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Varietal Improvement of Strawberry (*Fragaria* x *ananassa* Dutch.) Through Somaclonal Variation Using *In Vitro* Techniques

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ABSTRACT

Strawberry is a valuable, nutritious, and economically important fruit all over the world including Bangladesh. Therefore, there is a demand to develop a suitable variety of strawberry. For this purpose, leaf explants from *in vitro* grown strawberry plantlets were cultured onto MS medium supplemented with different concentrations and combinations of 2,4-D, NAA and BA for callus induction. The most effective combination was 2.0 mg/L NAA with 0.5 mg/L BA. Then, the calli proliferated in this medium were cultured in MS medium containing different concentrations and combinations of BA, BA + NAA and BA + KIN + NAA for shoot regeneration. The best media combination was 1.5 mg/L BA + 0.75 mg/L NAA + 0.5 mg/L KIN. The regenerated shoots were cultured onto MS medium with different combinations of auxins or in MS and ½ MS medium without plant growth regulators (PGRs). The highest rooting performance was recorded in MS medium without PGRs. The plantlets were then gradually acclimated and successfully transferred to the field for evaluation. Somaclonal variations in different morphological characters such as plant height, no. of leaves/plant, petiole length, no. of stolon/plant, stolon length, no. of nodes/stolon, canopy size, no. of clusters/plant, fruit shape, no. of fruits/plant, average fruit wt. (g), fruit wt/plant (g), were noticed. Some of the somaclones exhibited better performances of the above mentioned characteristics than those of micropropagated mother plants and were well adapted to Bangladesh agro-climatic condition and were cultivated commercially in the winter season by many farmers.

Keywords: Acclimatization, Callus, Micropropagation, Plantlets , Regeneration.

INTRODUCTION

Strawberry is a nutritious and economically important fruit mainly grown in temperate and sub-temperate regions. The cultivated strawberry, *Fragaria* x *ananassa* Duch. is a natural hybrid of *Fragaria chiloensis* L. P. Mill. and *Fragaria virginiana* Duch. and belongs to the family Rosaceae sub-family Rosoideae along with blackberries and raspberries. There are two main types of strawberry cultivars: short-day or June bearing and ever bearing. Temperature may interact with photoperiods in all types of strawberries. Basically, cool temperatures promote and hot temperatures inhibit flowering (Rieger, 2006). The temperature sensitivity is the greatest in short-day cultivars. Climatic condition of Bangladesh in winter, specifically from November to March, seems to be suitable for commercial cultivation of strawberry. There are many strawberry genotypes grown in

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tropical and sub-tropical environment but fruit of these genotypes are mostly unpalatable. Though some genotypes of strawberry are imported in our country from India or other countries, it is important to get more adaptable varieties for large-scale production. Due to lack of breeding facilities and having important position in the world agriculture, strawberry has drawn the attention of scientists for its genetic improvement since long past. Plant tissue culture tools have been used for increasing the speed and efficiency of the breeding process, to improve the accessibility of existing germplasm and to create new variations for crop improvement through micropropagation, anther culture, *in vitro* selection, embryo rescue, somaclonal variation, somatic hybridization and transformation. Plants regenerated from calli exhibit great genetic variability in agronomic traits that is known as somaclonal variation (Larkin and Scowcroft, 1981). Somaclonal variation can broaden the genetic variation in crop plants; many plant characters can be altered including plant height, yield, number of flowers per plant, early flowering, grain quality, resistance to diseases, insect and pests, cold, drought, and salt (Jain *et al.,* 1998; Patnaik *et al.,* 1999). Reproducible protocol for the callus induction and shoot regeneration using leaf and petiole explants was standardized for strawberry cv. Chandler (Kaushal *et al.,* 2004). In Bangladesh, there are some limitations for the cultivation of strawberry such as lack of genetic diversity, lack of institutional initiative, collection of diversified germplasm, etc. That's why, in the present study, we focused on the varietal improvement of strawberry through somaclonal variations using *in vitro* technique targeting adaptive to agro-climatic conditions of Bangladesh.

MATERIALS AND METHODS

Planting materials of strawberry (*Fragaria* x *ananassa* Dutch. cv. Hokowase (old Japanese short-day cultivar) were originally collected from the Faculty of Agriculture, Yamagata University, Japan, and then grown at research field of the Department of Botany, Rajshahi University (RU), Bangladesh (Figure 1a).

Shoot tips (Figure 1b) and nodal segments (Figure1c) were collected from the research field of the Department of Botany, RU. Then, the explants were surface sterilized with the help of savlon, Tween-80 and 0.1% $HgCl_2$. Sterilized shoot tips and nodal segments were cultured on MS medium supplemented with 1.5 mg/L BA + 0.5 mg/L KIN + 0.5 mg/L GA_3 for shoot proliferation (Figure 1d). Leaf segments (Figure 1e) from *in vitro* grown strawberry plants measuring 6-8 mm were excised aseptically and cultured in test tubes containing 10-12 mL of MS medium supplemented with various concentrations of 2,4-D, NAA and BA either alone or in combinations for callus induction and incubated in the dark for 2-3 weeks. When the calli attained a size of about 10-15 mm in diameter, they were rescued and subcultured on the same or different Plant Growth Regulators (PGRs) supplemented media for maintenance. During callus culture, percentage of explants induced callus, the degree of callus development, the callus color and nature were recorded. Then, the selected calli were placed on medium supplemented with various concentrations and combinations of PGRs for shoot regeneration. The percentage of calli producing shoots and total number of shoots/callus were counted in each treatment. The shoots from selected calli were excised and transferred on multiplication medium for further growth. The plantlets obtained from each individual callus were further multiplied.

When the regenerated shoot apices reached 4-5 cm in length with 5-6 well-developed leaves, they were rescued from the culture vessels and separated from each other and cultured individually in tubes containing 10-12 mL of rooting medium with different combinations of auxins or in MS and ½MS media without PGRs for root induction. Rooted plantlets were gradually acclimatized and were successfully

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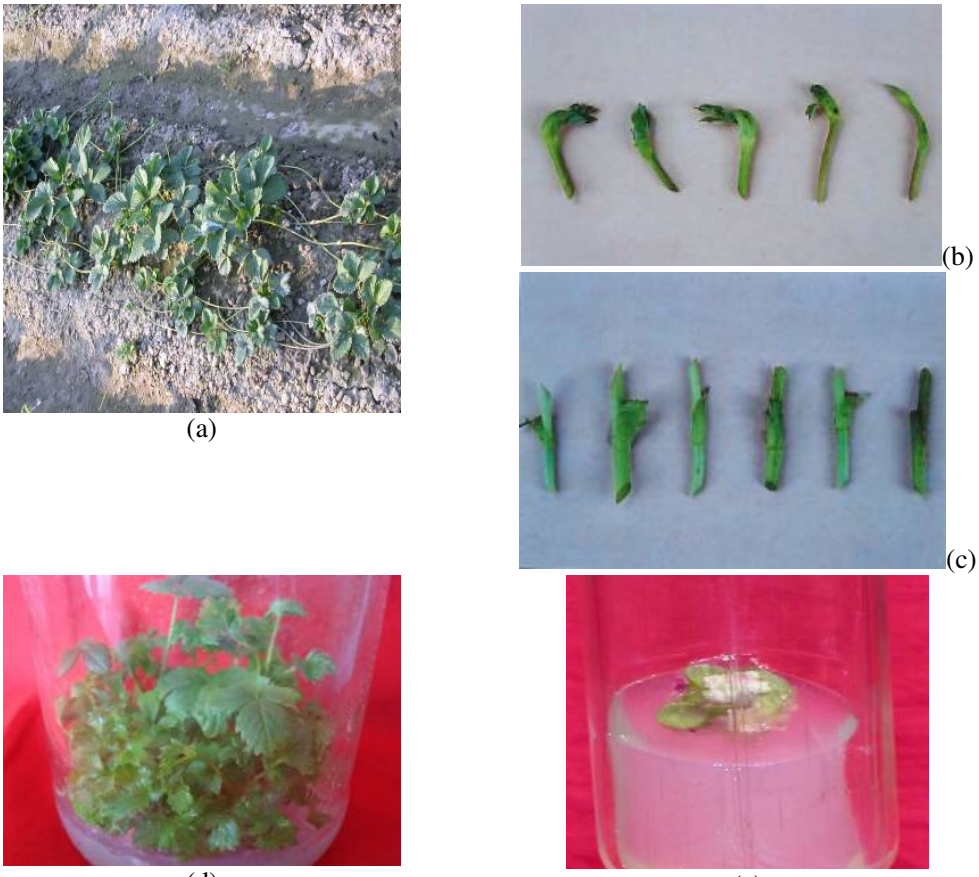

Figure 1. Planting materials for establishment of *in vitro* culture: Source plants (a); Shoot tip explants (b); Nodal segment explants (c); *In vitro* plantlets (d) Leaf segments for callus initiation (e).

established in the field. Prior to transfer to the field, the culture tube caps were removed and the open culture vessels were kept inside the growth chamber. Then, they were taken out from the controlled environment of growth chamber and kept in room temperature to bring them in contact with the normal temperature for acclimatization. After hardening, the plantlets were brought out of the culture vessels carefully and washed thoroughly under running tap water to make it agar gel free. The plantlets were dipped in a fungicide (0.1% Bavistin (Carbendazim) solution, BASF Aktiengesellschaft, Germany) for ten to fifteen minutes to kill any microbes attached to the roots, and were transferred to plastic pots under shady place and covered with polythene sheet. Finally, they were transplanted in the field. Data on different morphological characters such as plant height, no. of leaves/plant, petiole length, no. of stolon/plant, stolon length, no. of nodes/stolon, canopy size, no. of clusters/plant, fruit shape, no. of fruits/plant, average fruit wt. (g), and fruit wt/plant (g) were collected and the wide range of somaclonal variations was recorded.

Primary Somaclone Selection

Considering major somaclonal variations based on the abovementioned morphological characters, some plants were primarily selected. The selected plants were again multiplied through *in vitro* techniques or micropropagation.

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Secondary Somaclone Selection

In vitro plantlets derived from primary selected plants were acclimatized and established in the field in the similar way and again some somaclone were selected. Among the different somaclones, three types were significantly different from each other based on the mentioned characters.

RESULTS AND DISCUSSION

Callus formation is controlled by the level of plant growth regulators (auxins and cytokinins) in the culture medium. Leaf segments from *in vitro* grown strawberry (*Fragaria* x *ananassa* Dutch.) plants were used to induce callus supplemented with different concentrations and combinations of 2,4-D, NAA, 2,4-D + BA and NAA+BA. The explants showed callus development in most of the culture media combinations. However, the effects of different PGR combinations on the degree and types of callus formation were different. Among the different PGR combinations, MS medium supplemented with 2.0 mg/L NAA with 0.5 mg/L BA was found to be the most effective in terms of % of explants induced to develop callus and the degree of callus development (Table 1; Figure 2b and 2c). Auxin alone, NAA at 1.5 and, 2.0 mg/L, 2,4-D at 2.0 mg/L, 2,4-D + BA at 2.0 + 0.5, 3.0 + 0.5 and

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	Degree of callus development	Callus color	Callus nature	Adventitious shoot formation
2,4-D					
1.0	46.00	+ [a]	Cre [d]	S [f]	— [i]
1.5	66.66	++ [b]	Cre	S	—
2.0	73.33	++	Cre	S	—
3.0	60.00	++	Cre	LC [g]	—
NAA					
1.0	66.66	++	LCre [e]	LC	—
1.5	73.33	+++ [c]	LCre	LC	—
2.0	80.00	+++	LCre	LC	—
3.0	66.66	++	Cre	C [h]	—
2,4-D + BA					
2.0+0.5	80.00	+++			—
2.0+1.0	73.33	++			—
3.0+0.5	86.67	+++	All light	All loosely	—
3.0+1.0	80.00	+++	creamy	compact	—
4.0+0.5	66.66	++			—
4.0+1.0	46.67	+			—
NAA+BA					
2.0+0.5	**93.33**	+++			—
2.0+1.0	86.67	+++			—
3.0+0.5	80.00	+++	All white	All compact	—
3.0+1.0	73.67	++	brown		—
4.0+0.5	53.33	++			—
4.0+1.0	40.00	+			—

[a] Little callusing , [b] Moderate callusing, [c] Highly callusing, [d] Creamy, [e] Light creamy, [f] Soft, [g] Loosely compact, [h] Compact, [i] No response

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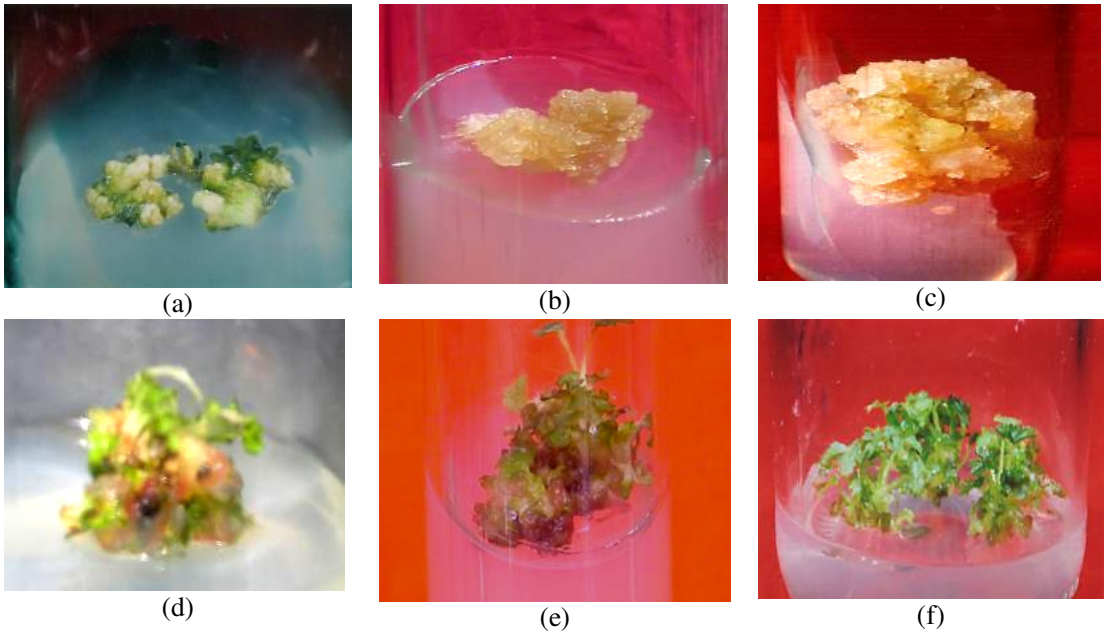

Figure 2. Callus induction and shoot regeneration: Initial culture of leaf segments for callus induction (a); Callus induction from leaf segment in media supplemented with 2.0 mg/l NAA with 0.5 mg/l BA (**b, c**); Multiple shoots regenerated from leaf derived callii in media contained 1.5 mg/l BA + 0.75 mg/l NAA + 0.5 mg/l KIN (d**, e**) Multiplication of regenerated shoots (f).

3.0 + 1.0 mg/L and NAA + BA at 2.0 + 1.0, 3.0 + 0.5 mg/L were also found to be effective PGR combinations for callus formation. To generate somaclonal variability, induction, maintenance, and regeneration of calli are prerequisites because of various abnormalities that occur in the genetic constituent during callus culture in artificial conditions and are ultimately exhibited in the regenerated plants (Larkin and Scowcroft, 1981; Shamima *et al.,* 2003).

In previous studies, it has been observed that leaf tissues of strawberry are highly regenerable (Jones *et al.,* 1988; Liu and Sanford, 1988; Nehra and Stushnoff, 1989; Nehra *et al.,* 1990; Jelenkovic *et al.,* 1991; Popescu *et al.,* 1997; Passey *et al.,* 2003). In addition, calli derived from leaf produced more shoots compared to calli derived from petiole (Popescu *et al.,* 1997). In this investigation, calli proliferated in 2.0 mg/L NAA with 0.5 mg/L BA were cultured on MS medium supplemented with different concentrations of BA alone or different concentrations and combinations of BA + NAA and BA + NAA + KIN for shoot regeneration. Among the different combinations, the highest response to shoot regeneration was noticed in media contained 1.5 mg/L BA + 0.75 mg/L NAA + 0.5 mg/L KIN (Table 2; Figure 2d and 2e)**.** The kind of PGR 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, tested hypocotyls, runners, petioles, and lamina. Only young fully expanded leaves were used in the lamina study. They determined in preliminary tests that BA and 2,4-D were the most effective PGR to use. Various combinations of BA, IBA, 2,4-D, KIN, NAA, TDZ, CH, and KNO_3 have all been reportedly used in callus induction and plant regeneration studies in strawberry (Liu

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Table 2. Effect of different concentrations and combinations of BA with NAA and KIN 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 shoot regeneration medium (mg /l)	Morphogenic response after 5 weeks of subculture	
	Percentage of calli induced shoot regeneration	Number. of shoots/callus
BA + NAA + KIN		
0.5 + 0.1+0.5	16.3	4.1
0.5 + 0.5+0.5	20.4	5.2
0.5 + 0.75+0.5	—	—
0.5 + 1.0+0.5	—	—
0.5 + 1.5+0.5	—	—
BA + NAA + KIN		
1.0 + 0.1+0.5	22.4	6.1
1.0 + 0.5+0.5	30.3	8.9
1.0 + 0.75+0.5	32.4	9.2
1.0 + 1.0+0.5	13.2	3.9
1.0+ 2.0+0.5	—	—
BA + NAA + KIN		
1.5 + 0.1+0.5	36.8	11.6
1.5 + 0.5+0.5	46.5	11.7
1.5 + 0.75+0.5	**60.7**	**16.7**
1.5 + 1.0+0.5	44.2	12.6
1.5 + 1.5+0.5	—	—
BA + NAA + KIN		
2.0 + 0.1+0.5	6.1	1.5
2.0 + 0.5+0.5	13.5	3.7
2.0 + 0.75+0.5	16.6	4.2
2.0 + 1.0+0.5	—	—
2.0 + 1.5+0.5	—	—

and Sanford, 1988; Nehra *et al.,* 1990; Goffreda *et al.,* 1995). Liu and Sanford (1988) reported using casein hydrolysate (CH) and potassium nitrate on leaf explants of 'Allstar' strawberry. Both stimulated the production of callus and shoot and reportedly had an additive effect.

The microshoots of strawberry inoculated in MS and ½MS media without plant growth regulators were induced to develop root without developing any callus at their base. When cultured in MS rooting medium without PGR, all cultured shoots developed roots within 10-15 days of inoculation, whereas 86% of the shoots were induced to develop root in ½MS rooting medium without PGR. Addition of auxin in rooting media accentuated rooting, but also microcuttings developed callus at their base, which hampered their field establishment. Similar results on the rooting and subsequent field establishment were also reported by Boxus (1974), Owen and Miller (1996), and Jimenez-Bermudez and Redondo-Nevado (2002). Then, the rooted plants were gradually acclimatized and transferred to the *ex vitro* condition for field evaluation (Figure 3).

Somaclonal variation has been successful in identification of new varieties in sugarcane, sorghum, tomato, wheat, celery, flax and *Pelargonium* (Skirvin and Janick, 1976; Compton and

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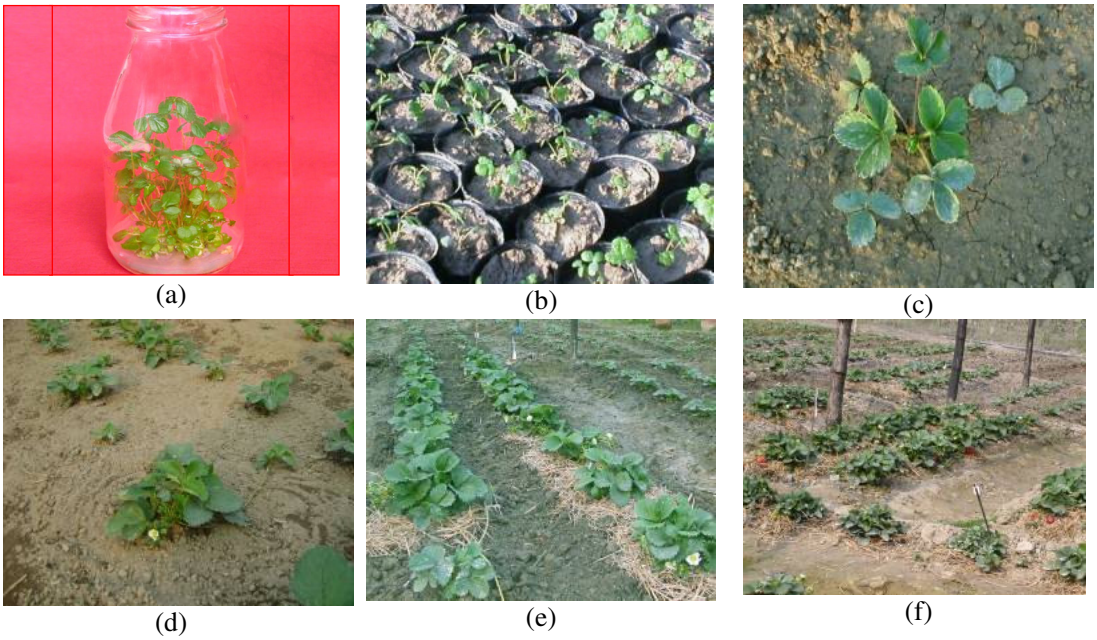

Figure 3. Field establishment of the *in vitro* grown strawberry plantlets: Acclimatization of *in vitro* grown plantlets (**a**); Plants after transplantation on to plastic pot after 15 days (**b**); A strawberry plant at 20 days after transplantation into the field (**c**); Strawberry plants, 30 days after transplantation into the field (**d**); Experimental field at 45 days after transplantation (**e**) Strawberry field at 75 days after transplantation (**f**).

Veilleux, 1991; Sears *et al.,* 1992; Duncan *et al.,* 1995; Karp, 1995). Strawberries are also amenable to *in vitro* somaclonal variation (Battistini and Rosati, 1991; Kaushal *et al*., 2004). In the present investigation, somaclonal variations in different morphological characters were observed among the *in vitro* callus derived plants (Figure 4). Wide ranges of variations for different quantitative and qualitative characters such as plant height, no. of leaves/plant, petiole length, no. of stolon/plant, stolon length, no. of nodes/stolon, canopy size, no. of clusters/plant, fruit shape, no. of fruits/plant, average fruit wt. (g), fruit wt/plant (g) showed the very high coefficient of variability. On the basis of superiority of the abovementioned characters compared to mother plants, some somaclones were selected and, among them, three varieties were named as variety RABI-1, RABI-2 and RABI-3. Among these three varieties, RABI-3 was the best for cultivation in Bangladesh agro-climatic conditions and was commercially cultivated during November–March in the following years by many farmers. In this period, Bangladesh has 20-25 ^{0}C daytime and 10-15 ^{0}C nighttime temperature and 8 hours+ direct sunlight per day, but not more than 14 hours which is very essential climatic requirement for strawberry cultivation.

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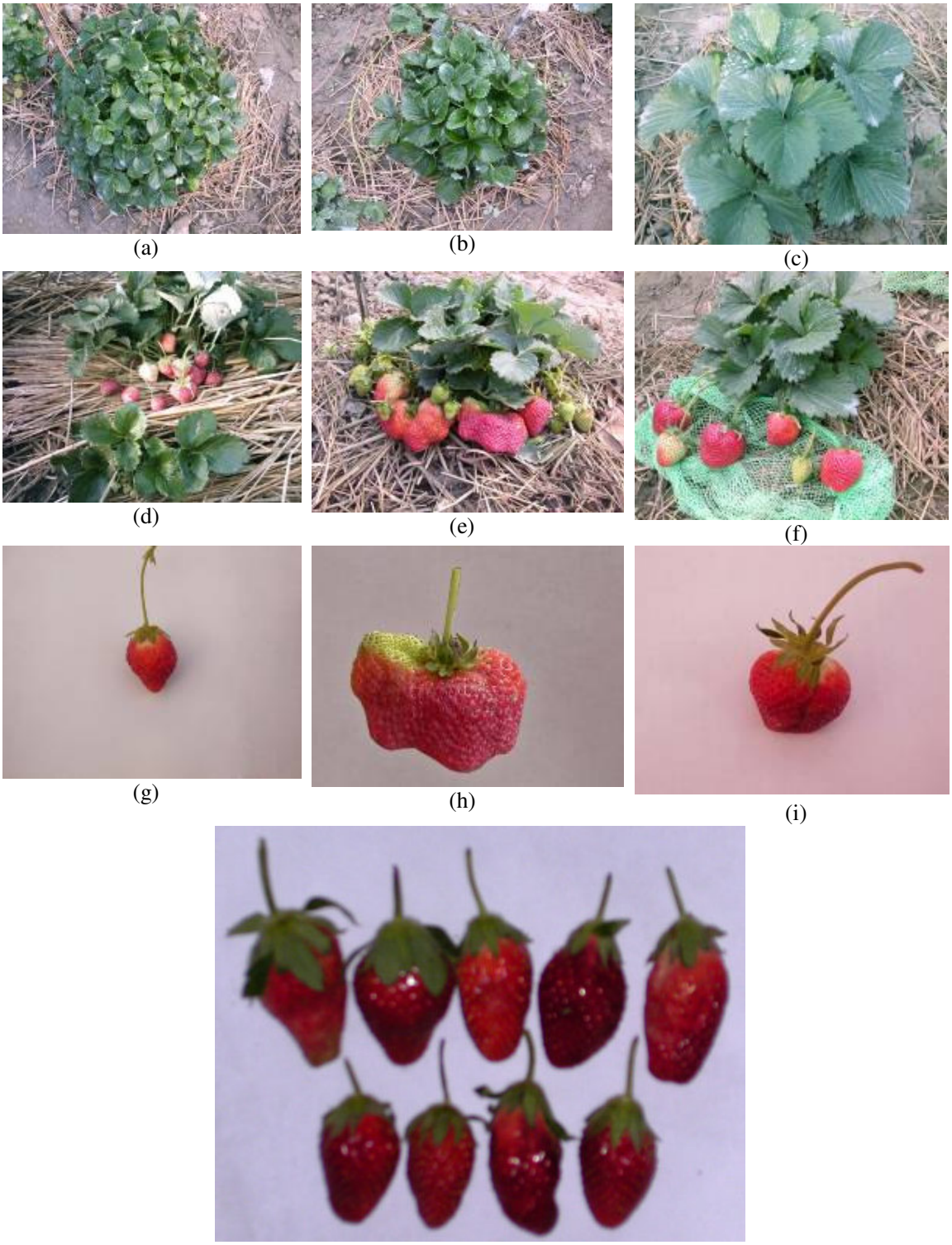

Figure 4. Somaclonal variation in different morphological characteristics: Three significant plant types named RABI-1, RABI-2 and RABI-3 (**A, B, C**); Plant types with fruits (**D, E, F**); Single fruit (**G, H, I**) Abnormalities in fruit shapes in other somaclones (**J**).

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اصلاح ژنتیکی رقم های توت فرنگی (*Fragaria x ananassa* Dutch.) از طریق تغییرات سوماکلون با روش های درون شیشه ای

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چکیده

در سرا سر جهان، توت فرنگی میوه ای با ارزش و سرشار از عناصر غذایی است که ازنظر اقتصادی مهم است. بنا بر این برای تولید یک رقم مناسب از این گیاه همواره تقاضا وجود دارد. برای این منظور، نمونه های ریز از برگ توت فرنگی های کشت شده در درون شیشه تهیه شد و روی محیط کشت MS که برای القای تشکیل پینه با غلظت ها و ترکیب های مختلف 2,4-D، NAA وBA غنی شده بود کشت شدند. موثر ترین ترکیب غنی سازی شامل ۲ میلی گرم در لیترNAA با نیم میلی گرم در لیتر BA بود. سپس ، پینه به دست آمده در این محیط روی محیط کشت MS که دارای غلظت ها و ترکیب های موادی شاملBA ، NAA+BA ، BA + KIN + NAA برای باززایی ساقه بود کشت شدند. بهترین ترکیب برای محیط رشد شامل ۱/۵ گرم در لیترBA و 0.5 mg/L NAA + 0.75 mg/L KIN بود. ساقه های باززایی شده روی محیط کشت MS دارای ترکیبات مختلف اکسین ها یا روی محیط کشت MS یا MS ½ بدون تنظیم کننده های رشد(PGRs) کشت شدند. بیشترین رشد ریشه در محیط کشت MS بدون تنظیم کننده های رشد مشاهده شد.سپس گیاهچه ها به تدریج با شرایط خو گرفته (acclimated) و برای ارزیابی با موفقیت به مزرعه منتقل شدند. تغییرات سوماکلون در ویژگی های شکلی مختلف مانند بلندی گیاه، تعداد برگ در بوته، طول دمبرگ، تعداد شاخه خزنده در بوته، طول شاخه خزنده، تعداد گره ها در هر شاخه خزنده، تعداد خوشه ها در بوته، شکل میوه، تعداد میوه در بوته، میانگین وزن هرمیوه بر حسب گرم و میانگین وزن میوه در بوته ثبت شد. در مورد این ویژگی ها، بعضی سوماکلون ها عملکرد و رشد بهتری از ریز-ازدیادی (micropropagated) بوته های مادری نشان دادند و به خوبی با شرایط اقلیمی-کشاورزی بنگلادش سازگار ی داشتند و بسیاری از کشاورزان آن ها را به صورت تجارتی در فصل زمستان کشت کردند.

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