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MICROPROPAGATION OF STRAWBERRY (*Fragaria x ananassa* Duch.)

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ABSTRACT

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The experiment was conducted from March 02 to September 25, 2010 at Plant Tissue Culture Laboratory of Genetic Engineering and Biotechnology Department, Jessore Science and Technology University, Jessore, Bangladesh to clarify the effective concentration of growth regulators for micropropagation of strawberry. Response of medial additives like adenine sulfate (120mgL^{-1}) and coconut water (150 mgL^{-1}) were also investigated. The concentration of cytokinin-auxin was ranged from 1.0-3.0 mgL^{-1} and 0.1-0.3 mgL^{-1} respectively. Addition of adenine sulfate, gibberellic acid and coconut water was insignificant than combine concentration of cytokinin and auxin regarding usable shoots. But shoot length was the highest (2.4 cm) which is significantly different than that of gibberellic acid and coconut water. Maximum number of nods was also observed in the presence of adenine sulfate. For rooting the shoots were transferred to MS medium with four different combination of IBA (0.1, 0.2, 0.5, 1.0) mgL^{-1} and IAA (0.1, 0.2, 0.5, 1.0) mgL^{-1} individually. Low concentration of IAA produces maximum number of roots which was 16.5 and significantly different than that IBA or IBA IAA combination. But the highest root length was found in the combined treatment of IBA and IAA (0.1+0.1) mgL^{-1}.

Keywords: *micropropagation, strawberry, adenine sulfate, MS medium*

INTRODUCTION

Strawberry (*Fragaria x ananassa* Duch.) is a natural hybrid of *Fragaria chiloensis* L.P. Mill. and *Fragaria virginiana* Duch. Strawberries (food of youth) have traditionally been a popular delicious fruit for its flavor, taste, fresh use, freezing and processing and are highly valued as dessert fruit. *Fragaria x ananassa* is one of the most fascinating fruits of the world, which is a rich source of vitamins and minerals and has fabulous flavor and tantalizing aroma. It contains numerous important dietary components and is a rich source of vitamin C (Driscoll's 2004). It also contains significant levels of ellagic acid, which is thought to be an anti-carcinogenic (ICAR news 2005). However, Strawberries have introduced in Bangladesh recently and getting popularity and cultivate in very small scale. Different regions of Bangladesh are suitable to cultivate strawberry in terms of photoperiod, temperature and humidity. It is the time to research for improving strawberries varieties which are cultivated in our environment. Multiplications by a vegetative method have different obstacle (Boxhus 1974). Karhu and Hakala (2007) found that strawberries can be propagated in vitro condition by tissue culture methods where as micropropagation is a very useful technique of improvement of plant. They observed that micropropagated strawberry plants were comparatively better in different characters (crown size, number of runners, flowering time and yield of berries) than conventionally propagated runner plants. Keiho *et al.* 2003 studied the effect of cytokinin at different concentration on strawberry plantlets that's micropropagated from axillary buds and found the rate of the mutation of leaf characteristics increased concomitant with micropropagation period for plantlets of *Fragaria x Ananassa* Duch. *cv*. Hokowase micropropagated strawberries on the MS medium with BA 0.5 mg/L and KIN 1.0 mg/L. But leaves of the micropropagated plant were abundant in compared with plantlets propagated with runners, because plant height was low and the leaf area index was small. Moreover, there was a problem in quality that the yield was smaller than normal and the average weight of individual fruit was small. Toyonoka and Nyoho were being studied by MS medium with BA 0.125 mg/L and KIN 0.25 mg/L and stated that would be adequate for micropropagation by axillary buds because the propagation ratio is equivalent. In addition, when propagated at this concentration, 'Toyonoka' were acclimated without transplantation to the rooting medium, so labor reduction is possible. Thus, cytokinin adversely influenced micropropagated plantlets and the next generation of 'Hokowase' compared with 'Toyonoka' and 'Nyoho'. Considering above mentioned fact this study was conducted to find out the optimum concentration of cytokinins (BA Benzyladenine and Adenine) and auxins (IBA and NAA, Alphanaphthalene acetic acid) for high multiplication rate of strawberry, effects of additives (coconut water, adenine) on high frequency shoot proliferation, effects of gibberelic acid (GA_3) on the structure of the strawberry plant and response of different concentration and combination of auxins on root induction.

MATERIALS AND METHODS

The experiment was conducted at Plant Tissue Culture Laboratory of Genetic Engineering and Biotechnology Department, Jessore Science and Technology University, Jessore, Bangladesh. The nodal segments of mature strawberry plant were used as explant that's collected from Sathe Nursery, Jainagar, Ishardi, Pabna, Bangladesh.

Sterilization of plant material

At first, explants were washed under running tap water for 20-25 min. Then they were rinsed with sterilized distilled water. Finally, explants were subjected to incision into appropriate sizes (around 1-1.5 cm long), and then transferred to the laminar hood where the processed explants were sterilized in 0.1% $HgCl_2$ with 2 drops of tween 80 for five minutes followed by three rinsing with sterile distilled water for 4 minutes each. Afterwards explants were resized by scalpels and forceps. Trace of water remaining on the surface of the explants was soaked with sterilized filter paper. Thus surface sterilization was completed and the material was ready for inoculation on appropriate nutrient medium.

Media for Micropropagation

MS (Murashige & Skoog, 1962) basal media supplemented with different concentrations and combinations of growth regulator were prepared as a media for Micropropagation.

Media for shoot proliferation

For shoot proliferation, nodal segments of mature strawberry plant were taken. Node was cultured on MS basal medium supplemented along with the concentration of cytokinin, auxin, additives and GA_3 (Table 1). After mixing all stock solutions and growth regulators at appropriate volume, 3% sucrose was added. The pH of the medium was adjusted to 5.7-5.8 and then agar (0.7%) was added and dissolved. The media were dispensed in the 40×150 mm glass bottles in a volume of 20-25 ml. Each treatment consisted of 10 glass bottles. The media were sterilized by autoclaving at 121ºC for 20minutes. Culture media were dispensed in the glass bottles and sterilized as mentioned above. About 3-4 weeks period was required for shoot proliferation.

Media for root induction

For rooting, regenerated shoots were sub-cultured in solidified MS medium supplemented along with (Table 2) concentration of IBA or IAA and their combinations. After mixing all stock solutions and growth regulators at appropriate volume, 3% sucrose was added. The pH of the medium was adjusted to 5.7-5.8 and then agar (0.7%) was added and dissolved. The media were dispensed in the 40×150 mm glass bottles in a volume of 20-25 ml. Each treatment consisted of 10 glass bottles. The media were sterilized by autoclaving at 121ºC for 20minutes. Culture media were dispensed in the glass bottles and sterilized as mentioned above. About 3-4 weeks period was required for root induction.

Data collection and statistical analysis

Weekly Visual observation of culture was made and frequency of culture showing plantlet, shoot and root formation and multiplication was recorded. The data pertaining to shooting and rooting per culture were analyzed by using Duncan's new multiple range tests (Duncan 1955).

Transfer to the soil

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 sprayed with fungicide and planted to normal and sterilized soil in polybags. After 7 days the hardened plantlets were planted in soil.

RESULTS AND DISCUSSION

In this experiment, nodal segments of mature strawberry (*Fragaria* x *ananassa* Duch.) were inoculated on MS medium supplemented with combination of cytokinins (BA) and auxins (NAA or IBA) with or without Gibberelic acid (GA_3), Adenine and with or without additives like coconut water for morphogenic responses.

Effect of different concentrations and combinations of cytokinins, auxin, Gibberelic Acid (GA_3) and medial additives on shoot formation

A number of combinations of growth regulators were employed for shoot proliferation. Herein experiment different treatments of cytokinin (BA) ranging from 1.0-3.0 mg/L (1.0, 2.0, 3.0 mgL^{-1}) along with auxin (0.1, 0.2, 0.3, mgL^{-1}) were employed respectively. Data were collected after 4-6 weeks of inoculation. The highest number of usable shoot was observed at the concentration of T7 (1.0 mgL^{-1} BA with 0.1 mg/L NAA) (Fig2.A and Table 3) which was 13.4. The highest shoot length was observed at the concentration of T6 (2.0 mgL^{-1} BA with 0.2 mgL^{-1} NAA +120 mgL^{-1} Adenine sulfate) (Fig2.C and Table 3) which was 2.4 cm. Adenine were used in the treatments of T1 (1.0mgL^{-1} BA with 0.1mgL^{-1} NAA +120 mgL^{-1} Adenine sulfate +150 $ml.L^{-1}$ coconut water) (Fig2.B) shoot number was 11.3 and T6 (2.00mgL^{-1} BA with 0.2mgL^{-1} NAA +120 mg/L Adenine) (Fig2.C) shoot number was 9.2. The maximum length of shoot and shoot number was produced on the adenine containing medium. Adenine sulfate was used in the medium at the concentration of 120 mgL^{-1}. In this experiment after using adenine the explants were multiplied to form many shoots. High concentration of Adenine sulfate with low concentration of BA and NAA produce significant number of shoots and maximum length of shoots. The shoots look strong, healthier and vigorous (Fig1.C).

On the other hand other treatments of this experiment produce multiple shoots which were narrow, small and less healthy than shoots which were produced on adenine containing medium (Fig1.A and Fig1.B). The nodal segments of strawberry were cultured on basal medium supplemented with various combinations of cytokinins

and auxins. Of these, relatively high concentration of BA with low concentration of NAA was found to be the best for shoot regeneration (Islam *et al.* 2007). A high level of auxin to cytokinin is root promoting, where the opposite condition i.e., an increased concentration of cytokinin and low concentration of auxin (a medium in which the ratio of cytokinin to auxin is greater than 1 favors shoot bud induction (Maheshwari & Kumar, 2006). In shoot induction, effectiveness of cytokinin (BA) along with NAA or IBA was proved to be superior to the other concentrations for in vitro regeneration of sugarcane varieties viz. Isd-20, Isd-2/54 (Mamun *et al.* 2004).

Miers & Thoms, 2007 reported in their study cytokinins, viz. 2.22–17.74 mM N6-benzyladenine (BA), 2.32–19.58 mM 6-fufurylaminopurine (kinetin), 2.46–9.84 mM N6-2-isopentenyl adenine (2iP) were used alone or in combination with auxins to obtain the most suitable growth hormonal level for the proliferation of shoots in established explants. Both BA (2.22–4.44 mM) and 2iP (2.46–4.92 mM) with IBA (4.9 mM) had positive, but not significant effect on shoot formation with only single shoot per explant.

Table 1. Treatment employed for shoot regeneration of the experiment

Treatments	Growth regulators (BA) $mg.L^{-1}$	Growth regulators (NAA) $mg.L^{-1}$	Additives Coconut water $ml.L^{-1}$	Adenine sulfate $mg.L^{-1}$	Growth regulators (GA_3) $mg.L^{-1}$
01.	1.0	0.1	-	-	-
02.	2.0	0.2	-	120	-
03.	1.0	0.1	150	120	-
04.	1.0	0.1	150	-	1.0
05.	2.0	0.1	150	-	1.0
06.	2.0	0.2	150	-	1.0
07.	3.0	0.3	150	-	1.0

Table 2. Treatment employed for root induction of the experiment

Treatments	Growth regulators (IBA) $mg.L^{-1}$	Growth regulators (IAA) $mg.L^{-1}$
01.	0.1	-
02.	0.2	-
03.	0.5	-
04.	1.0	-
05.	-	0.1
06.	-	0.2
07.	-	0.5
08.	-	1.0
09.	0.1	0.1
10.	0.2	0.2
11.	0.5	0.5

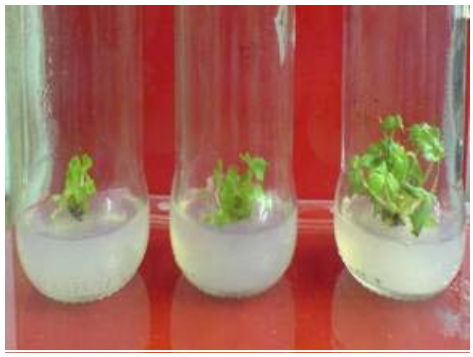
Table 3. Effect of growth regulators on shoot of Strawberry (*Fragaria* x *ananassa* Duch.). Data were taken 4-6 weeks after culture

Treatment	Growth regulators concentration ($mg.L^{-1}$)					No. of Shoots. Mean	Length (cm) of shoots. Mean
	BA	NAA	Adenine Sulfate	GA_3	Coconut water ($ml.L^{-1}$)		
T7	1.0	0.1	-	-	-	13.4^{a}	2.2^{ab}
T1	1.0	0.1	120	-	150	11.3^{ab}	1.4^{c}
T6	2.0	0.2	120	-	-	9.2^{abc}	2.4^{a}
T3	2.0	0.1	-	1.0	-	7.0^{bcd}	1.4^{c}
T2	1.0	0.1	-	1.0	150	5.9^{cd}	1.5^{c}
T4	2.0	0.2	-	1.0	150	4.3^{d}	1.8^{bc}
T5	3.0	0.3	-	1.0	150	3.6^{d}	1.5^{c}

Table 3. Shows that the combined treatment of BA and NAA were very much effective for shoot formation of Strawberry (*Fragaria* x *ananassa* Duch.). As the combined concentration of BA and NAA (1.0 + 0.1) mgL^{-1} shows the best results of shoot formation. The average length of shoots was high in T6 treatment. After four-six weeks these shoots were then transferred to rooting medium.

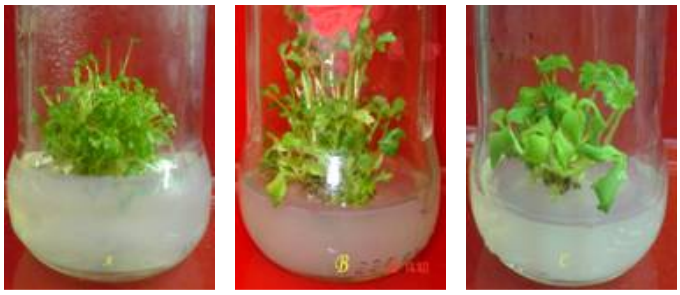
Table 4. Effect of growth regulators on root proliferation of Strawberry (*Fragaria* x *ananassa* Duch.). Data were taken 4 weeks after culture

Treatment	Growth regulators concentration ($mg.L^{-1}$)		No. of Roots. Mean	Length (cm) of Roots. Mean
	IBA	IAA		
T5	-	0.1	16.5a	3.4a
T3	0.5	-	16.3a	2.6bc
T4	1.0	-	12.3ab	1.7d
T8	-	1.0	9.6bc	3.2ab
T2	0.2	-	7.9bcd	1.7d
T10	0.2	0.2	7.3bcd	3.4a
T11	0.5	0.5	6.9cd	2.0cd
T1	0.1	-	6.7cd	2.3cd
T9	0.1	0.1	5.5cd	3.6a
T6	-	0.2	4.8cd	3.3ab
T7	-	0.5	3.5^{d}	3.2^{ab}

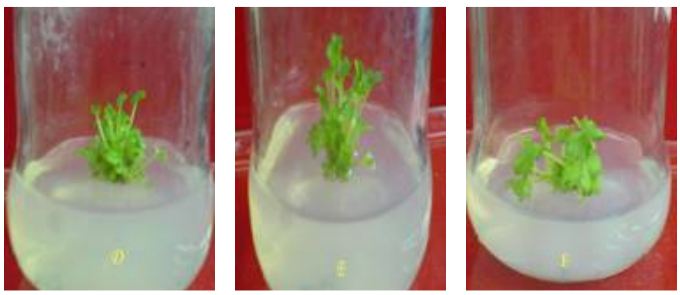


A **B** **C**

Figure 1. (A) BA+NAA, (B) BA+NAA+GA_3 and (C) BA+NAA+Adenine sulfa

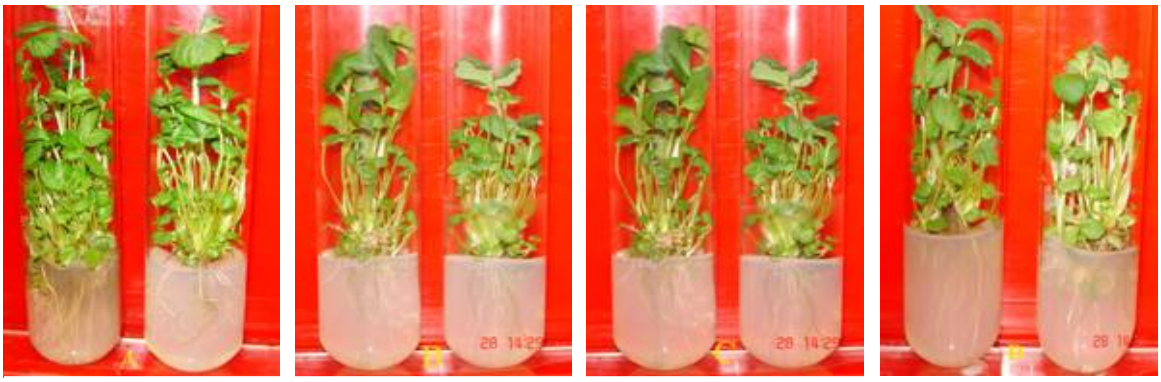


A **B** **C**



D **E** **F**

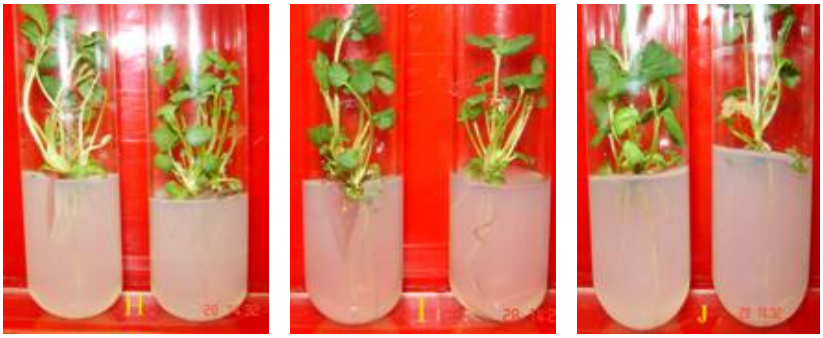
Figure 2. (A) 1.0 mgL^{-1}BA+0.1 mgL^{-1}NAA, (B) 1.0 mgL^{-1}BA+0.1 mgL^{-1}NAA+120 mgL^{-1}Adenine+150 mlL^{-1} CW, (C) 2.0 mgL^{-1}BA+0.2 mgL^{-1}NAA+120 mgL^{-1} Adenine, (D) 2.0 mgL^{-1}BA+0.1 mgL^{-1}NAA+1 GA_3 mgL^{-1}, (E) 1.0 mgL^{-1}BA+0.1 mgL^{-1}NAA+1 GA_3 mgL^{-1}+150 mlL^{-1} CW, (F) 2.0 mgL^{-1}BA+0.2 mgL^{-1}NAA+1 GA_3 mgL^{-1}+150 mlL^{-1} CW



A **B** **C** **D**

Figure 3. (A) IAA 0.1 mgL^{-1}, (B) IBA 0.5 mgL^{-1}, (C) IBA 1.0 mgL^{-1}, (D) IAA 1.0 mgL^{-1}

E **F** **G**



H **I** **J**

Figure 3. (E) IBA 0.2 mgL^{-1}, (F) (IBA 0.2+IAA 0.2) mgL^{-1}, (G) (IBA 0.5+IAA 0.5) mgL^{-1}, (H) IBA 0.1 mgL^{-1}, (I) (IBA 0.1+IAA 0.1) mgL^{-1} and (J) IAA 0.2 mgL^{-1}

Plate-4. Regenerated plantlets of strawberry in plastic pots after 7 days of transplantation

Mathur *et al.* (2008) reported in their study best rooting response was observed on MS basal media containing 40 or 80 mg/L adenine sulphate. Root emergence (2-3 per shoot) became evident on this medium within 3-6 days of transfer. Higher level of adenine sulphate also favoured better root elongation. When IBA (1.5 mg/L) was also supplemented in adenine sulphate containing medium the rooting response was delayed by 10-12 days but number of roots per shoot was significantly enhanced. The elongation of these roots was, however, very slow in comparison to those growing in presence of adenine sulphate alone. Addition of BAP in the rooting medium was generally found inhibitory for rooting. The rooted plantlets grown on 40-80 mg/l adenine sulphate containing medium also exhibited the formation of typical musli fingers towards the end of the culture passage.

CONCLUSION

Plant tissue culture is the primary branch of plant biotechnology. It offers the recovery of disease resistant, salinity resistant, stress tolerance of high yielding varieties. Nevertheless, to elucidate in vitro propagation of Strawberry (*Fragaria x ananassa* Duch.) with a view to determine the most suitable medium compositions for the best response, standardization of growth regulators for maximum germination, shoot proliferation and selection of suitable auxin concentrations for root induction in vitro from nodal segments. BA used as cytokinin and NAA used as auxin in seven-treatment combination. The concentration of cytokinin/auxin was ranged from 1.0-3.0 mg/L and 0.1-0.3 respectively. GA_3 1.0 mg/L, adenine 120.0 mg/L additives like coconut water 150 $ml.L^{-1}$ was also used with the combination of cytokinin-auxin. Addition of adenine sulfate, gibberellic acid and coconut water was insignificant than combine concentration of cytokinin and auxin regarding usable shoots. All the treatments produce usable shoots. The highest number of usable shoot was observed at the concentration of 1.0 mg/L BA with 0.1mg/L NAA. The highest shoot length was observed at the concentration of 2.0 mg/L BA + 0.2 mg/L NAA +120 mg/L adenine. Shoot length was the highest (2.4 cm) which is significantly different than that of gibberellic acid and coconut water. In this experiment, as the concentration of growth regulators decreased shoot formation increased. After four to six weeks the well developed in-vitro shoots were sub-cultured on MS medium supplemented with the same concentration of growth regulators. After four to six weeks of sub-culture, these were transferred to the rooting medium. For rooting the shoots were transferred to MS medium with four different concentration of IBA (0.1, 0.2, 0.5, 1.0), and IAA (0.1, 0.2, 0.5, 1.0 mg/L) individually. The combination of IBA and IAA were also used on the concentration of 0.1, 0.2 and 0.5 mg/L. After four weeks the highest numbers of roots were observed in IAA 0.1 mg/L.

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