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In vitro response of strawberry (*Fragaria x ananassa* Dutch.) for callus induction and shoot regeneration

M Rezaul Karim[1*], M Abdul Aziz[1], Uthpal Krishna Roy[1], M Aminul Hoque[2], M Monzur Hossain[1]

[1]*Department of Botany, University of Rajshahi, Rajshahi 6205, Bangladesh*

[2]*Department of Agronomy and Agricultural Extension, University of Rajshahi, Rajshahi 6205, Bangladesh*

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Abstract

Effect of growth regulators on *in vitro* responses of strawberry for callus induction and shoot regeneration has been investigated. MS (Murashige and Skoog) medium supplemented with 2,4-diclorophenoxyacetic acid (3.0mg/l) and 6-Benzyl adenine (0.5mg/l) were found most effective in terms of percentage to callus inductions and degree of callus development. Calli derived strawberry explants were cultured in shoot formatting media supplemented with different concentrations of plant growth regulators. The combination of BA (1.5 mg/l) and α-Napthalene acetic acid (0.75mg/l) showed highest percentage of shoot induction (55.6±0.81) and multiple shoot regeneration. Combination of BA, NAA and KIN (6-Furfuryl amino purine/kinetin) found to be most efficient in shoot induction (60.7%) and multiplications (16.7±0.48). After successful shoot regeneration, regenerated shoots were cultured for root induction in MS_0. The developed plantlets through *in vitro* culture technique were acclimated and successfully transferred to *in vivo* condition for further evaluations.

*Corresponding Author: M Rezaul Karim ✉ rezabotany@yahoo.com

Introduction

Strawberry is one of the most popular and valuable and nutritious fruit of the world due to genetic heterozygosity, adaptability and plasticity of the plants (Losina-Losinskaja, 1926; Staudt, 1999*a*). Strawberries are grown in geographically diverse areas ranging from the low-altitude tropics to subtropics to high altitude continental areas (Darrow, 1966). The production and consumption of strawberry is increasing day by day because of its food value and other importance (http//www.vegparadise. com/highest perch 45.html # uses). Strawberry belongs to the genus *Fragaria*, tribe Potentilleae of the Rosaceae family (Staudt, 1962, 1989, 1999*b*; Naruhashi and Iwata, 1988). About nineteen (19) or so wild species of *Fragaria* have four levels of ploidy with a base chromosome number of x = 7. *Fragaria* x *ananassa* (Garden strawberry) is an octoploid (Darrow, 1966) species (2n=8x=56). It is a natural hybrid of *Fragaria chiloensis* L. P. Mill. and *Fragaria virginiana* Duch. (Staudt and Dickore, 2001) Strawberries are perennial, stoloniferous herbs, meaning that they spread via stolons or runners (Nfapp, 2003). Though strawberry is grown in geographically diverse areas, it is a new crop in context of Bangladesh (Sakila *et. al.*, 2007) and for large scale production it is urgent need to improve varieties adapted to agro-climatic condition in Bangladesh. In recent years, novel tissue culture technique named somaclonal variation through callus induction and indirect regeneration method is being used for varietal improvement in various crops. There are some early reports on *in vitro* response on callus induction and regeneration of strawberry (Larkin and Scowcroft, 1981; Jain *et. al.,* 1998 and Patnaik *et. al.*, 1999, Kaushal *et. al.*, 2004). Therefore, the present investigation has been designed to develop an efficient protocol for *in vitro* callus induction and regeneration of strawberry for somaclonal variation.

Material and methods

For callus induction, two or three weeks old immature *in vitro* leaves were collected from Plant Breeding and Gene Engineering Laboratory of Rajshahi University of Bangladesh. Explants was surface sterilization by using mercuric chloride ($HgCl_2$) as surface sterilizing agent, tween-80 and savlon (ACI Pharma, Bangladesh) as surfactant cum detergent. Then the leaves were cut into small segments (0.5-1.0cm) and notched by scalpel in laminar air flow and were cultured on a semisolid MS (Murashige and Skoog, 1962) medium supplemented with different concentrations (1.0, 1.5, 2.0 and 3.0) of 2,4-diclorophenoxyacetic acid (2,4-D) and α-Napthalene acetic acid (NAA) alone and different concentrations and combinations (2.0+0.5, 2.0+1.0, 3.0+0.5, 3.0+1.0, 4.0+0.5 and 4.0+1.0) of 2,4-D (2,4-diclorophenoxyacetic acid)+BA (6-Benzyl adenine) and α-Napthalene acetic acid (NAA)+6-Benzyl adenine (BA). Then the cultures were incubated in dark condition around 15 days for callus induction. The responded calli were further sub cultured on MS (Murashige and Skoog, 1962) media containing low concentrations and combinations of BA, NAA and KIN (6-Furfuryl amino purine/kinetin) for shoot regeneration. The pH of all media was adjusted to 5.7 before addition of agar and sterilized by autoclaving for 20minutes at 15lb. pressure and at 121°C temperature. The cultures were maintained at 25°C±2°C (room temperature) under the cool white fluorescent lights for 16 hours photoperiod at 2000-3000 lux.

Results and discussion

The leaf explants of strawberry induced to callus development in all of the culture media formulations. The highest 93.33±0.87% explants induced to develop callus in this medium of single NAA at 1.5mg/l but combination with 2,4-D+BA at 3.0+0.5mg/l and NAA+BA at 2.0+0.5mg/l concentrations. lowest callus induction (46.00±0.89%) were found in singly 2,4-D at 1.0mg/l and in 2,4-D+BA medium at 4.0+1.0mg/l (46.67±0.42%). besides, NAA+BA at 4.0+1.0mg/l concentration showed so poor (40.00±0.29%) callus induction, that is less than 2,4-D at 1.0mg/l concentrations of callus induction. Average callus formation was found in singly 2,4-D medium at

2.0mg/l (86.67±0.56%) and NAA with 2.0mg/l (86.66±0.46), but in 2,4-D+BA was found 86.67±0.77% at 2.5+0.5mg/l and NAA+BA medium were found 80.0% performance at 2.0+1.0 and 3.0+0.5mg/l respectively which is represent in table 1. Mention a couple of previous findings about best callus induction media already found in strawberry.

Table 1. Effect of different concentrations of 2,4-D, NAA alone and combinations of 2,4-D+BA, NAA+BA in MS medium on callus formation from *in vitro* grown strawberry leaf explants. In each treatment, 15 explants were incubated in the culture medium and the data were recorded after four weeks incubation in dark.

Growth regulator supplements (mg/l)	% of explants Induced callus + Standard deviation	Degree of callus development	Callus colour	Callus nature	Adventitious shoot formation
2, 4-D					
1.0	46.00 ± 0.89	+	Cre	S	-
1.5	66.66 ± 0.82	++	Cre	S	-
2.0	86.67 ± 0.56	+++	Cre	S	-
3.0	60.00 ± 0.84	++	Cre	LC	-
NAA					
1.0	66.66 ± 0.12	++	LCre	LC	-
1.5	93.33 ± 0.81	+++	LCre	LC	-
2.0	86.66 ± 0.46	+++	LCre	LC	-
3.0	66.66 ± 0.25	++	Cre	C	-
2,4-D + BA					
2.0 + 0.5	86.67 ± 0.77	+++		All loosely	-
2.0 + 1.0	73.33 ± 0.52	++	All light creamy	compact	-
3.0 + 0.5	93.33 ± 0.67	+++			-
3.0 + 1.0	80.00 ± 0.19	+++			-
4.0 + 0.5	66.66 ± 0.55	++			-
4.0 + 1.0	46.67 ± 0.42	+			-
NAA+BA					
2.0 + 0.5	93.33 ± 0.87	+++	All white brown		-
2.0 + 1.0	80.00 ± 0.68	+++		All compact	-
3.0 + 0.5	80.00 ± 0.43	+++			-
3.0 + 1.0	73.67 ± 0.98	++			-
4.0 + 0.5	53.33 ± 0.57	++			-
4.0 + 1.0	40.00 ± 0.44	+			-

- = No response
+ = Little callusing (0-50 %)
++ = Moderate callusing (51-80 %)
+++ = Highly callusing (81-100 %)

Cre = Creamy
LCre = Light Creamy
S = Soft
LC = Loosely Compact
C = Compact

However, the effect of different plant growth regulators formulations on the degree and types of callus formation were very different. Among the different PGR formulations 2.0mg/l NAA with 0.5mg/l BA was found to be the most effective media formulation in terms of percentage (%) of explants induced to develop callus and the degree of callus development. Leaf derivative callus of strawberry were over all showed effectiveness in all types of media formulations. With combination of BA + NAA regulators were shown the best performance of shoot regeneration (55.6±0.81%) and shoot multiplications (15.6±0.27) at 1.5+0.75mg/l. Low performance (shoot induced rate 8.7±0.82% and multiple shoot number 2.10±0.65) showed in 2.0+0.75mg/l and second highest (shoot induced rate 40.4±0.22 % and multiple shoot number 12.3±0.14) were found at 1.5+0.5mg/l concentrations respectively. But any response has been found in 1.0+1.0, 2.0+0.50,

1.0+1.00mg/l and 2.0+1.0mg/l concentrations (Table 2).

Table 2. Effect of different concentrations and combination of BA with NAA in MS medium on shoot regeneration from *in vitro* grown leaf derived strawberry calli. At least 20 calli were rescued and subcultured. Data were recorded after 5 weeks of subculture.

PGR supplements in callus induction medium	PGR supplements in shoot regeneration medium (mg/l)	Morphogenic response after 5 weeks of subculture	
		% of calli induced shoot regeneration + standard deviation	No. of multiple shoots/callus + Standard deviation
BA + NAA	BA+NAA		
	1.0 + 0.50	11.3 ± 0.21	2.9 ± 0.55
	1.0 + 0.75	9.2 ± 0.13	2.6 ± 0.34
	1.0 + 1.00	--	--
	BA+NAA		
	1.5 + 0.50	12.3 ± 0.22	3.0 ± 0.12
	1.5 + 0.75	18.3 ± 0.81	4.5 ± 0.23
	1.5 + 1.00	6.5 ± 0.64	2.2 ± 0.54
	BA+NAA		
	2.0 + 0.50	--	--
	2.0 + 0.75	8.7 ± 0.82	2.10 ± 0.65
	2.0 + 1.00	--	--
	BA+NAA		
	1.0 + 0.50	25.6 ± 0.72	7.6 ± 0.72
	1.0 + 0.75	13.4 ± 0.33	3.2 ± 0.13
	1.0 + 1.00	--	--
	BA+NAA		
	1.5 + 0.50	40.4 ± 0.22	12.3 ± 0.14
	1.5 + 0.75	55.6 ± 0.81	15.6 ± 0.27
	1.5 + 1.00	38.5 ± 0.65	11.8 ± 0.85
	BA+NAA		
	2.0 + 0.50	15.3 ± 0.56	3.4 ± 0.90
	2.0 + 0.75	22.4 ± 0.69	5.4 ± 0.57
	2.0 + 1.00	--	--

-- = No response

More over in combination of BA + NAA + KIN at different concentration was showed similar response to BA+NAA. The best induced shoot regeneration rate was found 60.7±0.22% and multiple shoot number was 16.7±0 .48 at 1.5+1.5+0.5mg/l combinations. Second highest response were showed (shoot induced rate 60. ±0.22% and multiple shoot number 16.7±0 .48) at 1.5+1.5+0.5mg/l and lowest performance (shoot induced rate 6.1±0.49% and multiple shoot number 1.5±0.73) were found in 2.0+0.1+0.5mg/l medium. Any responses have not found at 0.5+1.5+0.5mg/l and 2.0+0.5+0.5mg/l concentrations. In these different combination and concentration of BA+NAA showed better response at 1.5 + (0.5-1.0mg/l) and BA+NAA+KIN showed better performance at 1.5+ (0.1-1.5mg/l) + 0.5mg/l and BA + NAA showed very response for shoot initiation and multiplications at 2.0+ (0.50-1.0mg/l) shown in table 3. So it is found that plant growth regulators formulations in callus induction as well as regeneration media are critical factors for successful indirect regeneration of strawberry through callus.

Whereas, the calli proliferated in NAA + BA supplemented callusing media have very high regeneration potential. The calli developed in NAA+BA supplemented callusing media showed the highest response to indirect shoot regeneration when regeneration media contained 1.5mg/l BA+0.75 mg/l NAA+0.5 mg/l KIN shown in plate 1.

Table 3. Effect of different concentrations and combinations of BA with NAA and KIN in MS medium on shoot regeneration from *in vitro* and field grown leaf derived strawberry calli. At least 20 calli were rescued and sub cultured. Data were recorded after 5 weeks of subculture.

PGR supplements in callus induction medium	PGR supplements in shoot regeneration medium (mg/l)	Morphogenic response after 5 weeks of subculture	
		% of calli induced shoot regeneration + Standard deviation	No. of multiple shoots/callus + Standard deviation
BA + NAA + KIN	BA + NAA + KIN		
	0.5 + 0.1 + 0.5	16.3 ± 0.82	4.1 ± 0.85
	0.5 + 0.5 + 0.5	12.2 ± 0.64	3.6 ± 0.69
	0.5 + 0.75 + 0.5	20.4 ± 0.77	5.2 ± 0.74
	0.5 + 1.0 + 0.5	14.4 ± 0.59	3.9 ± 0.64
	0.5 + 1.5+ 0.5	--	--
	BA + NAA + KIN		
	1.0 + 0.1+ 0.5	22.4 ± 0.58	6.1 ± 0.58
	1.0 + 0.5 + 0.5	19.2 ± 0.42	4.7 ± 0.66
	1.0 + 0.7 5+ 0.5	30.3 ± 0.66	8.9 ± 0.12
	1.0 + 1.0 + 0.5	24.4 ± 0.91	6.2 ± 0.58
	1.0+ 2.0+0.5	32.4 ± 0.51	9.2 ± 0.72
	BA + NAA + KIN		
	1.5 + 0.1+ 0.5	36.8 ± 0.81	11.6 ± 0.69
	1.5 + 0.5 + 0.5	28.2 ± 0.67	8.4 ± 0.10
	1.5 + 0.75 + 0.5	46.5 ± 0.15	11.7 ± 0.42
	1.5 + 1.0 + 0.5	37.4 ± 0.19	10.5 ± 0.37
	1.5 + 1.5 + 0.5	60.7 ± 0.22	16.7 ± 0 .48
	BA + NAA + KIN		
	2.0 + 0.1+ 0.5	6.1 ± 0.49	1.5 ± 0.73
	2.0 + 0.5+ 0.5	--	--
	2.0 + 0.75 + 0.5	13.5 ± 0.41	3.7 ± 0.81
	2.0 + 1.0 + 0.5	9.4 ± 0.34	2.1 ± 0.75
	2.0 + 1.5 + 0.5	16.6 ± 0.80	4.2 ± 0.92

-- = No response

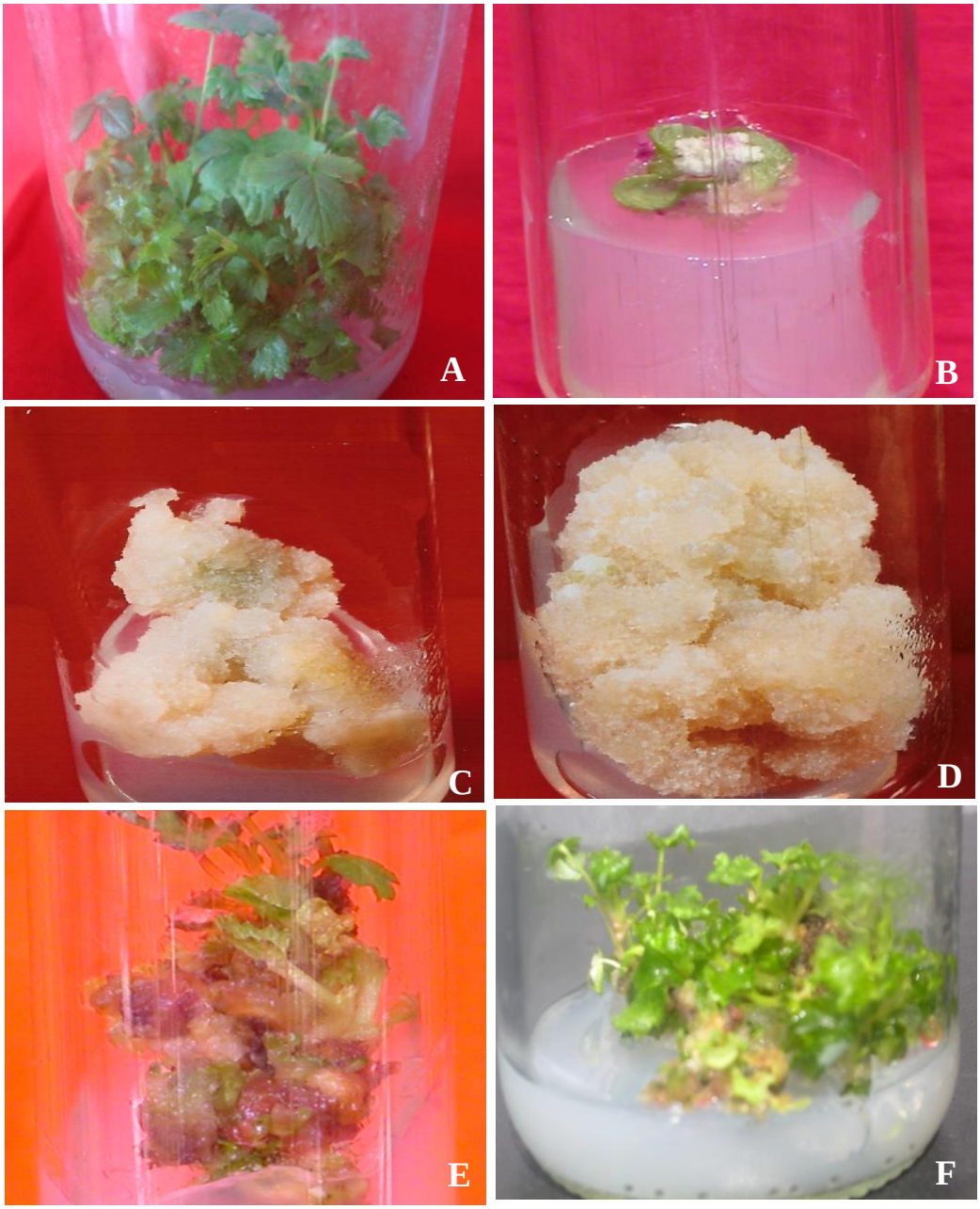

Fig 1. *In vitro* callus induction of strawberry from the leaf explants.

A. Source of leaf explants for callus induction. **B**. Initiation of callus from *in vitro* grown leaf, 15 days after culture. **C**. Callus developed from *in vitro* grown leaf in MS with PGA, 2 weeks after culture. **D**. Callus developed from *in vitro* grown leaf in MS with PGA, 4 weeks after culture. **E**. Multiple shoots regenerated in MS medium with PGA. **F.** Multiplication of regenerated plantlets from leaf derived calli of strawberry.

Mention a couple of previous findings about best regeneration media already found in strawberry Shamima *et. al.,* 2003 has shown that plant growth regulators concentrations and selections are vital for strawberry callus induction and regeneration. Besides, various formulations of BA, IBA, 2,4-D, KIN, NAA, TDZ, CH, and KNO_3 have been reported about callus induction and plant regeneration in strawberry plants (Liu and Sanford 1988 and Goffreda *et. al.,* 1995). Liu and Sanford (1988) reported using casein hydrolysate (CH) and potassium nitrate on leaf explants of 'Allstar' strawberry. Best callus and shoot production in their study was achieved with a combination of BA, IBA, CH, and KNO_3 by Nehra *et. al.,* 1990. In the present investigation, auxin in combination with cytokinin (NAA-BA) was found the most effective for callus induction. Passey *et. al.*, 2003 studied seven commercial cultivars of strawberry using leaf disks, petioles, roots, and stipules as explants material. The leaf disks had the highest regeneration rates for all cultivars with greater than 90% of explants producing shoots. Shamima *et. al.,* 2003 cultured *in vitro* derived leaf explants onto modified MS with PGR incubated in dark for 4 weeks and then transferred under light after subculturing onto fresh regeneration medium. She reported dark treatment of initial culture accentuates indirect regeneration from strawberry leaf, which is concomitant to the results of the present study. The kind of plant hormone and the amount used is as varied as the protocols for regeneration of strawberry. Nehra and Stushnoff (1989) were successful with IAA and BA, while six years later, Finstad and Martin (1995) touted the success of 2,4-D and BA. Jelenkovic *et. al.* (1991) studying different cultivars than Nehra or Finstad studied, tested hypocotyls, runners, petioles and lamina.

Present observation shows that the high incidence of callus induction and regeneration for somaclonal variations could be used in breeding programme for improvement of strawberry in Bangladesh.

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