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Callus culture from leaf blade, nodal, and runner segments of three strawberry (*Fragaria* sp.) clones

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Abstract: The present investigation was conducted to study the effects of auxin and cytokinin on callus induction from leaf blade, nodal, and runner segments of 3 strawberry clones. Among the 32 media combinations tested, 4.0 mg/L NAA + 1.5 mg/L IBA yielded the highest percentage of callus in all types of explants. The rate of adventitious shoot regeneration from callus was also influenced by explant type and concentration of BA in MS medium. The highest shoot regeneration rate was observed in leaf derived callus of clone Rabi-01 on media having 3 mg/L BA. Adventitious shoots were rooted, acclimatized, and subsequently transplanted to the field and observed until fruit production.

Key words: Auxin, cytokinin, callus, shoot regeneration, strawberry

Üç değişik çilek (*Fragaria* sp.) klonunda yaprak sapı, boğum ve stolon parçalarından kallus kültürü

Özet: Bu araştırmada, üç değişik çilek klonuna ait yaprak sapı, boğum ve stolon parçalarından kallus elde edilmesi üzerine oksin ve sitokininin etkileri ortaya konulmuştur. Denenen otuz iki besin ortamı kombinasyonu arasında 4,0 mg/L NAA + 1,5 mg/L IBA, tüm eksplant tiplerinde en yüksek kallus oluşum oranını vermiştir. Kallustan adventif sürgün oluşum oranı, MS besin ortamındaki IBA konsantrasyonundan ve eksplant tipinden etkilenmiştir. En yüksek sürgün oluşum oranı Rabi-01 klonuna ait yapraktan elde edilen kalluslarda ve 3 mg/L BA içeren ortamlar üzerinde elde edilmiştir. Adventif sürgünler köklendirilmiş, dış koşullara alıştırılmış ve daha sonra da araziye aktarılarak meyve üretimine kadar gözlenmişlerdir.

Anahtar sözcükler: Oksin, sitokinin, kallus, sürgün rejenerasyonu, çilek

Introduction

The strawberry (*Fragaria* sp.) is one of the most popular fruits in the world. It is eaten raw or used in making juice, desserts, jam, syrup, and wine. The leaves are used in blended herbal tea. Leaves and roots are believed to have medicinal properties. The fruit juice is used externally to counteract sunburn, skin blemishes, and discolored teeth (1).

Callus culture can be done from different vegetative organs such as the leaf, root, node, stem,

petiole, shoot tip, cotyledon, embryo, and floral bud. Young vegetative organs are more effective for callus induction. According to Krul and Mowbray (2), explant source is one of the most important parameters for successful long-term cell culture. Plant cell cultures provide an attractive route for producing high-value plant-derived products, such as flavorings, fragrances, alkaloids, colorants, and pharmaceuticals that are very expensive to synthesize chemically and that occur naturally only at very low concentrations (3). Successful callus culture also depends on the type of plant growth regulator. Cytokinins and auxins are known to promote callus formation in tissue culture (4-6). Auxin has a wide range of effects on plant growth and morphogenesis. A natural auxin of higher plants is involved in regulating cell elongation, cell division, and differentiation (7). Cytokinin can promote cell enlargement in certain tissues (8,9).

Organogenesis of strawberry has been reported by several workers (10-13). Different tissues of diverse genotypes have been used as sources of explants, namely anthers (14,15), leaf disk (16-19), immature embryo (20), cotyledons (21,22), and petiole (23,24). In most cases, regeneration efficiency is low and also depends on the genotype.

The present study was conducted to determine the appropriate cytokinin-auxin combination to establish a mass production system of friable callus and its regeneration ability using different vegetative organs from 3 strawberry clones.

Materials and methods

Three strawberry clones (developed by the Plant Breeding and Gene Engineering Lab, Department of Botany, University of Rajshahi, Bangladesh) viz. Rabi-01, Rabi-02, and Rabi-03 were used in the present experiment. Runner tips (1-1.5 cm) were collected from field grown stock plants and washed with Tween-80 for 20 min to remove surface contaminants. Afterwards, explants were rinsed several times with sterile distilled water. Surface sterilization was done within a laminar air flow cabinet by dipping the runner tips in 0.1% $HgCl_2$ solution (w/v) for 5 min. From sterile runner tips, terminal buds (3-4 mm) were dissected and cultured on basic MS (25) culture medium containing 3% sucrose, 0.8% agar. The pH was adjusted to 5.8 before adding agar and autoclaving (121 °C, 1.06 kg/cm^2, 15 min). The culture vessels containing explants were incubated in a growth chamber under a 16/8 h light/dark cycle at 25 ± 2 °C. The aseptic shoots obtained after 2 weeks of culture were used as the source of explants for subsequent experiments. Young, fully expanded leaves and nodal segments were collected from in vitro grown cultures. The leaves were aseptically sliced into 0.5 cm^2 pieces, discarding their margin. Longitudinally sectioned runner and nodes were placed on the medium with cut surface down. One explant was placed in each test tube containing 12 mL of the appropriate medium.

To study the effects of auxin and cytokinin on callus induction, different vegetative parts (leaf, runner, and nodal segments) of 3 strawberry clones were cultured on 32 different callus induction media (Table 1). Three-week-old calli were subcultured on the plant regeneration media. The regeneration media had 3 different concentrations (1.5, 3.0, 6.0 mg/L) of BA. After 12 weeks of culture, regenerated micro shoots were transferred to rooting medium with MS+0.5 mg/L IBA. In all media, 8 g/L Bacto-agar (DIFCO) and 3% sucrose were used. The pH was adjusted to 5.8 before autoclaving. The explants were incubated for 3 weeks at 25 ± 1 °C under cool-white fluorescent lamps with a 16-h photoperiod. After 6 weeks, well developed plantlets were removed from culture tubes, washed thoroughly with distilled water to remove any trace of agar and then transferred to plastic bags containing sterile garden soil. After that, plants were maintained in a nursery for better establishment. Well developed plants were planted in the field. Each treatment was repeated 9 times. Data were analyzed using SAS and Excel software.

Results and discussion

Leaf blade, nodal and runner segments were cultured on MS basal medium supplemented with various levels of 2, 4-D and NAA, alone or in combination with BA and Kin, to study their callus induction ability. Results obtained are illustrated in Table 1 and Figure 1A-F. The results reveal that media M_{16} yielded the highest percent of callus from leaf (72%), nodal (89%), and runner (87%) segments. The results also reveal that the concentrations of 2, 4-D and NAA had significant effects on callus induction.

Table 1. Effects of 2,4-D, NAA, Kin, and BA on callus induction from leaf, node, and runner segment explants of 3 strawberry clones.

	Media combination	Leaf				Runner				Nodal segments			
		Rabi-01	Rabi-02	Rabi-03	Mean	Rabi-01	Rabi-02	Rabi-03	Mean	Rabi-01	Rabi-02	Rabi-03	Mean
	NAA												
*M_1	0.5	0	0	0	0.0^{p}	18	15	19	17.3^{p}	15	20	15	16.7^{m}
M_2	1	20	22	25	22.3^{no}	41	40	45	42.0^{l}	44	40	42	42.0^{h}
M_3	2	31	30	35	32.0^{kl}	83	80	85	82.7^{cd}	85	80	81	82.0^{b}
M_4	4	37	38	40	38.3^{hi}	88	85	78	83.7^{bcd}	81	80	85	82.0^{b}
	NAA+Kin												
M_5	1.0+1.0	35	30	33	32.7^{kl}	42	40	45	42.3^{l}	35	38	36	36.3^{j}
M_6	1.0+1.5	36	35	38	36.3^{ji}	47	45	50	47.3^{k}	40	45	42	42.3^{h}
M_7	2.0+1.0	40	42	40	40.7^{gh}	78	75	74	75.7^{e}	75	72	73	73.3^{cd}
M_8	2.0+1.5	45	45	46	45.3^{ef}	76	75	78	76.3^{e}	75	75	79	76.3^{c}
M_9	4.0+1.0	55	56	55	55.3^{c}	85	84	85	84.7^{bcd}	85	84	83	84.0^{ab}
M_{10}	4.0+1.5	60	60	61	60.3^{b}	88	82	86	85.3^{abc}	85	80	85	83.3^{b}
	NAA+BA												
M_{11}	1.0+1.0	32	30	31	31.0^{l}	62	60	64	62.0^{gh}	55	52	55	54.0^{g}
M_{12}	1.0+1.5	37	37	35	36.3^{ji}	65	65	66	65.3^{fg}	59	55	59	57.7^{fg}
M_{13}	2.0+1.0	42	42	40	41.3^{gh}	76	78	75	76.3^{e}	75	78	70	74.3^{cd}
M_{14}	2.0+1.5	47	45	47	46.3^{ef}	81	80	82	81.0^{d}	83	80	80	81.0^{b}
M_{15}	4.0+1.0	70	71	70	70.3^{a}	88	88	85	87.0^{ab}	85	82	85	84.0^{ab}
M_{16}	4.0+1.5	72	70	74	72.0^{a}	90	88	89	89.0^{a}	88	86	88	87.3^{a}
	2,4-D												
M_{17}	0.5	0	0	0	0.0^{p}	15	10	15	13.3^{q}	25	20	21	22.0^{l}
M_{18}	1	27	25	28	26.7^{m}	45	40	45	43.3^{l}	45	44	40	43.0^{hi}
M_{19}	2	45	40	41	42.0^{g}	65	60	61	62.0^{gh}	61	62	60	61.0^{f}
M_{20}	4	52	55	50	52.3^{d}	75	70	75	73.3^{e}	75	70	71	72.0^{d}
	2,4-D+Kin												
M_{21}	1.0+1.0	20	18	22	20.0^{o}	28	25	27	26.7^{o}	25	25	28	26.0^{k}
M_{22}	1.0+1.5	25	22	20	22.3^{no}	32	33	30	31.7^{mn}	25	26	28	26.3^{k}
M_{23}	2.0+1.0	34	35	36	35.0^{jk}	52	50	52	51.3^{j}	46	45	48	46.3^{h}
M_{24}	2.0+1.5	36	37	37	36.7^{ij}	53	55	52	53.3^{j}	47	45	48	46.7^{h}
M_{25}	4.0+1.0	54	52	50	52.0^{d}	83	85	85	84.3^{bcd}	80	79	82	80.3^{b}
M_{26}	4.0+1.5	55	56	59	56.7^{c}	86	88	87	87.0^{ab}	81	82	85	82.7^{b}
	2,4-D+BA												
M_{27}	1.0+1.0	25	22	25	24.0^{mn}	29	32	30	30.3^{n}	26	28	25	26.3^{k}
M_{28}	1.0+1.5	28	25	28	27.0^{m}	34	35	36	35.0^{m}	28	25	30	27.7^{k}
M_{29}	2.0+1.0	42	43	45	43.3^{fg}	56	58	60	58.0^{i}	58	59	55	57.3^{fg}
M_{30}	2.0+1.5	49	45	51	48.3^{e}	59	62	63	61.3^{hi}	59	60	62	60.3^{f}
M_{31}	4.0+1.0	54	58	55	55.7^{c}	68	65	70	67.7^{f}	68	70	65	67.7^{e}
M_{32}	4.0+1.5	58	55	62	58.3^{bc}	71	73	75	73.0^{e}	70	72	75	72.3^{d}
	Mean	39.9^{a}	39.4^{a}	38.7^{a}		61.6^{a}	61.3^{a}	60.0^{a}		58.8^{a}	58.7^{a}	58.0^{a}	

* = Media code; Means with the same letter are not significantly different. $P \leq 0.05$

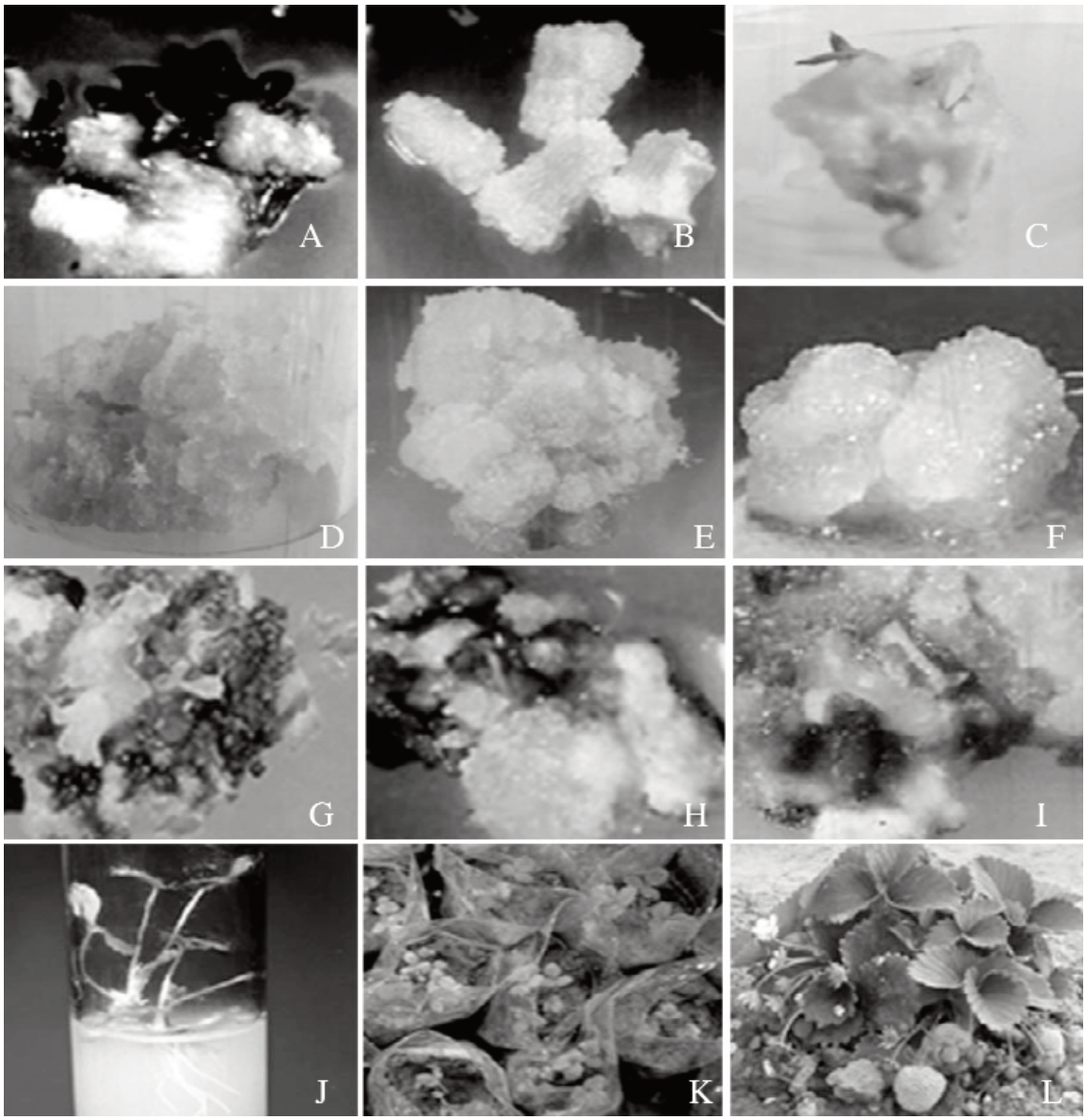

Figure 1. Callus initiation from leaf (A), runner segments (B), and nodal segments (C) on callus induction medium after 3 weeks of culture. Proliferation of callus obtained from leaf (D), runner (E), and nodes (F) on 4 mg/L NAA+1.5 mg/L IBA medium after 6 weeks of culture. Shoot regeneration from leaf (G), runner (H), and node (I) derived calli after 3 months on shoot regeneration medium. Microshoots with root on root initiation medium (J). Acclimatized plantlets (K). Callus derived plants with fruits in field (L).

With increasing concentrations of 2, 4-D and NAA, the callus induction rates increased significantly. Of the 2 auxins, 2, 4-D yielded better results for callus initiation when used singly. Kin had no significant effects in combination with 2, 4-D and NAA in terms of increasing callus percentage. In contrast, BA had a positive effect in combination with 2, 4-D and NAA, and significantly increased the callusing rate.

Runner segments yielded a mass of compact white soft callus, whereas nodal segments produced white, soft callus with a smooth, wet-looking surface, and leaf blade produced a light yellow, compact and granular callus (Figure 1). Of the 3 explant types, runner segments produced the maximum callusing rate (60.0%-61.6%) irrespective of genotype in all 3 clones, followed by nodal segments (50.0%-58.8%),

Table 2. Effects of BA on shoot regeneration from leaf, node, and runner derived callus of 3 strawberry clones.

MS+BA (mg/L)	Clone	% of callus produce shoots		
		Leaf	Node	Runner
1.5	Rabi-01	25	15	18
	Rabi-02	15	5	5
	Rabi-03	20	8	10
0.3	Rabi-01	45	35	30
	Rabi-02	25	15	18
	Rabi-03	30	20	20
6.0	Rabi-01	30	22	20
	Rabi-02	20	10	10
	Rabi-03	18	15	12

while leaf segments yielded the lowest percentage (38.7%-39.9%) of callus formation. No significant variation was observed among genotypes in all cases.

However, many factors such as genotype, composition of the nutrient medium, and physical growth factors such as light, temperature, humidity, and endogenous supply of growth regulators are important for callus induction (26). Several reports have been published about the effects of plant growth regulators on callus culture in different plants such as tomato (27,28), potato (29,30), and maize (31). In the present study, it was observed that NAA - BA was the best combination for callus induction in strawberry. In our experiment, runner segments and nodal explants produced considerably more callus than leaf explants, indicating that the source of explant is an important factor in determining the rate of success. This suggests that levels of endogenous hormones or their sensitivity may vary between different vegetative organs. Kim and Kim (3) demonstrated that endogenous supply of growth regulators and type of explants have a great influence on callus culture in Sheridan grape.

Calli derived from the 3 sources of explants (leaf, nodal, and runner segments) were subcultured for shoot regeneration in MS medium supplemented with different concentrations (1.5-6.0 mg/L) of BA. After 10-12 weeks of inoculation, shoot regeneration started (Figure 1G-I). Leaf derived calli yielded the highest percentage of regenerating shoots (Table 2) and also the highest number of shoots per explant (data not shown). These were followed by nodal and runner derived calli. Of the 3 clones, Rabi-01 responded the best, followed by Rabi-03 and Rabi-02 with respect to shoot regeneration from the induced calli in all cases. The medium fortified with 3 mg/L BA produced the maximum frequency of shoot regeneration, and the frequency of regeneration decreased with the decrease (1.5 mg/L) or increase (6.0 mg/L) in BA level. Three-week-old calli yielded a higher plant regeneration percentage than 2- or 4-week-old calli (data not shown). Over 90% of the regenerated shoots could be readily rooted when cultured on medium containing 0.5 mg/L IBA only (Figure 1J). Well rooted plantlets were successfully acclimatized and transferred to the field (Figure 1K, L). No abnormalities were found in callus derived plantlets under field condition in their vegetative growth and fruit production.

The findings of the present study demonstrate that plant regeneration may be possible from calli developed from leaf, nodal, and runner segments of 3 strawberry clones.

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