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Research Article
In vitro Propagation of Kiwi from Nodal Segments and Development, a New Rooting Technique of Micro Shoots

Chitta Ranjan Deb and Philip Kadunlung Gangmei

Department of Botany, Nagaland University, Lumami, 798 627 Nagaland, India

Abstract

Background and Objective: *Actinidia deliciosa* (A. Chev.) popularly known as Kiwi fruit is an important horticultural plant grown in different parts of the world. Common practice of propagation of the species is through stem cutting but due to poor rooting of stem cuttings limits the production of clonal planting materials . Present study was aimed at development of *in vitro* propagation protocol of the species for production of clonal planting materials. **Materials and Methods:** A successful attempt was made to develop an efficient *in vitro* propagation protocol using *in vivo* source nodal segments, an efficient rooting technique and primary hardening using some low cost substrata as agar alternative. **Results:** About 7 week old nodal segments of *in vivo* source collected during November were cultured on MS medium fortified with sucrose (3%, w/v), Polyvinylpyrrolidone (300 mg L^{-1}), benzyl adenine (BA) (6 μM), where within 6 days of culture morphogenic response initiated followed by average 4.2 shoot buds formation in 75% cultured nodal segments. The unidonal nodal segments from the resultant shoot buds on initiation medium proliferated and developed multiple micro shoots on MS medium enriched with sucrose (3%) and BA (3 μM) where as many as 6.2 micro shoots developed in 58.3% culture per cycle. The well developed foliated and defoliated micro shoots (4-5 cm long) were rooted on nutrient medium containing indole butyric acid (9 μM) where 50 and 56.3% rooting response registered, respectively accompanied by 5 and 7 numbers of roots/shoot. The rooted plantlets were hardened on 1/4th strength liquid medium containing sucrose (1%) and chopped coconut coir for 7-8 weeks followed by transferred to potting mix where ~82% transplants survived. **Conclusion:** Outcome of the present work resulted a new route for efficient and low cost propagation protocol of Kiwi fruit.

Key words: Alternative low cost substrata, clonal propagation, defoliation and rooting, kiwi, nodal segments

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Corresponding Author: Chitta Ranjan Deb, Department of Botany, Nagaland University, Lumami, 798 627 Nagaland, India Tel: +91-9436006808

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Vegetative tissue based clonal planting material production through conventional means to trap the genetic gain for horticultural important plants are most cost effective technique, however, due to poor rooting of cutting is the limitation[1-5]. In contrary, tissue culture techniques provide rapid and dependable method for production of genetically uniform clonal planting material in short tenure[4-11]. This technique has been successfully used for propagation and conservation of species with reproductive limitations[8,12-14]. Different vegetative explants like leaf, bulb/pseudo-bulb, roots, axillary buds/nodal segments, inflorescence have been used for propagation of many agronomically important species[8-9,11,13-17]. Amongst the different explants, nodal segments with axillary buds has been found to be most effective method for producing clone[14,18].

Agar has been extensively used in plant tissue culture and adds to the production cost due to its high cost. In the past some efforts have been put into for screening low cost substrata as agar alternative with the objective to reduce the production cost[19,20]. These substrata were used with differential success in different stages of culture. Past studies have shown that use of alternative substrate like forest litter, coconut husk, polyurethane foam etc. reduce the production cost as well improve the survival of transplants in the potting mix[19,20].

Actinidia deliciosa or 'Kiwi fruit' belongs to family Actinidiaceae is a woody climber shrub, dioecious grow in temperate cold mountainous region and cultivated for its delicious fruits. Of many species of the genus *Actinidia, A. deliciosa* and *A. chinensis* are the 2 cultivated for fruits. Success in marketing of cultivars of *A. deliciosa* has initiated breeding programmes of the species for improved cultivars. However, dioecious nature and long juvenile period, poor rooting of the species limits the breeding programmes. The first commercial cultivation of Kiwi fruit began in New Zealand during 1970s. In India, Kiwi fruit was introduced in 1960s in parts of the Himalayan region from New Zealand and USA. However, the commercial potential and climate suitability has led to its spread to other parts of the country including Nagaland. In the past some efforts were made with limited success for producing *in vitro* planting materials[21,22].

Initial phase of the present study experienced that production of shoots from the stem cutting is quite easy in the nursery but very difficult to induce functional roots and becomes a hurdle in using stem cutting for propagating in the nursery. Subsequently study was planned to develop *in vitro* culture protocol for production of clonal planting materials from nodal segments of *in vivo* source with special focus on developing effective *in vitro* rooting mechanism of micro shoots. Besides this it was also focused on use low cost substrata as agar alternative in the hardening stage for effective transplantation and reduce production cost.

MATERIALS AND METHODS

Plant materials and processing: *Actinidia deliciosa* nodal segments were collected from *in vivo* source from the cultivated farm at Aosakhelimi village, Zunheboto district, Nagaland, India (Altitude 1495 MASL, 094°31'35.6" E and 26°08'42.2" N). Plants materials were collected round the year at monthly interval from the young shoots of ~7 weeks after sprouting. Nodal segments were cut off from the branches, wrapped in fresh banana leaf with sprinkled water to keep moistened. Scales etc. were carefully removed and washed under running tap water and stored at 4°C in the refrigerator till used. About 2 cm size nodal segments were surface sterilized by scrubbing by soft brush with 'Labolene' (a commercial laboratory detergent at 1:100, v/v) followed by washing under running tap water for 10-15 min, followed by sterilization with aqueous solution of $HgCl_2$ (0.3%, w/v) for 3-5 min followed by washing 4-5 times with sterile water.

Tissue culture medium: For culture initiation MS medium[23] was used as nutrient medium. Nutrient medium was supplemented with different concentrations of PGRs like BA and KN (0-16 μM) singly and in combination of NAA (3 μM), different organic carbon sources (viz., dextrose, glucose and sucrose at 0-4%, w/v), three different antioxidants: PVP, CA and AC at different concentrations (0-700 mg L^{-1}). The medium was gelled using 0.8% (w/v) bacteriological grade agar and pH was adjusted to 5.8 with 0.1 N NaOH and HCl before autoclaving at 1.05 kg cm^{-2} pressure and 121°C for 20 min. In each borosilicate test tube (150×25 mm) ~15 mL medium was dispensed and plugged with non-absorbent cotton before autoclaving.

Initiation of cultures: Sterilized nodal segments were cultured on fortified MS medium as mentioned above. For each treatment 20 explants and in each test tube segment was cultured and maintained at 25±2°C under cool fluorescent light at an intensity 40 μmol m^{-2} sec^{-1} for 12/12 h (light/dark) photo periods unless mentioned otherwise. All the cultures were sub-cultured at 5-6 weeks interval. The cultures were monitored regularly and data collected at weekly interval. *In vitro* response was evaluated based on the percentage of explants responded and number of propagules formed in the culture after specific period of time.

Plant regeneration and culture proliferation: The micro shoots sprouted/developed directly from the cultured nodal segments were maintained for another 2 passages under optimum initiation medium for further growth. The shoot buds/micro shoots were separated, leaves separated and uninodal segments cultured on fresh MS medium containing different carbon sources like glucose, dextrose and sucrose (0-4%), PGRs (BA and KN, 0-15 µM) singly for plant regeneration and culture proliferation.

Rooting of the regenerates: In some cases on regeneration medium few roots developed but roots were not fully developed and shorter in length. About 4-5 cm long micro shoots with well expanded leaves from the regeneration medium were selected for inducing roots following different technique as stated below:

- One set of micro shoots was pulse treated with IBA and NAA solution (0-50 µM) singly for 0-24 h (0, 1/2, 1 and 24 h) followed by culturing on MS plain medium containing sucrose (3%). For plus treatment cut ends of the micro shoots was dipped in PGRs solution for the stipulated duration and transferred to fresh plain nutrient medium
- While in another set, the PGRs (NAA and IBA, 0-15 µM) were directly incorporated in the rooting medium for inducing of roots
- In the third set, effort was made to study the effect of foliation/defoliation of micro shoots on *in vitro* rooting. For the purpose, the leaves of the micro shoots were removed using sterile surgical blade and cultured on the optimum rooting medium and maintained under normal laboratory condition

***In vitro* hardening of the regenerates:** The well rooted plantlets were subjected to primary hardening. For the purpose the regenerates were transferred on 1/4th strength MS medium adjunct with sucrose (1%) and maintained in the normal laboratory condition for 7-8 weeks. In the last 2 weeks of hardening process a part of the culture vials were exposed to normal day light for 4 h a day followed by transferred to normal laboratory condition. Besides agar gelled medium, regenerates were also maintained on other agar substitutes like chopped coconut coir, saw dust, forest litter for 6-8 weeks with the objective to look for agar alternative.

Substrata for hardening medium: Dry coconut husk, forest leaf litter were chopped into small pieces (~0.1-0.5 cm). These were soaked in laboratory detergent for 1-2 h and rinsed with water several times and sun-dried for 4-5 h. Besides coconut coir and forest litter, saw dust was also used as substratum. Saw dust was collected from the local carpentry workshop. The substratum was sun dried and soaked in laboratory detergent for 2-3 h and rinsed thoroughly under running water followed by sun dried. About 10-12 g of chopped coconut husk, forest litter and saw dust were transferred to test tubes separately and autoclaved at 121°C temperature for 30 min at the pressure of 1.05 kg cm^{-2}.

About 12-15 mL 1/4th strength MS liquid medium with sucrose (1%) was dispensed in all the test tubes and autoclaved at 121°C temperature, 1.05 kg cm^{-2} pressures for 20 min. These media filled test tubes with different substrata were used for hardening of the rooted regenerates under normal laboratory conditions.

Potting mix and transplantation of the regenerates: Top soil from forest was crushed to small granules, mixed with fine sand and dried rotten wood powder at 2:1:2 ratios. Potting mix was transferred to black poly bags and plastic cups. The primarily hardened plantlets were transferred to poly bags and plastic cups filled with potting mix. The plantlets hardened with alternative substrata were transferred along with the substrata (without washing the roots). The planted poly bags were covered with holed transparent bigger poly bags and nurtured alternate weekly with 1/10th MS salt solution for one month. The covered poly bags were removed from the pots and maintained in partial shade ca.50% for 2-3 weeks and finally exposed to normal day light. The acclimatized transplants was transferred to the experimental bed and monitored regularly for three months.

Experimental design and statistical analysis: Completely randomized experimental design was followed. In each experiment 20 explants were cultured for each treatment and all the experiments were repeated 5 times. *In vitro* response was evaluated based on the percentage of explants responded and data was presented as mean ± standard error and one way ANOVA and compared using Least Significant Difference (LSD) test at $p \leq 0.05$.

RESULTS

Development of *in vitro* protocol for production of clonal planting materials of Kiwi fruit was achieved through different stages and every step was affected by different factors. Different steps of the process are presented in (Fig.1a-h).

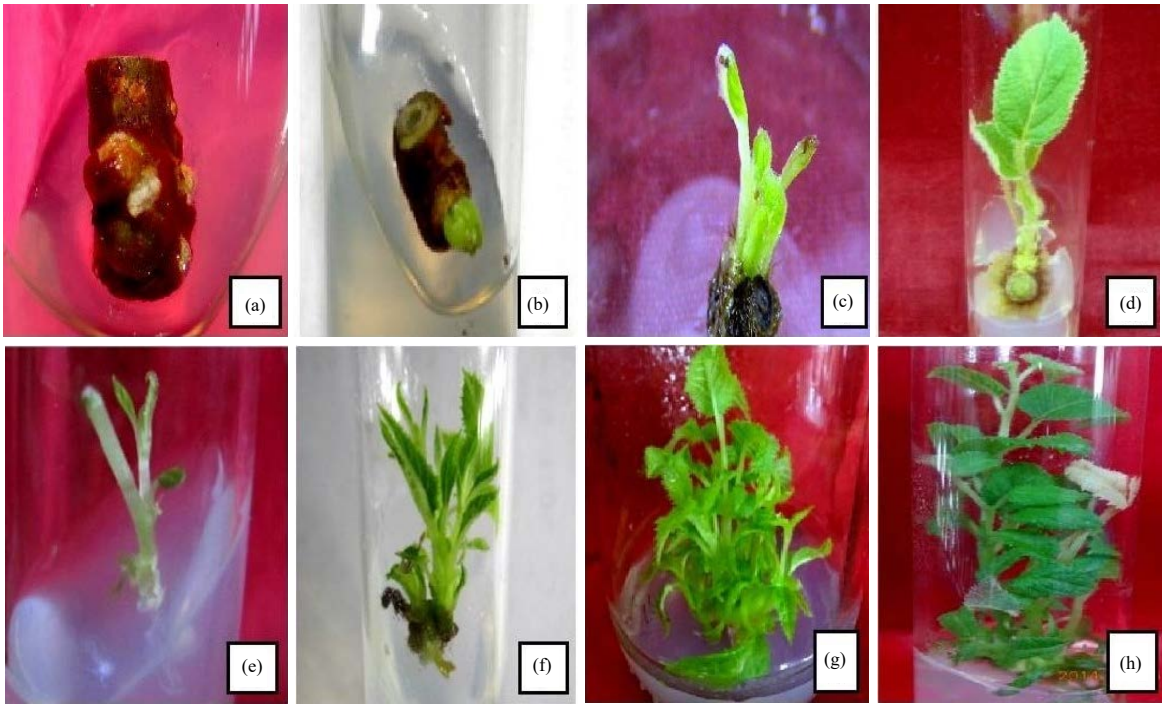

Fig. 1(a-h): Direct organogenesis of *in vivo* nodal explant of *A. deliciosa* cultured on initiation medium showing various developmental stages, (a) Swelling of nodal explant on initiation medium, (b) Meristematic loci/shoot buds developed from nodal segments with undifferentiated leaflets, (c) Young shoot buds releasing leaves, (d) Differentiated shoot bud with fully opened leaves, (e) Cultured nodal explants from *in vitro* source on regeneration medium, (f) Multiple shoot buds developed from cultured nodal segment, (g) Multiple micro shoots with fully open leaves on regeneration medium and (h) Healthy micro shoots ready for rooting

Table 1: Effect of season of Kiwi nodal segment collection on *in vitro* morphogenic response

Month of collection	Time for initial response (days)*	Response (%) (±SE)#	Morphogenic pathway**	Type of response@
January	A	50.0 (1.2)^{c}	Sb	Dark green shoots, broad leaves with moderate growth rate
February	A	41.7 (1.2)^{d}	Sb+Ca	Shoots light green, long internodes and smaller curly leaves
March	B	37.5 (1.1)^{e}	Ca+Sb	Light green shoots, unhealthy growth rate with curly leaves
April	C	20.8 (1.1)^{g}	Ca+Sb	As above, with stunted in growth rate
May	C	25.0 (0.8)^{h}	Sb+Ca	Callused, curly smaller leaves, shoots with long internodes and necrotic
June	C	16.7 (0.9)^{h}	Ca+Sb	As above
July	C	08.3 (0.6)^{j}	Ca+Sb	Shoots light green, slow growth rate, light to brownish smaller leaves
August	C	12.5 (0.8)^{l}	Ca+Sb	Shoots with long internodes, curly light green leaves
September	B	25.0 (1.3)^{f}	Sb+Ca	Light green smaller leaves, unhealthy growth rate
October	B	50.0 (1.3)^{c}	Sb+Ca	Dark green shoots, light green leaves, slower growth rate
November	A	75.0 (1.5)^{a}	Sb	Dark green shoot buds, broad dark green leaves, healthy growth
December	A	62.5 (1.2)^{b}	Sb	Dark green shoots and leaves but moderate growth rate

Newly flushed shoot of ~6-8 weeks old, *A: 0-5 days, B: 5-10 days, C: 10-15 days, #±SE: Standard error from mean, data represents mean of 5 replicates, data with the same letters in the column are not significantly different at 5% level, **Ca: Callus, Sb: Shoot bud, Ca+Sb: Callus+Shoot bud, @MS medium supplement with sucrose (3%, w/v), BA (6 μM) and PVP (300 mg L^{-1})

Initiation of culture

Seasonal effect of explants collection: Newly flushed shoots (~7 weeks old) of *A. deliciosa* were collected from the mature plants at monthly interval (Table 1) for three successive years. The *in vitro* morphogenetic response was greatly influenced by various factors like season of explants collection, strengths of basal medium, quality and quantity of organic carbon sources, PGRs, antioxidants in the initiation medium. Explants collected during midsummer (July) found to be least responsive (8.3%) and most tissues degenerated due to necrosis while, winter season was found to be suitable for *in vitro* morphogenesis (Table 1). First sign of

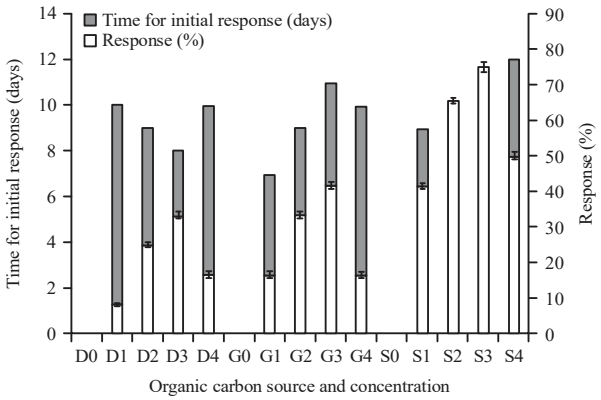

Fig. 2: Effects of quality and quantity of organic carbon sources on *in vitro* morphogenic response of Kiwi nodal segments from *in vivo* source

Table 2: Effect of quality and quantity of antioxidants on *in vitro* initiation culture of Kiwi nodal segments from *in vivo* source

Antioxidant type and concentration (mg L^{-1})*				
AC	CA	PVP	Response (%) (±SE)#	Morphogenic response@
0	0	0	-	Release of phenolic and browning of medium
100	-	-	33.3 (0.7)^{f}	Release of phenolic exudates and explants degenerated gradually
300	-	-	50.0 (1.5)^{c}	Exudation of phenolic and lighter browning of medium
500	-	-	41.7 (1.1)^{d}	Dark green shoot bud with broad leaves, no browning, healthy growth
700	-	-	25.0 (1.2)^{g}	Controlled browning, light green shoots, long and curly leaves
-	100	-	41.7 (1.1)^{d}	Shoot bud slight brown with curly light green leaves, stunted growth
-	300	-	50.0 (1.2)^{c}	Swollen ends, dark green shoots with smaller leaves
-	500	-	41.7 (1.4)^{d}	Controlled browning, healthy shoots with curly leaflets
-	700	-	33.3 (0.9)^{f}	Slight brownish shoot buds with curly leaves, controlled browning
-	-	100	41.9 (1.2)^{d}	Leaves dark green and curly and shoots with long internodes
-	-	300	75.0 (1.5)^{a}	Healthy shoots, dark green leaves, controlled browning
-	-	500	66.7 (1.8)^{b}	Moderate browning, shoots dark green with stunted growth
-	-	700	25.0 (0.6)^{g}	Poor morphogenesis with small green leaves

*AC: Activated charcoal, CA: Citric acid, PVP: Polyvinylpyrrolidone, #±SE: Standard error from mean, data represents mean of 5 replicates, data with the same letters in the column are not significantly different at 5% level, @MS medium supplemented with sucrose (3%) and BA (6 μM)

morphogenesis was recorded within 6 days of culture as swelling of cultured nodal segments (Fig. 1a, b). Of the different seasons studied, explants collected during February to October yielded poor *in vitro* response, where in most of the cases fewer shoot buds formed. Explants collected during winter (November-January) supported healthy shoot buds formation with broad, dark green leaf (Table 1, Fig. 1c). Under the given conditions nodal explants collected during November exhibited optimal *in vitro* morphogenic response (75%).

Nutrient medium fortified with different antioxidant (AC, CA and PVP) at different strengths to control phenolic exudation. Under antioxidant controlled medium due to severe phenolics exudation tissues turned necrotic and degenerated, incorporation of antioxidants improved the condition. Of the different quality and quantity of antioxidants used, PVP at concentration 300 mg L^{-1} was found to be ideal where healthy morphogenesis was achieved (Table 2). Compared to PVP, other 2 antioxidants across the concentrations (AC and CA) found to be less effective. At lower concentrations of all the 3 antioxidants failed to reduce the browning of medium while at higher concentrations (>300 mg L^{-1}) though the browning could be stopped but affected adversely the morphogenic response.

Effects of organic carbon: Presence of organic carbon in the initiation medium was prerequisite for successful induction of morphogenic response. The morphogenetic response was very poor on medium fortified with dextrose and glucose across the concentrations (Fig. 2). Of the different concentrations of different organic carbons tested, 3% was found to be most effective, where at this concentration dextrose invoked 33.3% response within 8 days of culture initiation followed by glucose (41.6% in 11 days) and sucrose (75% in 6 days). At lower concentration of sucrose, fewer shoot buds formed while at higher concentration, the response was comparatively poorer.

Effects of PGRs: For invoking morphogenic response BA and KN were incorporated singly (0-16 µM). Of the 2 cytokinins tested at different concentrations, KN was found to be inferior across the concentrations compared to BA where in most of the cases only one shoot bud invoked/node and fewer explants registered *in vitro* response (Table 3). On BA rich medium explants exhibited swelling and cracking as first sign of response after 6 days of culture which differentiated into shoot buds within 3 weeks of culture (Fig. 1a-c). Under the given conditions optimum response was recorded on BA (6 µM) fortified medium where as many as 4 shoot buds/node was resulted in 75% of explants cultured (Table 3) on MS medium with sucrose (3%), PVP (300 mg L^{-1}). The shoots buds elongated on the same initiation medium in subsequent sub-culture (Fig. 1c-d). The shoot buds converted into micro shoots within 2 passages of 5-6 weeks intervals on the optimum initiation medium with well expanded leaves (Fig. 1d).

Besides singly treatments, combined treatments of BA-NAA and KN-NAA were also studied for *in vitro* initiation of culture as shown in Table 3. Of the 2 combinations, BA-NAA combination was found superior over KN-NAA combination though both the combinations were far inferior compared with singly treatments of both BA and KN. The synergistic effect of BA-NAA and KN-NAA exhibited varied morphological responses, such as calli formation, development of somatic embryos and shoot buds development. Whereas, in absence of cytokinins tissue/explants gradually degenerated. Under the given experimental conditions, BA-NAA (6+3 µM, respectively) in combination exhibited better morphogenic response where as many as 3.4 shoot buds resulted within 15 days on culture initiation (Table 3).

Culture proliferation and plant regeneration: The micro shoots with fully expanded leaves were cropped from initiation cultures. The uninodal segments were excised out and cultured for culture proliferation and plant regeneration (Fig. 1e) on MS medium fortified with different quality and quantity of organic carbon sources viz. dextrose, glucose and sucrose (0-4%) and PGRs like BA and KN (0-15 µM) and cultured under normal laboratory condition for culture proliferation and plant regeneration for 2-3 passages.

Effects of organic carbon and PGRs: Shoot buds cultured on MS medium conjunct with three different organic carbon sources (0-4%, w/v). Of the 3 carbon sources tested, sucrose outperformed the other two in multiple shoot formation where as many as ~6 shoot buds with ~58%

Table 3: Effect of quality and quantity of cytokinins (BA and KN) and NAA on direct organogenesis of nodal explants of *Actinidia deliciosa* from *in vivo* source

Cytokinins concentration (µM)					
BA	KN	NAA	Time for initial response (d)	Response (%) (±SE)*	No. of loci formed/node
0	0	0	6	-	-
2	-	-	6	50.0 (1.3)^{c}	3.2
4	-	-	5	51.2 (1.5)^{c}	3.0
6	-	-	6	75.0 (1.5)^{a}	4.2
8	-	-	7	58.3 (2.3)^{b}	2.0
10	-	-	8	50.0 (1.3)^{c}	3.0
12	-	-	8	41.7 (1.4)^{d}	3.0
14	-	-		33.3 (0.9)^{e}	1.0
16	-	-	9	25.0 (1.2)^{g}	1.0
-	2	-	7	33.3 (0.9)^{e}	1.0
-	4	-	8	25.0 (1.5)^{g}	2.2
-	6	-	6	41.7 (2.1)^{d}	1.0
-	8	-	7	50.0 (2.1)^{c}	1.0
-	10	-	8	41.7 (2.3)^{d}	3.2
-	12	-	11	33.3 (1.5)^{e}	1.0
-	14	-	10	33.3 (1.7)^{e}	1.0
-	16	-	13	25.0 (2.0)^{g}	1.0
3	-	3	12	25.0 (0.5)^{g}	2.0
6	-	3	15	37.5 (0.7)^{f}	3.4
9	-	3	14	43.8 (1.1)^{d}	1.0
12	-	3	13	31.3 (0.9)^{e}	2.0
15	-	3	11	25.0 (0.7)^{g}	2.0
-	3	3	14	18.8 (0.5)^{h}	0.0
-	6	3	10	25.0 (0.8)^{g}	2.2
-	9	3	9	43.8 (1.4)^{d}	2.0
-	12	3	9	50.0 (0.9)^{c}	0.0
-	15	3	13	31.3 (0.8)^{e}	1.0

*±SE: Standard error from mean, data represents mean of 5 replicates, data with the same letters in the column are not significantly different at 5% level

Table 4: Effects of cytokinins on culture proliferation of Kiwi

Cytokinin concentration (µM)			
BA	KN	Response (%) (±SE)*	Mean No. of micro shoot formed/shoot
0	-	00.00	0.0
3	-	58.3 (1.2)[a]	6.2
6	-	50.7 (1.1)[b]	4.2
9	-	33.3 (1.2)[d]	2.4
12	-	33.3 (1.1)[d]	3.2
15	-	41.7 (2.1)[c]	0.0
-	3	50.6 (2.1)[b]	0.0
-	6	33.3 (1.2)[d]	1.0
-	9	50.0 (1.4)[b]	2.2
-	12	41.4 (1.4)[c]	3.0
-	15	33.3 (1.1)[d]	1.0

*±SE: Standard error from mean, data represented the mean of 5 replicates, data with the same letters in the column are not significantly different at 5% level

Table 5: Effect of pulse treatment with IBA and NAA and treatment duration on *in vitro* rooting of micro shoots of *Actinidia deliciosa*

Auxins concentration (µM)				
IBA	NAA	Pulse treatment duration (h)	Response (%) (±SE)*	Average No. of roots/shoot
0	0	-	-	-
10	-	½	06.2 (0.2)	2
	-	1	12.5 (0.2)	4
	-	24	25.0 (0.3)	3
20	-	½	12.5 (0.3)	2
	-	1	31.2 (0.5)	3
	-	24	37.5 (0.7)	4
30	-	½	25.0 (0.3)	2
	-	1	56.2 (0.6)	5
	-	24	43.7 (0.6)	4
40	-	½	25.6 (0.8)	2
	-	1	51.3 (0.6)	4
	-	24	37.5 (0.7)	3
50	-	½	18.7 (0.7)	2
	-	1	43.8 (0.5)	4
	-	24	37.8 (0.8)	3
-	10	½	18.7 (0.6)	1
-		1	18.7 (0.6)	1
-		24	31.2 (0.6)	2
-	20	½	18.7 (0.60	1
-		1	25.6 (0.4)	3
-		24	31.2 (0.8)	2
-	30	½	25.0 (0.6)	2
-		1	43.7 (0.6)	3
-		24	31.2 (0.6)	4
-	40	½	37.5 (0.7)	2
-		1	50.0 (0.5)	4
-		24	43.7 (0.7)	3
-	50	½	25.0 (0.6)	2
-		1	37.5 (0.7)	3
-		24	31.2 (1.1)	2

*±SE: Standard error from mean, data compiled after 7 week of culture

response (data not presented). At higher concentration (>3%) of sucrose regenerated shoot buds exhibited curly growth. Other two organic sources found to be not suitable for culture proliferation. Of the 2 PGRs tested, BA was better over KN where 6.2 shoot buds developed per explants in 58.3% culture at concentration of 3 µM within 5 weeks (Table 4, Fig. 1 f, g). Micro shoots thus produced were further sub-cultured for another 2 passages for culture proliferation (Fig. 1g, h). In contrary on similar concentration with KN, 50% shoot buds proliferated to micro shoots where as many as 2.2 shoot buds per node developed.

Rooting of micro shoots: Micro shoots (~4-5 cm long) with fully developed leaves (Fig. 1h) from regeneration medium were selected for rooting under different conditions as stated in materials and methods (Table 5, 6). Besides incorporation

Fig. 3(a-j): Rooting of micro shoots, hardening and transplantation of Kiwi regenerates, (a) Defoliated micro shoot on rooting medium after 3 weeks of culture showing initiation of rooting, (b) Micro shoot with normal leaves on rooting medium, (c) Healthy rooting of defoliated shots, (d) Callus formation in the basal part of micro shoot at higher concentration of PGRs, (e) Well rooted plantlets transferred for hardening, (f) New shoots and roots developed during hardening stage, (g) Hardened plantlets after ~7-8 weeks of hardening (with secondary roots) ready for transfer to potting mix, (h) Defoliated primary hardened plantlets transferred to potting mix, (i) Transplants started producing new leaves and (j) Transplants established in potting mix

of PGRs directly in the rooting medium, micro shoots were also pulse treated with IBA and NAA (0-50 µM) for 0-24 h and maintained on MS plain medium. Of the two PGRs tested for pulse treatment, treatment with IBA (30 µM) for 1 h supported better rooting with 5 roots per micro shoot in 56.2% of culture (Table 5). While, pulse treatment with NAA at 40 µM for 1 h was found to be better treatment where as many as 4 roots per micro shoot developed in ~50% of cultured micro shoots within 7 week of culture (Table 5).

Compared to pulse treatment, direct incorporation of IBA and NAA in the rooting medium was found to be beneficial (Table 6). Of the different concentrations of IBA and NAA (0-15 µM) incorporated directly in the medium, IBA was found to be better choice which was similar to pulse treatment. Under the given conditions, IBA at 9 µM supported 5 roots formation with average 2.1 cm root length in 50% culture, while on NAA (12 µM) enriched medium 4 roots induced in 43.8% culture (Table 6).

Besides normal leaves, defoliated micro shoots were also cultured on IBA and NAA fortified medium for root induction. In general defoliated micro shoots were found to be better over micro shoots with intact leaves for rooting both on per cent response as well as number of roots and root length (Table 6, Fig 3a-c). In defoliated micro shoots rooting initiated within 3 weeks of culture (Fig. 3a) compared to 5 weeks in micro shoots with normal leaves (Fig. 3b). Of the 2 PGRs tested, more number of roots, better root length and healthy root growth was achieved on medium enriched with 9 µM IBA where 7 roots per shoot in 56.3% (compared to 5 roots in 50% culture with intact leaves) where average root length was 2.5 cm (Table 6, Fig. 3b, c). At lower concentrations rooting performance was poorer while at higher concentrations with rooting there was callusing at the basal parts of micro shoots (Fig. 3d). The well rooted micro shoots were taken out from the rooting medium and transferred to hardening condition (Fig. 3e).

Primary hardening of regenerates and transplantation to potting mix: The well rooted plantlets were washed with luke warm water to remove traces of agar from the roots, transferred to 1/4th strength MS liquid plain medium containing 1% sucrose containing different substrata (Fig. 4). The cultures were maintained for 7-8 weeks in the normal laboratory conditions followed by exposing the cultures to the normal day light for 4 h a day in the last 2 weeks. Within 6-7 weeks of transfer in the hardening condition plantlets started producing new shoots, roots and secondary roots which is the characteristics of kiwi plant in natural condition

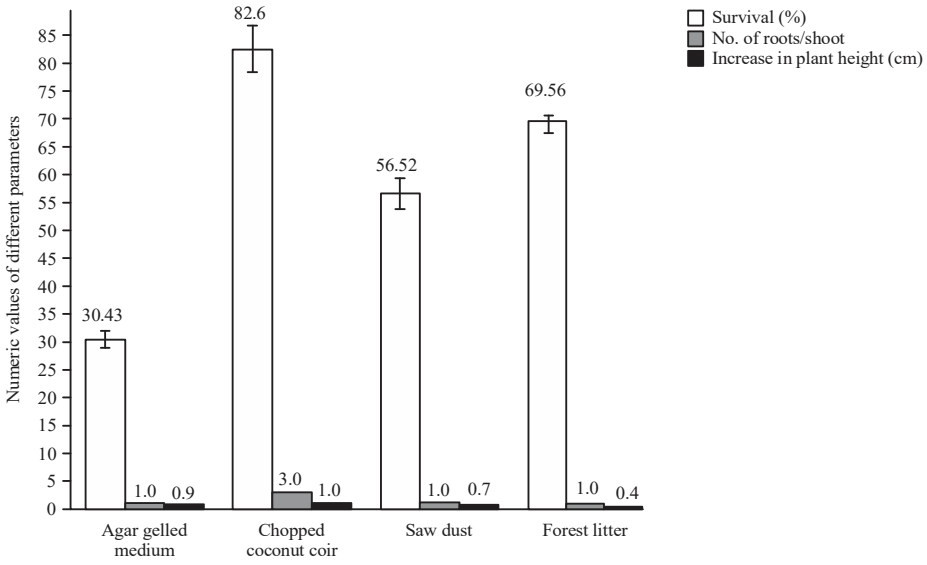

Fig. 4: Effects of different substrata on *in vitro* hardening of Kiwi plantlets

Table 6: Effect of defoliation of micro shoots and auxins on *in vitro* rooting of *Actinidia deliciosa*

Auxins concentration (μM)		Micro shoots with normal leaves			Defoliated micro shoots		
NAA	IBA	Response (%) (±SE*)	No. of roots/ micro shoot	Average root length (cm)	Response (%) (±SE[a])	No. of roots/ shoot	Average root length (cm)
0	0	-	-	-	-	-	-
3	-	18.7 (1.2)[f]	2	1.2	12.5 (1.1)[h]	1	1.4
6	-	25.0 (1.3)[e]	3	1.5	25.0 (1.2)[f]	3	1.5
9	-	37.5 (1.1)[c]	3	1.9	31.3 (1.6)[d]	4	2.8
12	-	43.8 (1.7)[b]	4	2.0	50.0 (1.8)[b]	5	2.1
15	-	31.3 (1.4)[d]	3	1.7	43.8 (1.6)[c]	3	1.6
-	3	16.7 (1.2)[f]	2	1.3	18.8 (1.6)[g]	2	2.2
-	6	37.5 (2.1)[c]	4	1.9	31.3 (1.6)[d]	4	2.0
-	9	50.0 (2.1)[a]	5	2.1	56.3 (1.9)[a]	7	2.5
-	12	43.8 (1.3)[b]	4	2.0	50.0 (1.7)[b]	5	1.9
-	15	31.2 (1.7)[d]	3	1.6	43.7 (2.3)[c]	4	2.0

Cultures maintained on MS medium supplement with sucrose (3%) and PGRs have been directly incorporated in the nutrient medium, data collected after 6 weeks of culture, *±SE: Standard error from mean, data with the same letters in the column are not significantly different at 5% level

(Fig. 3 f, g). The cultures exposed to normal day light was hardier compared to plants maintained only to the normal culture condition. It was observed that cultures exposed to normal day light produced secondary roots from the stem, glands and scale in the stem (Fig. 3g) which was achieved in about 82.6% regenerates within 7-8 weeks (Fig. 4). Of the different substrata used, chopped coconut coir was found to be most suitable for hardening where 82.6% regenerates survived and as many as 3 new shoots developed during the hardening process accompanied with increase in plant height (1.0 cm). Compared to coconut coir, agar gelled medium, saw dust and forest letter were found to inferior for the purpose (Fig. 4).

Primarily hardened plantlets were transferred to poly bags and plastic cups filled with potting mix (Fig. 3 h-i). Plantlets hardened with alternative substrata were transferred along with the substrata. Transplants were covered with holed transparent bigger poly bags and nurtured alternate weekly with 1/10th MS salt solution for one month. The covered poly bags were removed from the pots and maintained in partial shade ca.50% for 5-6 weeks and finally exposed to normal day light. The acclimatized transplants was transferred to the experimental bed and monitored regularly for 3 months. Within 2-3 weeks of transfer in the potting mix the new leaves started emerging (Fig. 3j). Established transplants were maintained for 2-3 months in the poly house where leaves turned dark green before transferring to the cultivated plot. Over 500 plants were transferred to the field of which ~70% transplants survived after 2 months of transfer.

DISCUSSION

Actinidia deliciosa is cultivated commercially for fruits in the orchards but limitation of expansion of cultivated area is due to shortage of quality planting materials. The species is propagated by stem cuttings/grafting. Grafting is a general commercial practice as *Actinidia* cuttings roots readily from hardwood cuttings but requires long time. Present study was undertaken for development of protocol for production of clonal planting materials as well as to develop efficient rooting technique. *In vitro* response is controlled by different physiochemical processes and interaction with PGRs and other factors. In the present study, culture was established from *in vivo* source nodal explants. The *in vitro* morphogenic response differed significantly from the explants collected during different seasons. Nodal segments collected during November supported 75% *in vitro* organogenesis followed by December while, least response was obtained during July-August. There was a gradual decline in response from the month of January till June and least during July. For initiation culture from *in vivo* source explants, season of explant collection is the foremost step as different vegetative organs exhibit differential *in vitro* morphogenic response in different growing season[7,9,13,24]. Similar trend was also reported in other woody species viz., in *Stevia*[25], in *Fragaria* and *Potentilla*[26], *Strobilanthes flaccidifolious*[7], *Cinnamomum tamala*[24]. This could be due to the fact that during summer with high relative humidity, favorable rainfall and temperature support production of more phenolic compounds *in vivo* which is leached in the medium but during winter browning of medium was comparatively less and favored morphogenesis. In present study first notable sign of response was swelling of explants followed by cracking at the cut ends followed by protruding axillary buds and after 3 weeks shoot bud with few dark green leaflets formation. In contrary, explants collected during summer, particularly in the months of July-August supported only 8-12% response (Table 1).

Tissue necrosis due to phenolic exudation from cultured tissues is a limiting factor for *in vitro* culture establishment. In the present study phenolic exudation could be substantially controlled incorporating AC, CA and PVP, optimum response was achieved with PVP (300 mg L^{-1}). While, >300 mg L^{-1} of all the 3 antioxidants did not support healthy organogenesis. Further, it was found that both AC and CA were inferior over PVP. Present report is in agreement with Deb and Arenmongla[7] where 300 mg L^{-1} was ideal for *in vitro* morphogenesis from nodal segments of *Strobilanthes flaccidifolious*, however, antioxidants like PVP and CA in combination at 200 and 100 mg L^{-1}, respectively could successfully control browning in *Pinus kesiya*[27].

Studies on kiwi in present study, optimum organogenesis (75%) was obtained on sucrose (3%) enriched medium where explants responded within 6 days of culture. At lower concentrations response declined and the finding is in agreement with past reports[7,13]). Role of sucrose as organic carbon in plant tissue culture was studied by several investigators[7,13,28]. Sucrose not only acts as an external energy source but also help to maintain osmotic potential of the culture which would permit the absorption of mineral nutrient present in medium, essential for optimal proliferation[29].

In *A. deliciosa* direct shoot bud formation was achieved from nodal explants on medium containing BA (6 µM) where 75% nodal segments responded positively and 4.2 shoot buds developed/node. Beside singly treatments of BA and KN, the synergistic effect of BA-NAA and KN-NAA were tested. The combination of BA and KN with NAA revealed the variation in their effect on explants. In the past the positive synergetic effect of BA and KN with auxins such as NAA, IBA and IAA for regeneration and proliferation was tested for horticultural plant species like pear shoot tip culture[30].

The shoot buds developed from nodal explants were cultured on proliferation medium and maintained for 2-3 passages where BA (3 µM) fortified medium yielded 6.2 micro shoots/cycle. Meenakshi *et al.*[31] reported the efficient role of BA on culture proliferation in banana. The quality of nutrient medium plays important role in culture proliferation but there is no standard basal medium which is equally effective for different taxa[7,13,32]. In kiwi full strength MS medium was found superior over other strengths for plant regeneration and differentiation. Besides nutrient medium and PGRs, present of organic carbon source is crucial factor of culture proliferation. In this study sucrose (3%) was found to be superior over other carbon sources. Several workers in the past reported the positive role of sucrose on culture proliferation[24,33].

Role of PGRs on *in vitro* rooting have been extensively studied in the past. For *in vitro* rooting of micro shoots of *A. deliciosa* different concentrations of IBA and NAA were used. The first root formation was recorded after 3 weeks of culture on NAA (1.0 mg L^{-1}) fortified medium, while other concentrations could invoke rooting of micro shoots only after ~6 weeks. Optimum response was observed at 9 µM IBA in 56.3% defoliated micro shoots with 7 roots per shoot compared to 50% response with 5 roots from foliated micro shoots after 5 weeks of culture. Further, it was found that foliated micro shoots promoted callusing at the base with fewer roots but in defoliated micro shoots there was no or very little callus formation. There was a negative relationship between callus formation and rhizogenesis and indicates that root development is direct organogenesis. This is possibly due

to fact that undifferentiated tissue/callus may interfere in development of normal vascular connection in the roots. It is believed that in the defoliated shoots, the endogenous auxin level was lesser compared to foliated shoots due to removal of leaves. Under this condition callus formation was negated and the available auxin was used for invocation of root meristem. This statement is based on the fact that at higher auxin concentration there was callusing from the base even from the defoliated micro shoots. In the past the stimulatory effect of IBA on rooting was reported in many species[7,34] and the present finding with *A. deliciosa* is in agreement with past reports.

Apart from direct incorporating PGRs in the medium, the micro shoots were also pulse treated with IBA and NAA (0-50 µM) for 0-24 h followed by transferring to PGRs free medium containing sucrose (3%). Of the different concentrations of PGRs and treatment periods, shoots pulse treated for one h with IBA (30 µM) supported 5 roots per shoot with 56.2% response against 43.7% shoots supported 3 roots per shoot under identical condition with NAA (30 µM). These findings suggest that for *in vitro* rooting of regenerated shoots of *Actinidia deliciosa,* IBA is an ideal PGR. Similar response was also reported by some past workers[7,35].

In vitro raised plantlets must experience the flavor of natural environment to get adjusted with its system through trial and error. For *in vitro* produced regenerates it is important that the plants get adapted to new conditions from controlled conditions which are tagged with limited gas exchanges, high relative humidity, low light intensity and up-taking of carbon sources (sugars) in the culture medium[19,20]. Abnormal characteristics of *in vitro* plantlets in leaves of unusual stomata structure, less development of cuticle or receptacle wax on the surface which contributes to excessive water loss resulting in the high mortality of transplants[36]. Under culture condition nutrients is readily available from exogenously supplied carbon in medium along with abundant moisture for physio-chemical activity. Past reports indicates that decrease in relative humidity in post transplant stage is detrimental for survival in the potting mix[19,20,36], besides this other factors like planting bed/ substrate, plantlets age and shading level affect the plantlets under greenhouse[37]. In *Actinidia deliciosa* well rooted plantlets were transferred to hardening condition with different substrata and maintained for 7-8 weeks. In the last 2 weeks of hardening, culture vials were exposed to the normal day light for 4 h followed by normal laboratory condition. It was found that the cultures exposed to normal day light during the hardening process were dark green, harder and produced secondary roots in the shoots which are characteristics of kiwi plants and adapted better post in transplant condition. Of the different substrata used for hardening, regenerates from partially chopped coconut coir registered better survival rate (82.60%) compared to 30.43% survival of transplants from agar gelled medium where rooted plantlets were softer, indicated from the shoots turning light greenish.

The transplants in poly-bags were nurtured with 1/10th MS salt solution for one month and covered initially by transparent holed polyethylene cover ensure high humidity, subsequently covers were removed progressively whenever leaves appeared to be wet. The polyethylene covers were withdrawn completely after 3 weeks of transfer followed by transferring to partially shade for 1 week before shifting to experimental garden. About 70% of transplants registered survival with sprouting few hairy dark green leaves after 6 weeks of transfer. In the past similar reports were published by Deb and Imchen[19], Deb and Pongener[20], however those reports were with orchids species. To best of our knowledge present report is the first successful attempt for primary hardening of *in vitro* raised non-orchid plantlets on agar alternative substrata .

Present study successfully attempted the development of *in vitro* low cost propagation protocol from nodal explants. Low cost substrata were used only during hardening stage. Further study will be focused on use of these substrata from initiation of culture to hardening stage.

CONCLUSION

Actinidia deliciosa popularly known as 'Kiwi fruit' an economically important horticultural species grown in different parts of the world including Nagaland. During present study *in vitro* micropropagation protocol was developed from *in vivo* nodal segments and optimized the different factors for *in vitro* production of clonal planting materials. During the study a new technique is developed for rooting of micro shoots by defoliation which offers better rooting and root growth. Further, a successful attempt was made for hardening of regenerates on medium containing low cost substrata in place of conventional agar which offers better survival of regenerates in potting mix and also reduces the production cost.

SIGNIFICANCE STATEMENT

An efficient protocol for production of clonal planting material of Kiwi and *in vitro* rooting of micro shoots was

developed from *in vivo* source nodal segments. Further, low cost substrata as agar alternative during hardening was used which reduced the production cost.

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