Clonal propagation of Strawberry (*Fragaria x ananassa* Duch.) through *in vitro* runner tip culture through incorporation of growth hormones

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Abstract

Runner tips explants of strawberry give rise to multiple shoots when cultured on MS medium supplemented with different concentrations and combinations of BAP with KIN or NAA or GA_3.The highest response of shoot multiplication was obtained on MS containing 2.5 mgl^{-1} BAP + 0.5 mgl^{-1} Kin + 0.5 mgl^{-1} GA_3. The maximum frequency of rooting (83%) and highest number of roots (3.49) was produced in medium containing 1.0 mgl^{-1} IBA. The well grown rooted plantlets were acclimatized and successfully established in autoclaved vermiculate soil and as well as natural condition. Using our established protocol, it is also possible to provide large numbers of micropropagated plantlets of this cultivars to produce high quality strawberry fruit for commercial cultivation practices.

Key words: Strawberry, in vitro, regeneration, acclimatization multiple shoots and roots,

1. Introduction

Strawberry is one of the member of the Rosaceae family which is genetically octoploid (2n=8x=56) in nature, is the most important soft fruit worldwide [1]. The cultivated strawberry (*Fragaria* X *ananassa* Duch.) is a natural hybrid between the Scarlet or Virginia strawberry (*F. virginiana* Duch.) and pistillatet South American (*F. chiloensis* L. Duch), [2]. It is a perennial, stoloniferous herb. Strawberries have traditionally been a popular delicious fruit for its flavor and taste. It can be consumed fresh, frozen or processed. It is produced in 71 countries worldwide on 506000 acres [3].

Strawberry is one of the most fascinating fruits of the world. People of all spheres like this fruit because of some unavoidable reason. Firstly, it's bright red color, juicy texture and tantalizing aroma attracts all very easily. Secondly, it contains comparatively high percentage of minerals including manganese, calcium, potassium, iron, copper, numerous dietary fibers, carbohydrates, unsaturated fatty acid, proteins and most of the nutritious elements essential for human being [5]. It is also a rich source of vitamins like B1, B2, C. Especially the fruit contains higher vitamin C concentration than orange or lemon. Finally, it has relatively high quantities of ellagic acid, which has a wide range of biological activity including anti-carcinogenic impact [4]. This dilactone of hexahydroxy diphenic acid is a natural phenol antioxidant, gradually getting popularity to use in

mental function, viral and bacterial infection and cancer treatment [3]. It is consumed in large quantities, either fresh or in prepared foods, such as preserved fruit juice, pies, ice creams, and milk shakes. Artificial strawberry flavor is also widely used in many industrialized food products. It is produced in 73 countries worldwide on 200,000 hectors and produced 31 lac metric tons strawberry per year [6].

In conventional cultivation, the strawberry (*Fragaria* X *ananassa* Duch.) species sexually propagated via runner seedlings. Nevertheless, such seedlings due to their weakness and susceptibility to pathological agents are not always suitable for this cultivation. Moreover, most of the strawberry cultivars are grown in the temperate parts of the world, although a few day-neutral variety can be grown in the sub-tropical regions. However, commercial cultivation is not popular in Bangladesh due to lack of proper cultivation knowledge, profitability and lack of available season friendly variety [7]. For better strawberry production, 10 to 20h photoperiod, 12 to 30°C day temperature and 12 to 24 short days are essential [8]. Bangladesh is a sub-tropical country with 15 to 25°C winter average day temperature, where almost two months include short photoperiod of 12 to 16 h [9]. Therefore, from the beginning through the whole winter season strawberry can be grown without that much excessive nursing and care. In present time, gradually framers are becoming interested in strawberry culture due to high demands and more profits. Since last few years, strawberries were cultivating but the main constrain of its cultivation was to maintain plant materials in hot summer of Bangladesh [10].

To solve problems of natural seedlings and make it available, nowadays the advantageous alternative to this conventional method seems to be the use of micro-propagated plants for cultivation [14]. Micro-propagation of strawberry has been applying

in large scale for commercial production since mid1970s [15]. *In vitro* techniques had first applied to strawberry meristems to generate large scale of plantlets using an efficient method of mass propagation [14][16]. Strawberries can be propagated *in vitro* condition by tissue culture methods whereas micro-propagation is a very useful technique to get improved plantlets. Micro-propagated strawberry plants were comparatively better in different characters (crown size, number of runners, flowering time and yield of berries) than conventionally propagated runner plants [11][12]. Although production of propagules through runner has been reported to contribute 90% of total Dutch strawberry production, the product in Elsanta cultivars was found to be susceptible to several fungal diseases [13].

However, strawberry was introduced in Bangladesh two decades ago which is becoming popular in recent years. Bangladesh Agricultural Research Institute (BARI) recently released a strawberry variety known as BARI Strawberry-1 [17]. Conventional propagation methods are slow, laborious, and expensive with many limitations and may not be recommended for commercial multiplication [18]. Commercial multiplication of the released variety in this country is essential for rapid extension. The significance of *in vitro* propagation is that it offers higher multiplication rate [19] of plantlets from a single individual in a relatively short span of time and space [20].

Moreover, the conventional way of production is not adequate to meet the commercial demand. Micropropagated strawberry plant has been introduced to prevent most of the plant and soil transmissible diseases [9]. Large-scale commercial propagation by *in vitro* techniques has been used widely in the strawberry industry [21].

Therefore, in our investigation by considering the view of the potential commercial value and market demand we established an efficient and cost-effective method of *in vitro* propagation of strawberry to ensure availability of planting materials for commercial cultivation practice.

2. Materials and Methods

2.1 Collection of explants and surface sterilization

Juvenile runner tips from strawberry mature and healthy plants (Fig. 1A) were collected from the strawberry garden of Jessore University of Science and Technology, Jessore 7408, Bangladesh. From the strawberry field we had collected around 5 cm runner tip explant with sterile scalpel and immediately dropped into distil water to avoid the cell damage and contamination. Collected explants were washed in a running tap water for 30 min and then thoroughly washed again by adding a few drops of Savlon (ACI Ltd. Bangladesh) and Tween-20 (UNI Chem, China) for 5 minutes followed by rinsing with three times autoclaved distilled water. Again, surface sterilized in a 0.1% mercuric chloride (MERCK, India) for 5 mins followed by rinsing them five times with autoclaved dH_2O inside the Laminar Air Flow Cabinet.

2.2 Inoculation

Small runner tips (0.5-1.0 cm) were inoculated on MS medium (Murashige, and Skoog, 1962) supplemented with different concentration of growth regulators (BA, NAA, KIN and GA_3). In addition to that, 30 gl^{-1} sugar were used as carbon source and 0.6% agar have used to solidify the medium. The pH of the medium was adjusted to 5.7 by using 1M KOH before autoclaving at 0.15 MPa in 121°C for 20 min. Being prepared the sterilized explants and solidified cooled medium we have inoculated them under laminar

air flow hood. Sterilized scalpel has used to inoculate the explants in a fashion that half of the explants should put inside the medium.

2.3 Incubation

The cultures were maintained in a growth chamber at 25 ± 2°C under photoperiod of 16 h/day and a light intensity of 2000-3000 lux provided by white fluorescence tube (Philips) including 55-60% relative humidity.

2.4 Sub-culture and multiple shoot regeneration

Subcultures were done every 2-3 weeks interval depending on the explants growth conditions. Nodal segments from the proliferated shoots were sub-cultured again for further multiple shoots regeneration.

2.5 Rooting and acclimatization

Regenerated multiple shoots were cut (approximately 2-3 cm long) and individual shoots were placed on MS solidified medium containing different single concentrations of IBA and IAA for root induction. Well grown *in vitro* rooted plantlets were taken out from culture tubes and washed thoroughly with tap water to remove the culture medium from the roots. Washed plantlets were planted to sterilized vermiculate soil in small pot and after acclimatization grown in a natural condition.

2.6 Data analysis

Surface sterilization, shoot regeneration, multiple shooting and rooting data were recorded during the experiments. In the data table we have presented those experimental data which have gotten from 15 explants inoculation at a time. After getting results we

have repeated the same hormone concentration three times to be sure about the homogeneity and appropriate hormone ratio. We have used SPSS software to measure the standard deviation of the data.

3. Results and Discussion

Runner tips explants of strawberry had cultured on MS medium with different concentration of BAP (0.5-2.5 mgl^{-1}), KIN (1.0-2.0 mgl^{-1}), and GA_3 (0.1-1.0 mgl^{-1}) (Table 1). Within 4-5 weeks of culture multiple shoots were directly initiated from the explants. The maximum number of shoots (7) regenerated using medium supplemented with BAP (2.0 mgl^{-1}) were greater than those observed in the medium including KIN and GA_3 singly, where 82% explants showed shoot proliferation (Table 1 and Fig. 1, B). After getting better response using BAP, different hormone concentration (0.5-2.5 mgl^{-1}) was applied to optimize optimum hormone concentration. In case of only BAP supplement in the medium, concentration (2.0 mgl^{-1}) was found optimum whether, lower or higher than this concentration showed lower shoot proliferation. every time we have experienced the same results.

On the other hand, runner tips explants were cultured on MS medium supplemented with different concentration and combination of BAP (1.0-2.5 mgl^{-1}) with KIN (0.1-0.5 mgl^{-1}) or BAP (1.0-2.5 mgl^{-1}) with GA_3 (0.05-0.5 mgl^{-1}) or BAP (1.0-2.0 mgl^{-1}) with NAA (0.1-0.5 mgl^{-1}). In addition to that, Medium combined with tri hormone combination BAP, KIN, GA_3 in different ranges had applied (1.0-2.5 mgl^{-1}; 0.5-1.0 mgl^{-1}; 0.5 mgl^{-1}) respectively. The numbers of shoots in medium with BAP+KIN+GA_3 combinations were greater than those observed in the medium supplemented with BAP + KIN or BAP + GA_3 or BAP + NAA treatments. Among all the BAP+KIN; BAP+GA_3; BAP+NAA combinations, (2.5+0.5) mgl^{-1}, (2.0+0.5) mgl^{-1}, (2.0+0.5) mgl^{-1} respectively in MS medium showed the highest response of 92%, 90% and 60% respectively. Comparing these three combination it was revealed that BAP+KIN combination was

better in perspective of short time response, shoots per explants and length of explants. Highest rate of response was obtained at 2.5 mgl^{-1} BAP + 0.5 mgl^{-1} KIN combination (Table 2) where in addition to 92% explants shoot proliferation, (7±0.23) shoots were developed in short time of 8-12 days. When BA concentration was decreased below 2.0 mgl^{-1}, the rate of shoot multiplication

reduced. In our present investigation, maximum number of shoots per explant (20 ± 0.23) and highest average length (3.5 ± 0.26) were recorded at BAP 2.5 mgl^{-1} + KIN 0.5 mgl^{-1} + GA_3 0.5 mgl^{-1}, which was found to be the best combination for high frequency of multiple shoot induction of strawberry (Table 2, Fig. 1, C). This hormone combination showed 98% shoot proliferation in 5-10 days. When BAP was supplemented with KIN or GA_3 or NAA the rate of shoot proliferation and the shoot length were increased but number of shoots were decreased. The high concentration of cytokinin reduced the number of micropropagated shoots [23]. Similar results have already been reported in *Fragaria indica* Andr. [24]. Also, this result is in consistent with the findings of in papaya [25] as well as in *Eucalyptus grandis* [26]. The shoot organogenesis varied from strawberry genotypes to genotypes [27]. Though some workers reported high concentration of BAP is the best for strawberry micro propagation [28] while other authors suggested 1.0 mg/l IAA + 1.0 mg/l BAP + 0.05 mg/l GA3; 0.5 mg/l BA + 0.1 mg/l GA3 + 0.1 mg/l IBA [29][30] and 0.5 mg/l BA + 0.1 mg/l IBA for strawberry micropropagation. Successful micro-propagation using meristem culture had given quality result in 0.5 mg/l BAP [31] with 77.5 % fully regenerated plant [31]. Even though in this study only MS medium had used some of the study reported the successful use of Knoxfield2**[32]** as a base medium with BAP supplement. In another study it was shown to use the walnut medium with (1.5-3) mg/l for walnut shoot proliferation [33]. This range is also quite similar and showing good effect of BAP though they did not use any KIN and GA_3. However, micro-shoots proliferation was 93.2% in five-six weeks duration according the study. Nas medium constrained with cooper and myo-inositol has impact on shoot proliferation and inclusion of polyamine growth regulators stimulate shoot proliferation [34]. Micropropagation of cherry cv. Lapin with BAP showed fast growth and proliferation [35] though KIN show the better result in elongation than proliferation which is similar in our study. Use of BAP show response in proliferation but KIN needs for elongation and combination of BAP, KIN and GA_3 show the best

sensitivity best elongation and fast-growing nature. In a nutshell we can state that hormone concentration BAP 2.5 mgl^{-1} + KIN 0.5 mgl^{-1} + GA_3 0.5 mgl^{-1} is the best combination for shoot proliferation than other studied combination. This combination gives the best outcomes and though it is not that much high concentration, which indicate the cost-effective shoot proliferation combination for strawberry micropropagation.

The developing shoots were elongated by sub-culturing on the same combinations of growth regulators. Later, elongated shoots were excised and used for root induction. Well grown shooted plantlets were cultured on different concentrations of IBA (0.5-2.0 mgl^{-1}) and IAA (0.5-2.0 mgl^{-1}) singly on ½MS solidified medium. Among the different treatments 1.0 mgl^{-1} IBA proved to be the most suitable for root induction with 83% of

rooting and 3.49 ± 0.05 roots per explant and the average root length being 3.18 cm (Table 3; Fig. 1, F). similar effects were observed of IBA in case of rooting of strawberry plantlets [3]. It has reported in strawberry root generation that using IBA in a 1mg/l concentration showed the best result in case of meristem culture [31]. Through incorporation of IBA, root induction, number of roots per plants and initiation of roots can be increased [31]. But in case of piper regeneration successful root induction about 80% plants were done using 1.5 mg/l IBA [35].

We have seen that higher concentration of hormone is not the best way of micropropagation, rather than, precise combination of different hormone is also very important. In all above described hormone combinations where lower or higher rate than the optimal concentrations we found fail to give high amount of shoot proliferation and it is also true in case of root induction and proliferation. Thoroughly analysis of all hormones combination both in shoot and root proliferation we found the best amount is BAP 2.5 mgl^{-1} + KIN 0.5 mgl^{-1} + GA_3 0.5 mgl^{-1} (Table 2, Fig. 1, C) for shoot and 1.0 mgl^{-1} IBA (Table 3; Fig. 1, F) for root proliferation.

Table 1: Effects of different concentration of BAP, KIN and GA_3 on multiple shoot regeneration from runner tips explants of strawberry.

Growth regulators conc.(mgl^{-1})	No. of explants inoculated	% of explants responded	Days to shoot formation	No. of shoots per culture (M±S.E.)	Highest length of shoots in cm (M±S.E.)
BAP					
0.5	15	20	18-22	3±0.57	1.90±0.05
1.0	15	40	15-20	5±0.58	2.10±0.19
1.5	15	70	12-15	5±0.45	2.51±0.57
2.0	**15**	**82**	**8-15**	**7±0.73**	**2.10±0.15**
2.5	15	71	12-15	5±0.66	2.03±0.03
KIN					
1.0	15	40	10-15	2±0.43	2.17±0.08
1.5	15	65	10-16	4±0.45	2.06±0.03
2.0	**15**	**73**	**8-15**	**5±0.21**	**2.20±0.06**
GA_3					
0.1	15	25	10-15	3±0.57	2.04±0.04
0.5	15	45	8-15	4±0.22	2.25±0.02
1.0	15	33	10-15	5±0.65	2.03±0.10

Note: M = Mean, S.E. =Standard Error

Table 2: Effects of different concentration and combination of auxin with cytokinin and GA_3 in MS medium on multiple shoot regeneration from runner tips explants of strawberry.

Growth regulators (mgl^{-1})	No. of explants inoculated	% of explants responded	Days to shoot formation	No. of shoots per culture (M±S.E.)	Highest length of shoots in cm (M±S.E.)
BAP+KIN					
1.5+0.5	15	65	10-15	3±0.58	2.20±.11
2.0+0.1	15	75	9-12	3±0.40	3.0±0.16
2.0+0.5	15	85	8-12	5±0.34	3.4±0.21
2.5+0.5	15	**92**	**8-12**	**7±0.23**	**3.5±0.66**
BAP+GA_3					
1.0+0.05	15	28	12-16	2±0.21	3.2±0.11
1.5+0.1	15	60	10-15	3±0.76	2.9±0.42
1.5+0.5	15	87	12-16	5±0.24	3.06±0.05
2.0+0.5	15	**90**	**12-16**	**8±0.58**	**3.3±0.24**
2.5+0.5	15	82	12-16	7±0.33	3.47±0.63
BAP+NAA					
1.0+0.5	15	28	12-20	2±0.55	2.0±0.26
2.0+0.1	15	57	12-20	4±0.32	2.3±0.27
2.0+0.5	15	60	10-15	5±0.35	2.56±0.38
BAP+KIN+GA_3					
1.0+0.5+0.5	15	80	7-12	12±.33	3.2±0.51
1.5+0.5+0.5	15	85	7-14	12±.66	2.5±0.88
1.5+1.0+0.5	15	72	6-15	14±.57	2.5±0.58
2.0+0.5+0.5	15	90	6-12	17±.39	3.4±0.32
2.5+0.5+0.5	15	**98**	**5-10**	**20±.23**	**3.5±0.26**

Note: M = Mean, S.E. =Standard Error

Table 3: Effects of different concentration of auxins (IBA, IAA) in MS medium on *in vitro*

root induction from regenerated shoots of strawberry after 7-8 weeks of culture.

Growth regulators conc. (mgl^{-1})	No. of shoot sub-cultured	Shoot derived from the explants of mature plants			
		% of rooting	Days to root generation	Average no. of roots	Average root length (cm)
IBA					
0.5	15	26	16-22	2.26±0.23	2.86±0.09
1.0	15	**83**	**12-20**	**3.49±0.05**	**3.18±0.04**
1.5	15	64	14-20	2.50±0.04	2.90±0.65
2.0	15	71	16-22	3.36±0.03	3.18±0.56
2.5	15	53	16-24	3.16±0.06	3.08±0.66
IAA					
0.5	15	39	18-26	2.04±0.35	3.18±0.30
1.0	15	56	16-22	3.45±0.33	3.50±0.50
1.5	15	64	16-26	3. 20±0.20	3.22±0.39
2.0	15	53	21-26	2.92±0.32	3.08±0.52
2.5	15	40	18-27	2.78±0.56	3.18±0.46

Note: M = Mean, S.E. =Standard Error

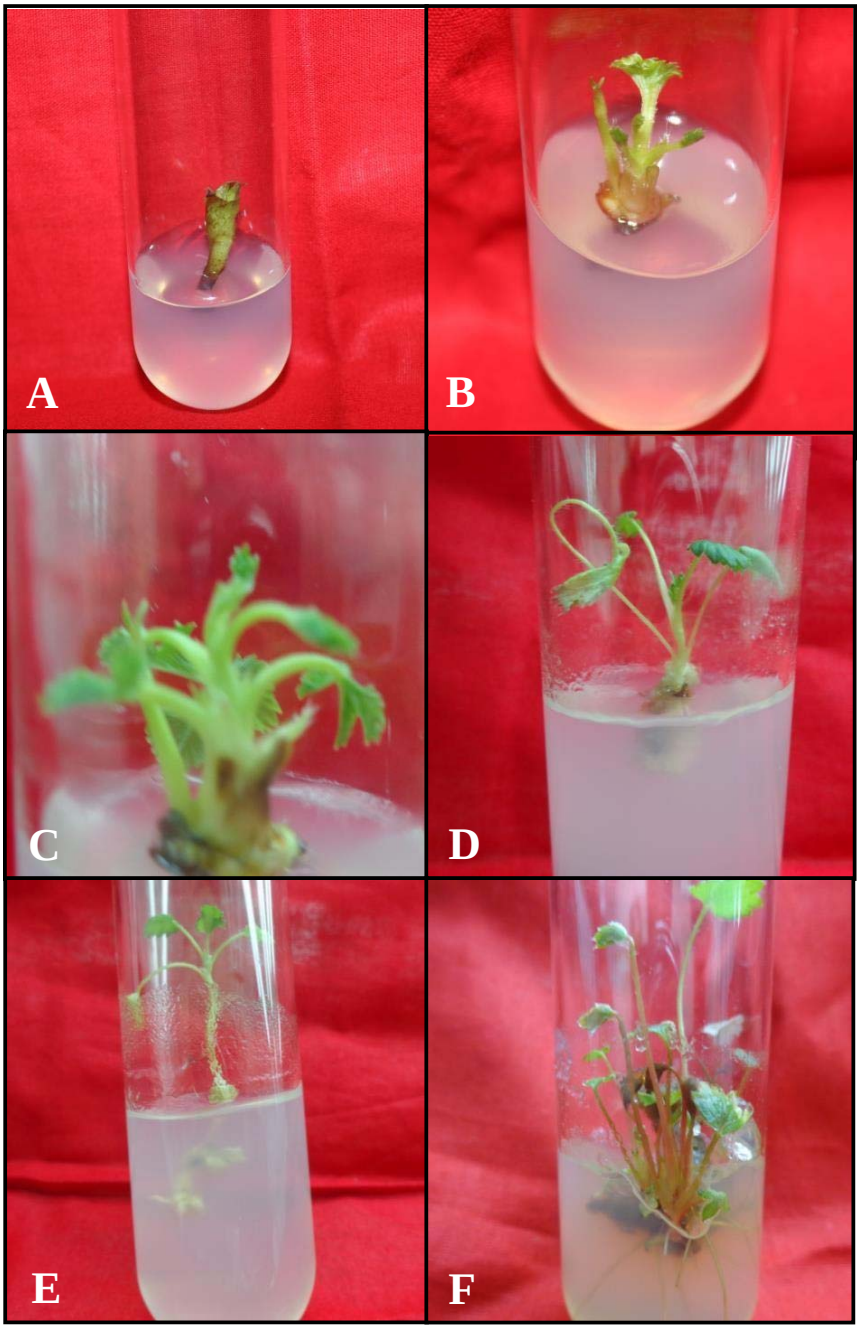

Figure 1: *In vitro* propagation of Strawberry plantlets from runner tips explants; A. Runner tips used as explants. B. Shoot proliferation on MS supplemented with 2.0 mgl^{-1} BAP after 3 weeks of culture. C. Proliferated of multiple shoots on MS+2.0 mgl^{-1} BAP+0.5 mgl^{-1} KIN+0.5 mgl^{-1} GA_3 after 4 weeks of culture. D and E. Establishment of shoot after 7 weeks of culture. E. Rooted shoots on 1.0 mg/l IBA after 7-8 weeks of culture.

4. Conclusion

From our investigation, it can be concluded that enormous strawberry plantlets even in off season can be produced from an explant within a short period of time by using our established protocol of micropropagation. Also, our investigation, provides a reliable and economical method of maintaining pathogen free plants. Although, very recently strawberry plants have been introduced in Bangladesh but within a short time this fruit has become very popular to whole country. There for requirements of healthy, disease free and high productive and cost-effective plantlets are increasing day by day. So, applying our above described micropropagation protocol we can easily fulfill our demand of strawberry plantlets for growing in commercial cultivation purposes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethical issues:

There are no ethical issues in the whole research work and in this manuscript. Our findings and this protocol is out of conflict.

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