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Research Paper

STUDY ON *IN VITRO* PROPAGATION OF TEA [*Camellia sinensis* (L). O. Kuntze] THROUGH DIFFERENT EXPLANTS

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Abstract

The present study has been carried out to establish a suitable protocol for micro propagation for utilizing for large scale propagation of tea plant through tissue culture from seeds, shoot tips and nodal segments. Seeds containing seed coat and without seed coat was inoculated in MS (Murashige and Skoog ,1962) media supplemented with different growth regulators. Removal of seed coat showed an early response for shoot formation. The highest (75%) shoot formation was obtained from seeds without seed coat treated with MS basal media + BAP 1.5 mg/l while KIN singly showed no response for shoot formation. However, in case of micro propagation from BT_2, BT_5, TV_{23} through shoot tips and nodal segments, explants were cultured in MS with 3.0 mg/l BAP and different concentrations of auxins and cytokinins. Among them 3.0 mg/l BAP and 0.05 mg/l IAA, (75% shoot tips and 66.67% nodal segments explants from BT_2) responded to highest shoot initiation. Shoot elongations were also observed in the same media when the regenerated shoots were sub cultured in a regular interval of 20-30 days. The highest mean length of shoot/explants for shoot tip (3.5 cm) and nodal segment (3.2 cm) explants were achieved on MS semisolid medium supplemented with 3.0 mg/l BAP and 0.05 mg/l IAA. Addition of 0.5 mg/l GA_3 on shoot elongation medium showed optimum result. Moreover, 48% rooting was obtained when micro shoots of BT_2 were treated with 300 mg/l IBA followed by 30 minutes and then transferred to potted soil and kept it natural environment. After proper hardening rooted plantlets were transferred to the field and 32% plantlets were found to survive.

Key words: *Camellia sinensis, in vitro* Shoot formation, growth regulators, MS medium.

INTRODUCTION

Tea, the most popular beverages in the world, obtained from the flush shoots of the plant *Camellia sinensis* (L). O. Kuntze. Conventional tea breeding is well established, though time-consuming and labors intensive due to its perennial nature and long gestation period (4-5 years). Additionally, tea breeding has been slowed by lack of reliable selection criteria. Vegetative propagation is standard, yet limited by slow multiplication rate, poor survivability of some clones, and need for copious initial planting material (Tahardi *et al*., 2003). Research on

tea micro-propagation has recently focused on exploring the potential of somatic embryogenesis as a more efficient means of plant manipulation and regeneration (Tahardi *et al*., 2003).

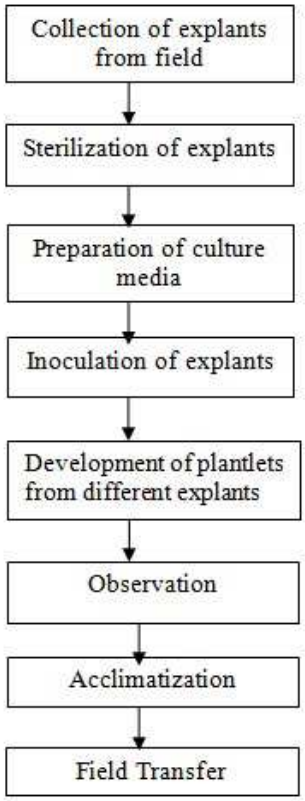
A suitable *in vitro* propagation method would remove many of problems related to the rooting of woody stem cuttings and also ensure the production of genetically uniform plants. With regard to species belonging to *Camellia,* different methods of tissue culture have been examined in several studies [Chen (1983), Haldeman *et. al.*, (1987), Kato, M. (1985) and Nakamura, Y. (1987)]. These works however, aim at establishment of primary culture to identify effects of explants, cultural conditions and varietal differences.

At present, the most parts of tea fields are planted with various clonal propagated cultivars. Fields performance of micro-propagated tea plants and the impact of cultural operations on growing tea plants have been reported previously (Marimuthu and Raj Kumar, 2001). For these reasons, tea plants were introduced into tissue culture for plant regeneration and genetic manipulation. Unlike other crops, reports are not available on the basic physiology of micro-propagated tea plants (Marimuthu and Raj Kumar, 2001).

Works on tissue culture of tea in Bangladesh are very limited. There have not so much research efforts have been made on it to establish a suitable micro propagation protocol as well as to develop its quality due to time consuming and high cost. For these reasons, we have a deep tendency to establish a suitable micro propagation protocol which gives a vast idea to tea growers for rapid propagation by using different explants such as seeds, nodal segment; shoot tips, internodal parts etc. The present study was done to develop a rapid and efficient micro propagation protocol using seeds, nodal segments and shoot tips from field grown tea [*Camellia sinensis* (L). O. Kuntze] plant through tissue culture.

MATERIALS AND METHODS

This experiment was conducted at both the Plant Genetic Engineering Lab. of the Department of Genetic Engineering and Biotechnology and Department of Food Engineering and Tea Technology, Shahjalal University of Science & Technology (SUST), Sylhet, Bangladesh. This experiment has been conducted through following flowchart.



The detail of methods employed during this study is given below:

COLLECTION OF EXPLANTS

The tea seeds were collected from Lakkatura Tea estate, Sylhet. Nodal segments and shoot tips were collected from experimental/research fields of Food Engineering and Tea Technology department. The all types of explants were aseptically excised and cultured on appropriate medium for micro propagation.

Table 1: Explants of different clones and seeds

Varieties	Explants	No. of Explants	Place of Collection
BT_2	Nodes, Shoot tips	20 + 20	FET Experimental field
BT_5	Nodes, Shoot tips	20 + 20	FET Experimental field
TV_{23}	Nodes, Shoot tips	20 + 20	FET Experimental field
Seed (Bi-clone)	Seed (With and without seed coat)	20 + 20	Lakkatura Tea Estate, Sylhet

Here, BT = Bangladesh Tea, TV = Tocklai Variety, seed (bi-clone)
FET = Food Engineering and Tea Technology

EXPLANT STERILIZATION AND *IN VITRO* CULTURE:

After collection of explants then these were washed thoroughly under running tap water for 30 min and then treated with liquid detergent (Tween 20) for 5 min, followed by dipping in savlon solution (5% v/v) for 5 min, followed by rinsing 5 - 10 times with distilled water. Then washing with 0.1% $HgCl_2$ for 10 minutes for final surface sterilization of explant. On the other hand, Seeds were washed under sterile conditions with sterile distilled water and imbibed overnight (for 16 h), in sterile distilled water. These imbibitions led to the swelling of the seeds and hastened the germination process by a week when compared to the non-imbibed control. Isolated explants were inoculated into test tube containing 20 ml of autoclaved MS semisolid medium with and without growth regulators for shoot regeneration. Data were recorded on the basis of morphogenic response of inoculated explants.

ACCLIMATIZATION AND FIELD EVALUATION:

For better rooting, plantlets were taken out very carefully from test tube and then transferred to small plastic pots containing garden soil, compost and sand in the ratio of 2:1:1. After transplantation, the plantlets were covered with large polythene sheet to maintain high humidity and kept them in growth chamber under artificial illumination and successfully acclimatized plants were transferred to the field.

RESULTS AND DISCUSSION:

Effect of different concentration of BAP and Kinetin on shoot formation from seed:

In vitro shoot formation from seed (with seed coat and without seed coat) on MS basal media supplemented with various concentrations of BAP and KIN were studied. The results of the treatments are summarized in the table 2.

Table 2: Effects of different hormones on seed (Without seed coat) germination of *Camellia sinensis* in MS semisolid medium.

PGR	Concentration mg/l	% of shoot formation (A.M ± S.E)
BAP	0.5	25.00±1.66
	1.0	53.33± 2.45
	1.5	75.00± 3.16
	2.0	60.00± 4.47
	2.5	40.00± 4.47
	3.0	35.00± 3.16
KIN	0.5	No shoot formation
	1.0	No shoot formation
	1.5	No shoot formation
	2.0	No shoot formation
	2.5	No shoot formation
	3.0	No shoot formation

A.M. = Arithmetic Mean and S.E. = Standard Error

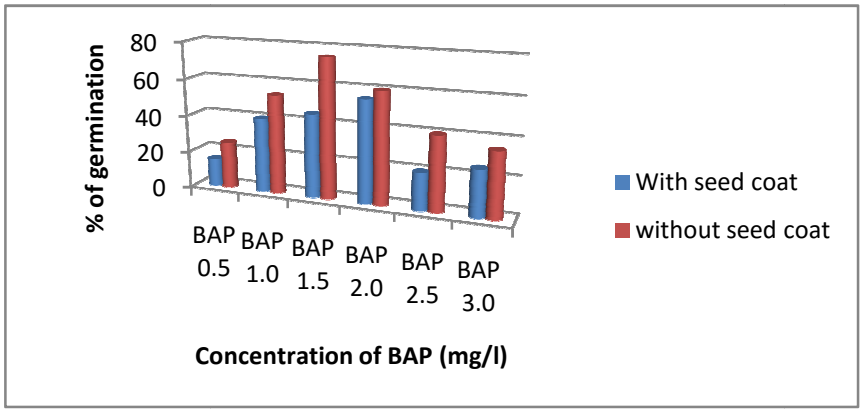


Fig. 1. Effect of seed coat on the shoot formation of *C. sinensis.*

Camellia sinensis contain two layers of seed coat. Some seed were inoculated with seed coat and some were without seed coat. The shoot formation percentages were higher in seeds without seed coat (fig.1). Among all the treatments, seeds treated with BAP 1.5 mg/l showed the highest (with seed coat 45 ± 2.16 % and without seed coat 75± 3.16 %) shoot formation (Fig.1). On the other hand, concentrations of BAP also showed various degree of response on shoot formation. But KIN alone showed no response for any kind of concentration (table 2).

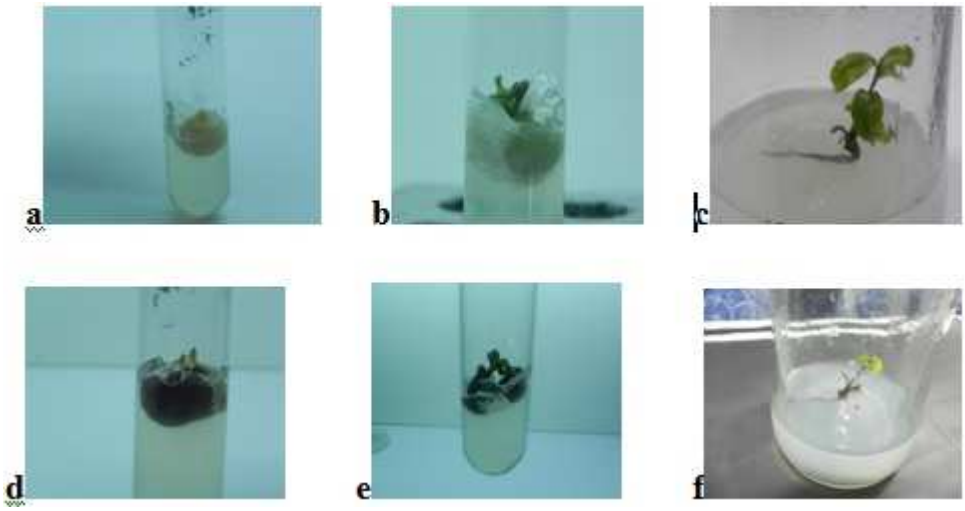


Fig. 2. Shoot formation of *C. sinensis* where a, b, c representing the rapid shoot formation of seed without seed coat and d, e, f representing the poor shoot formation of seed with seed coat. In each case seeds were inoculated with MS basal media supplemented with BAP 1.5 mg/l.

***In Vitro* shoots growth from shoot tips and nodal segments:**

Shoot tips and nodal segments of different varieties (BT_2, BT_5, and TV_{23}) of *Camellia sinensis* were cultured on MS semisolid media supplemented with various concentrations of hormone (BAP, KIN, TDZ) for shoot induction. It is generally recognized that an addition of cytokinin is necessary in shoot tip culture of woody plants (Dodds and J.H, 1983). Nakamura (1988) reported that the addition of cytokinins is essential for the growth of shoot tips of tea plant and he found more accelerated shoots with large leaves in MS medium with 3.0 mg/l BAP.

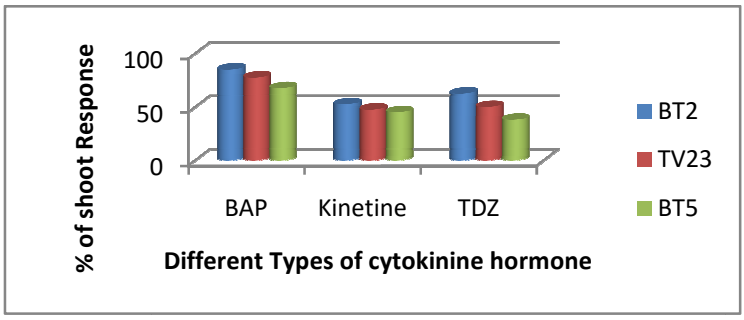


Fig.3. Effects of different types of cytokinin hormone on shoot regeneration from shoot tips of 3 different varieties of tea

Among three different varieties tested with different growth regulators, BT_2 was found to produce better response in different concentrations of BAP for shoot regeneration than BT_5, and TV_{23} (Fig.3). On the other hand, percentages of shoot formation from BT_5 and TV_{23} were moderate in respect of used different growth regulators (Fig.3).

The inoculated explants (shoot tips and nodal segments) were found to regenerate on MS media containing BAP alone but best shoot proliferation was observed on the combination of auxins and cytokinins. The best response was observed from shoot tips (Fig.4b) and nodal explants (3.4 cm/explants) in MS media with BAP 3.0 mg/l and IAA 0.05 mg/l, (Table 3). Multiple shoot formation was not occurred on all concentration tried during this study. Repeated sub culturing of the micro shoots in the same medium resulted profuse shoot stimulation and elongation. Combination of BAP with NAA or BAP alone was not found suitable than BAP with IAA for shoot Induction (Table 3).

Table 3: Effects of different growth regulators in MS semisolid media on morphogenic response of *Camellia sinensis* (L). O. Kuntze (BT_2) from shoot tips and nodal segments

Growth regulators (mg/l)					Shoot tips			Nodal Segments		
BAP	NAA	IAA	Kn	TDZ	% of explants forming shoots	Days required for shooting	Average length of shoot (cm)	% of explants forming shoots	Days required for shooting	Average length of shoot (cm)
3.0	0.1				18	40 - 45	1.1	20	40 - 45	1.5
3.0	0.5				14	35 – 37	1.5	24	38 - 40	2.0
3.0	1.0				24	33 – 37	2.2	26	30 - 35	2.2
3.0	**1.5**				**30**	**35 - 40**	**3.4**	**28**	**35 - 38**	**3.3**
3.0	2.0				22	33 - 38	2.5	27	33 - 38	3.0
3.0		0.01			24	28 - 30	1.9	28	30 – 32	1.2
3.0		**0.05**			**75**	**30 - 35**	**3.5**	**66.67**	**28 - 33**	**3.2**
3.0		0.10			20	33 - 34	2.0	22	30 - 33	1.0
3.0		0.50			12	32 - 36	2.2	16	30 - 32	1.3
3.0		1.00			16	30 - 33	1.8	08	27 - 29	1.0
3.0			0.1		10	30 – 32	1.5	08	28- 30	2.1
3.0			0.5		16	30 -33	2.7	12	28 - 33	2.0
3.0			**1.0**		**24**	**28 - 32**	**3.8**	**24**	30 - 37	**2.2**
3.0			1.5		22	30 -34	2.1	22	30 - 35	1.9
3.0			2.0		19	30 - 33	2.0	16	35 - 40	1.7
		0.01		3.0	32	40 -42	1.5	29	35 - 37	1.0
		0.05		3.0	28	35 – 40	2.2	28	30 - 32	1.3
		0.10		**3.0**	**36**	**32 - 36**	**2.8**	**32**	**30 - 33**	**2.6**
		0.50		3.0	24	30 - 35	2.4	22	33 - 37	2.2
		1.00		3.0	16	35 - 37	1.0	24	32 - 35	1.4

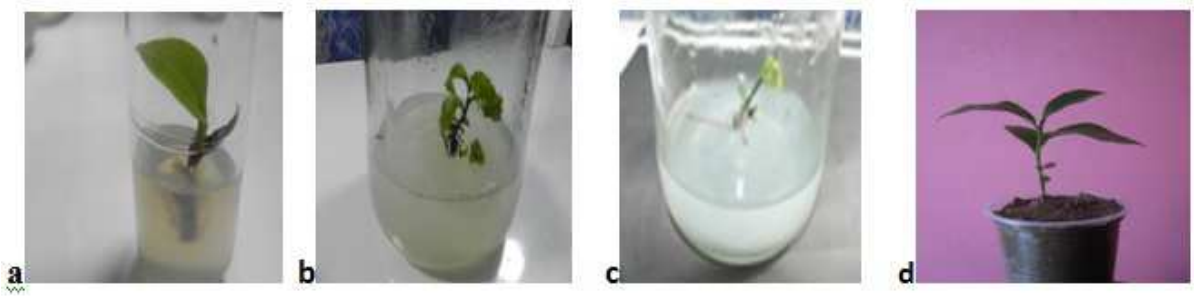


Fig. 4. Shoot induction and elongation from shoot tips of *C. sinensis*. MS media supplemented with BAP (3.0mg/l)

It was found that increased concentration of BAP reduced the number of shoots produced, showed necrosis and had shoot fasciations. Borchrtia et al. (2009) observed that the reduced number of shoot could be due to inhibition of adventitious meristem elongation due to the higher use of BAP concentration. In shoot tip culture of *Camellia japonica*, the appropriate concentration of BA was 0.5 -1.0 mg/l (Samartin *et al.,* 1984) and in *C. sinensis* it was 0.5 – 3.0 mg/l (Chen *et al*., 1983). It appears, therefore in the study, that the suitable strength of BAP ranges 1.5 – 3.0 mg/l in shoot tip culture of tea.

Table 4: Effect of IBA (300 mg/l) treatment on root formation

Treated time in 300mg/l IBA (min)	No. of shoots transferred to soil	% of shoots response in rooting	Days taken to root initiation	Mean Length of root/shoot (cm) after 60 days
10	8	-	-	-
20	8	25	62	1.5
30	8	48	60	2.4
40	8	20	68	1.0
50	8	necrosis	-	-

The simple and reliable method of micropropagation is *in vitro* cutting of nodal segments (Vieitez *et al*., 1989). In this experiment, the nodal segments with auxiliary bud were cultured in MS medium supplemented with different types of hormones singly or in combination (table 3). To find out the best hormonal combination for proliferation and elongation of shoot from nodal segment, a lot of combinations were practiced. It was found that the growth of shoots from nodal segment was accelerated on MS, when BAP (1.0 – 3.0 mg/l) was added. It appears, therefore, that the suitable concentration of BAP ranges 0.5 – 3.0 mg/l in the shoot tip and nodal segment culture of *Camellia sinensis*. To develop the better growth of shoot tips, Cytokinins combined with auxins at various concentrations were practiced. The combination of BAP (3.0 mg/l) and IAA (0.05 mg/l), however, stimulate the growth of shoot tips at a greater extent than the other combination tested (Table 3).

More than 48% regenerated shoots from shoot tip were rooted (Fig. 4b). In this present study, root induction was found from the explants of shoot tips, when proliferated shoots (2.5 – 3.0 cm) were treated with 300 mg/l IBA for 30 minutes and then transferred to plastic pots containing garden soil, compost and sand in the ratio of 2:1:1. After 60 days the percentages of rooted plant was noted and healthy plantlets were kept into the culture room at 28^0 C day/ 20^0 C night, 16 h day length and 70% humidity. Upon initiation of 1-3 leaves, the plantlets were transferred to another pot containing soil mix and covered with transparent polythene. These plantlets were exposed to environment for one hour daily and then again placed in growth room for another week. The final percentage of survival was 32% after 60 days. Rooting from shoots was also reported by different authors (Chen, J.S., 1984, Kato, M., 1985, Nakamura, Y., 1987, Samartin *et al.*1984). Sharma *et al.* (1999) reported that survival percentages depends on

maintaining a special designed hardening chamber with controlled soil pH, CO_2 enrichment, light conditions and relative humidity. The technique may adopt for large scale clonal/micro propagation and plantation for sustainable use of tea industry in Bangladesh.

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