vanilla planifolia* from the Rama Krishna Ashram, Mysore, India were used to initiate shoot cultures. All the growth regulators used in this study were obtained from Sigma, USA and other media components from Himedia, India. These sections (size, 2-3 cm) were washed in liquid detergent (Tween 20) and then rinsed in tap water for 10 min. The explants were surface sterilized with 0.15% (w/v) mercuric chloride for 10 min followed by 5 rinses in sterile distilled water. These explants were inoculated onto shoot initiation medium containing MS medium salts[12], supplemented with 8.87 μ*M* N^6-benzyladenine (BA) and 5.38 μ*M* α-naphthalene acetic acid (NAA), 3% (w/v) sucrose and gelled with 0.6% (w/v) agar.

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Cultures were incubated at 25 ± 2°C under 37.5 μmol m^{-2} s^{-1} light intensity and photoperiods of 16 hrs light. Proliferated shoots were separated and sub cultured periodically on MS basal medium containing BA (0.044 μ*M*) and indole-3-butyric acid (IBA) 0.49 μ*M*. Subsequently *in vitro* shoots were maintained on MS basal medium for 1 month and later used for shoot multiplication experiment. Explant with 1-3 nodes of 1 or 2 months old along with explants containing shoot tip and single node were used (Fig. 1).

For induction of multiple shoots, MS medium supplemented with zeatin (6-[4-hydroxy-3-methyl-but-2-enylamino] purine) at 2.28, 4.56, 9.12 and 11.4 μ*M* alone or in combination with BA at a concentration of 10.56 μ*M* was used. Similarly TDZ at a concentration of 2.27, 4.54 and 9.18 μ*M* along with coconut milk was incorporated into MS basal medium containing sucrose at 3%. The base of the *in vitro* grown shoots from nodal explants containing axillary bud were trimmed (final size 2.0 cm). Some shoot tips (2-3cm) with 1-3 nodes were also selected for experiments to see the influence of explant inoculation method (Fig. 1). The explants were inserted into the test media and incubated. The explants with sprouted axillary buds were later transferred onto vanilla shoot proliferation medium (VS) containing MS basal+3% sucrose+d-biotin (82 μ*M*)+BA (8.87 μ*M*)+NAA (5.37 μ*M*) for further shoot proliferation and elongation. Selected explants that responded well for either multiple shoot production or bulbous shoot bud (BS) formation were transferred to N69 medium[13] containing BA (1.1 μ*M*)+GA_3 (0.029 μ*M*)+folic acid (1132 μ*M*)+sucrose 2% and also onto simultaneous shoot multiplication and root forming (RS) medium which contained MS salts and vitamins along with myoinositol (100 mg l^{-1}), malt extract (25 mg $l^{-1)}$, sucrose (3% w/v) supplemented with IBA (9.80 μ*M*) for further growth and cultured for 30-45 days. Each treatment had 20 explants and the data was recorded after 45 days of culture. The data shown in the tables (1-5) represent the mean ± SE of 20 explants per treatment and repeated twice. Statistical analysis of data was done by Duncan's method[14]. *In vitro* grown shoots (3-4 cm) were excised and used for rooting on MS basal medium supplemented with 3% sucrose and 9.80 μ*M* IBA. Rooted plantlets were removed after 30-40 days from the media, freed of agar by washing in running tap water and planted on a sand compost mixture (1:2) and maintained at about 80% relative humidity under polyethylene hoods in the greenhouse. The plantlets were hardened for 30 days and then transferred to field.

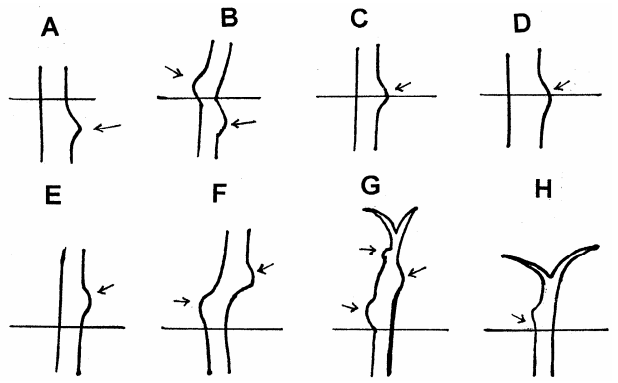

Fig. 1—Different types of *V. planifolia* explants and type of inoculation: A) Explant with single node below medium surface; B) Explant with two nodes one above and another below medium surface; C) Tender explant (1-month-old) node touching the medium surface; D) Matured or old stem (2-month-old) explant with single node touching the medium surface; E) explant with single node above medium surface; F) Explant with two nodes above medium surface; G) Explant with three nodes all above medium surface; & H) Explant with shoot tip and single node above medium surface.
Arrow indicates nodal region and horizontal line indicates medium surface

Results and Discussion

Shoot initiation was observed from axillary bud explants after 10 days of culture. Response to shoot formation varied with the type of inoculated explant and also the concentration of zeatin used in the media (Table 1). Only one shoot per bud was obtained after 30 days of culture irrespective of the type of explant and mode of inoculation (Fig. 1). In case of explant type F, G and H, all the axillary buds started to sprout in 10-20 days (Table 1). Type C explants from zeatin (9.12 μ*M*) medium, upon transfer to VS medium (Table 2) formed multiple shoots. However, maximum number of shoots (6.2 ± 1.2) with a shoot length of (1.2 ± 0.5 cm) was produced from type D explants at 9.12 μ*M* zeatin containing medium after 75 days incubation (Table 2). Zeatin in combination with BA induced BS formation in 30 days (Table 3, Fig. 2a). Continuous growth of these explants on the same medium for 75 days led to the production of more number of shoots per explant (Table 3). Maximum of 4.5 ± 0.9 shoots with a shoot length of 0.92 ± 0.6 cm, were produced with 100% shoot initiation response in type D explants at 9.12 μ*M* zeatin and 10.54 μ*M* BA (Table 3). Explants of types A, C, D, F and G from zeatin (4.56 μ*M*) with BA

Table 1—Effect of zeatin on % shoot response of *V. planifolia in vitro* (After 30 days)

Conc.of zeatin in medium (μ*M*)	Type of explant	% response	No.of shoots per explant	Shoot length (cm)	Time of BS emergence (days)
2.28	A	80.0	1.0 ± 0.0	0.37± 0.09	10
	C	50.0	1.0 ± 0.0	0.77 ±0.14	10
	D	50.0	1.0 ± 0	0.47 ± 0.48	12
	E	70.0	1.0 ± 0.0	0.31 7± 0.11	12
	F	60.0	1.0 ± 0.0	0.36 ± 0.12	12,16
	G	60.0	3.0 ± 0.1	0.43± 0.07	12,16,20
	H	60.0	1.0 ± 0.0	0.36 ± 0.11	10
4.56	A	100.0	1.0 ± 0.0	0.56± 0.15	10
	C	100.0	1.0 ± 0.0	1.25 ± 0.17	10
	D	100.0	1.0 ± 0	1.45 ± 0.18	12
	E	100.0	1.0 ± 0.0	1.41 ± 0.22	12
	F	80.0	1.0 ± 0.0	0.9± 0.18	12,16
	G	100.0	3.0 ± 0.15	0.65 ± 0.15	12,16,20
	H	100.0	1.0 ± 0.0	0.64 ± 0.13	10
9.12	A	100.0	1.0± 0.0	0.32 5±0.05	10
	C	100.0	1.0 ± 0.0	1.81 ± 0.24	10
	D	100.0	1.0 ± 0	2.06 ± 0.32	12
	E	100.0	1.0 ± 0. 0	1.76 ± 0.16	12
	F	70.0	1.0 ± 0.0	0.91 5± 0.14	12,16
	G	80.0	3.0 ± 0.1	0.62 7± 0.15	12,16,20
	H	90.0	1.0 ± 0.0	0.50 ± 0.20	10
11.4	A	60.0	1.0 ± 0.0	0.33 ± 0.05	12
	C	50.0	1.0 ± 0.0	0.75 ± 0.09	10
	D	50.0	1.0 ± 0	0.57 ± 0.05	12
	E	60.0	1.0 ± 0.0	0.31 ± 0.01	12
	F	30.0	1.0 ± 0.0	0.30 ± 0.10	12, 16
	G	40.0	1.0 ± 0.1	0.30 ± 0.15	12,16,20
	H	40.0	1.0 ± 0.0	0.30 ± 0.09	10

Type A, C, D, E, F, G = As in Fig.1, No. of explants =20, BS= Bulbous shoot

Table 2—Multiple shot initiation on VS medium from 30 days old axillary bud explants of *V. planifoia* initially cultured on zeatin containing medium

Conc.of zeatin in medium (μ*M*)	Explant type	*No. of shoot/explant Mean ± S.E.	Shoot length (cm) Mean ± S.E.
4.56	C	3.2 ± 0.6	0.92± 0.4
	D	3.5 ± 0.5	0.98± 0.3
9.12	C	5.6 ± 1.3	1.12± 0.6
	D	6.2 ±1.2	1.25± 0.5

*100% explants responded for multiple shoot production, Data recorded after 45 days

(10.54 μ*M*) medium upon sub culturing onto VS medium produced 17 ± 2.5 multiple shoots in type G explant (Table 4). The shoot growth of type F explants was maximum (1.25 ± 0.8 cm) along with maximum number of sprouts (3 ± 0.5).

When TDZ was used at 4.54 μ*M* in medium containing 10% CM, a maximum of 1.53 ± 0.3 BS per explant with (diam, 1.4 ± 0.1 cm) was obtained (Fig 2b). But in controls i.e. with 10% CM only 1 or 2 shoots were produced with a shoot length of 1.5 cm (Fig. 2c). A maximum of 30 ± 2.1 shoots per explant were produced on VS medium with BA 8.87 μ*M* +NAA 2.69 μ*M* from BS explants initially cultured on medium containing TDZ at 4.54 μ*M*+10% CM (Table 5). However, in control medium (without TDZ), a single shoot (length, 2.6 ± 0.1) was produced with roots. But no significant shoot elongation and opening of shoot buds was noticed in TDZ+CM medium possibly because TDZ is inhibitory to shoot elongation in apple[15] albizzia[16] and cassava[17]. Then the multiple shoots were transferred to N69 medium and RS medium for shoot proliferation and rooting of explants, respectively.

Table 3—Effect of zeatin in combination with BA (10.56 μ*M*) on shoot multiplication of *V. planifolia*

Zeatin conc. in medium (μ*M*)	Type of explant	% of shoot response	After 30 days culture period			After 75 days culture	
			No.of bulbous shoots	Time of BS emergence (days)	Diameter of bulbous shoot(cm)	No.of shoots/ explant	Shoot length (cm)
2.28	A	100	1.0 ± 0.0	8	0.4 ± 0.08	1.0± 0.65	0.25± 0.05
	B	100	1.0 ± 0.0	9	0.27± 0.06		
	C	50	1.5 ± 0.6	8	0.41± 0.1	2.0± 0.5	1.0± 0.08
	D	60	1.0 ± 0.2	10	0.75 ± 0.5	2.0± 0.2	0.65± 0.2
	E	60	1.2 ± 0.1	9	0.52 ± 0.05		
	F	10	2.0 ± 0.3	10, 14	0.3 ± 0.0	3.0± 0.72	0.80± 0.3
	G	100	3.0 ± 0.2	10,14,19	0.32 ± 0.05	1.5± 0.6	0.15± 0.08
	H	60	1.0 ± 0.0	9	0.45 ± 0.07		
4.56	A	100	1.0 ± 0.0	8	0.45 ± 0.05	1.3± 0.9	0.34± 0.11
	B	100	1.0 ± 0.0	9	0.47 ± 0.03		
	C	100	1.3 ± 0.1	8	0.52 ± 01.12	5.0 ± 0.8	1.25 ± 0.60
	D	100	1.2 ± 0.4	10	1.0 ± 0.16	4.5 ± 0.9	0.92 ± 0.6
	E	100	1.5 ± 0.4	9	0.52 ± 0.09		
	F	100	2.0 ± 0.2	10,14	0.35 ± 0.05	3.0 ± 0.3	1.12± 0.3
	G	80	3.0± 0.2	10,14,16	0.55 ± 0.12	1.5 ± 1.2	0.35± 0.07
	H	100	1.0 ± 0.3	9	0.27 ± 0.05		
9.12	A	100	1.0 ± 0.4	8	0.8 ± 0.11	2.0 ± 0.0	0.6± 0.05
	B	100	1.0 ± 0.3	9	0.45 ± 0.05		
	C	100	1.5 ± 0.3	8	0.3 ± 0.08	9.3 ± 1.1	1.69 ± 0.3
	D	100	2.0 ± 0.5	10	0.82 ± 0.09	5.0 ± 1.2	1.69± 0.4
	E	80	2.0 ± 0.3	9	0.39 ± 0.05		
	F	100	2.0 ± 0.2	10,14	0.4± 0.08	3.0 ± 0.1	0.80± 0.3
	G	80	3.0 ± 0.2	10.,14,19	0.31 ± 0.09	6.0 ± 0.3	0.75± 0.0
	H	100	1.0± 0.1	9	0.32 ± 0.05		
11.4	A	60	---	12	---	1.0 ± 0.1	0.4± 0.1
	B	60	1.0 ± 0.2	12	0.33 ±0.05		
	C	40	1.5 ± 0.2	12	0.37 ± 0.05	6.5 ± 1.2	1.2± 0.6
	D	40	1.0 ± 0.2	12	0.60 ± 0.08	4.0 ± 1.1	1.4± 0.5
	E	50	1.2 ± 0.1	10	0.37 ± 0.05		
	F	20	2.0 ± 0.1	12,16	0.40 ± 0.2	2.5 ± 0.8	0.7± 0.2
	G	80	3.0 ± 0.2	10,15,20	0.30 ± 0.05	1.5 ± 0.6	0.45 ± 0.2
	H	-	1.0 ± 0.1	--	--	--	--

No. of explants=20, Type F = explant with 2 nodes, Type G = explant with 3 nodes, BS = bulbous shoot bud

Zeatin induced rapid shoot induction from nodal explants compared to other cytokinins used earlier for the purpose[18]. But zeatin in combination with BA induced the formation of BS in less time (synergistic effect); some of them even proliferated into long shoots upon prolonged culturing on the same medium. The present study has shown that after obtaining substantial proliferation of shoots (18-32) per explant, transfer of multiplying clusters to a basal medium with lower salt concentration like that of N69 with lower BA (2.22 μ*M*) and lower sucrose (2%) was found essential for further growth of shoots as in case of other plants such as *Tagetus*[19]. In N69 basal with BA (2.22 μ*M*) and GA_3 (0.029 μ*M*), these growing clumps (shoot mass) not only proliferated but also gave rise to elongated shoots with broader leaves and root initials (Fig. 2d). The sub culturing of shoots, which were obtained from zeatin and TDZ along with CM (10%) containing media, onto the RS medium-containing IBA (9.80 μ*M)* produced maximum number of roots (4-5) per explant with a root length (3.8 cm) and long shoots with 5-6 nodes (Fig. 2e). These rooted plants showed good survival during hardening (Fig. 2f). According to earlier reports, 6-7 months time is required to produce 2-3 vanilla plantlets[5,18]. Even by using hormones incorporated agar medium and semisolid medium long incubation periods were required to get vanilla plantlets[11]. In the

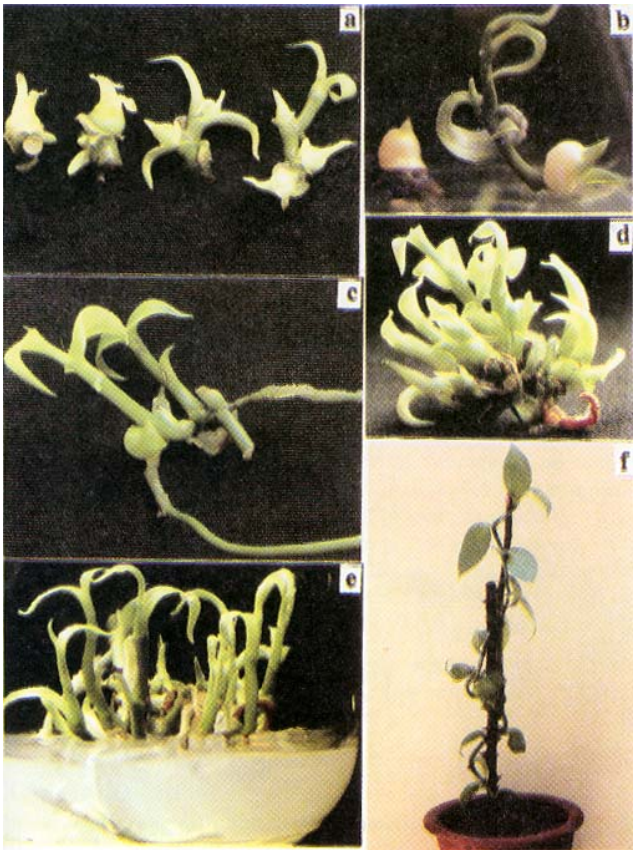

Fig. 2—a, Bulbous shoot buds formation from nodal explants (type 1 C, D, F, G) on medium containing BA (10.5 μ*M*) and zeatin (4.56 μ*M*); b, Globular bulbous shoot bud from nodal explant on medium containing TDZ (4.54 μ *M*); c, Long shoot induction from nodal explants on medium with 10% (v/v) CM; d, Rapid proliferation and elongation of shoots on N69 medium containing BA (2.22 μ*M*)+ GA3 (0.029 μ*M*); e, Long shoots formation along with roots on RS medium containing IBA (9.80 μ*M); &* f, Potted plant ready for field transfer

Table 4—Shoot proliferation response on VS medium from, axillary bud explants pretreated (30 days) with BA(10.56 (μ*M*) and zeatin in *V. planifoia*

Conc. of zeatin (μ*M*)	Explant type	*No. of shoots/ explant Mean ± S.E.	Shoot length (cm) Mean ± S.E.	Number of bulbous shoots
4.56	A	9.0 ± 1.2	0.58± 0.1	1.2 ± 0.08
	C	11.0 ± 1.8	0.95 ± 0.3	1.8 ± 0.2
	D	10.4 ± 2.2	0.97 ± 0.5	2.6 ± 0.8
	F	4.0 ± 0.8	1.25 ± 0.8	3.0 ± 0.5
	G	17.0 ± 2.5	1.50± 0.6	3.0 ± 0.4
9.12	A	4.0 ± 0.3	0.60 ± 0.1	1.0 ± 0.08
	C	12.0 ± 1.1	1.20 ± 0.2	2.0 ± 0.1
	D	5.6 ± 0.3	1.43 ± 0.7	1.6 ± 0.2
	F	4.2 ± 0.9	0.90 ± 0.1	1.0 ± 0.09
	G	8.0 ± 1.2	0.22 ± 0.1	1.0 ±0.05

*100% explants responded for multiple shoot formation, Data scored after 45 days

present report, high shoot multiplication rate (17-30) was achieved within 5-6 months, which varies with the hormones used. Another innovativeness of this study is bulbous shoot buds from nodal explants and subsequent high rate of multiple shoot formation on respective hormones incorporated media.

In conclusion, by using this protocol (Fig. 3) within 5-6 months, a maximum 30 shoots per explant could be obtained by culturing the explants initially in presence of TDZ+CM followed by their transfer to VS medium and their subsequent growth on N69 or RS medium to get plantlets with efficient field survival. This will be useful in multiplying of selected elite plantlets for efficient propagation. It is evident

Table 5—Effect of TDZ and coconut milk on shoot multiplication of *V. planifolia in vitro*

Medium	After 30 days culture period				30-day-old explants with BS transferred to VS medium (growth after 45 days)	
	% of response	Number of shoots per explant	Number of BS	Diameter of BS	Number of shoots per explant	Shoot length (cm)
CM 10%	60.0	1.0 ± 0.15	-	-	1.0 ± 0.0	2.6 ± 0.1
TDZ 2.27 μ*M*	50.0	-	0.5 ± 0.02	0.25 ± 0.02	1.5 ± 0.2	0.45 ± 0.6
4.54 μ*M*	50.0	-	0.5 ± 0.05	0.4 ± 0.06	3.0 ± 0.9	0.80 ± 0.12
9.18 μ*M*	60.0	-	1.0 ± 0.2	0.7 ± 0.04	3.5 ± 1.2	0.69 ± 0.08
CM 10% + TDZ						
2.27 μ*M*	100.0	_	1.2 ± 0.4	0.95 ± 0.09	28.0 ± 1.5	0.85 ± 0.09
4.54 μ*M*	100.0	-	1.53 ± 0.4	1.4 ± 0.12	30.0 ± 2.1	0.92 ± 0.2
9.18 μ*M*	100.0	-	1.2 ± 0.2	1.2 ± 0.12	25.0 ± 1.3	0.75 ± 0.11

BS= bulbous shoot bud, No. of explants= 20

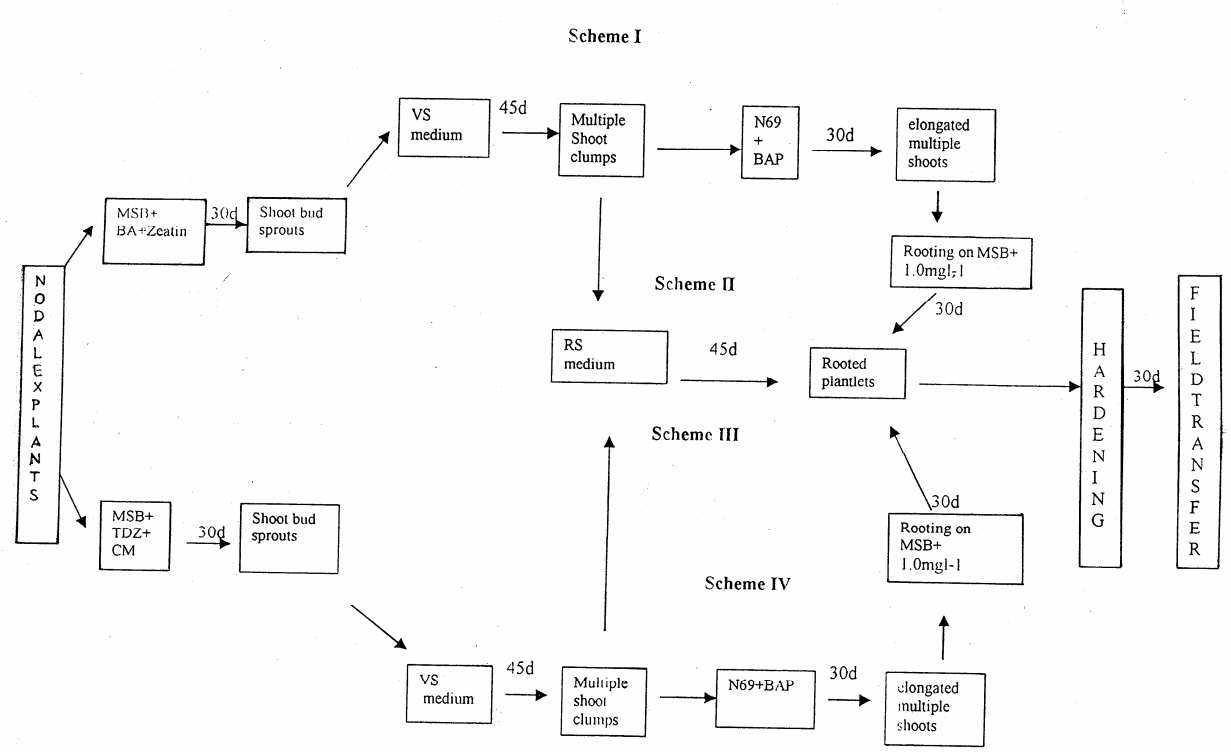

Fig. 3—Schematic representation of an effective protocol for Vanilla micropropagation

that inoculation of nodal explants onto BA+Zeatin containing medium (schemes 1 & 2 in Fig. 3) by touching the axillary bud portion to the medium surface (type C, as in Fig. 1) leads to the maximum number of multiple shoot formation (12 ± 1.1) along with explants containing three nodes (17 ± 2.5) upon subsequent growth on VS medium. Similarly, single node explants containing axillary bud produced maximum number of shoots (30 ± 2.1) on TDZ+10% CM containing medium (schemes 3&4 in Fig. 3) upon subsequent growth on VS medium.

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