

***In vitro* Regeneration of Strawberry Plant from Leaf Explants via Callus Induction**

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Abstract

The present investigation was carried out to develop an efficient *in vitro* regeneration protocol for strawberry plant using leaf explants. The highest percentage of callus induction was obtained on MS medium supplemented with 2.0 mg/l BAP and 0.5mg/l IBA. The highest numbers of shoots were regenerated from the callus on MS medium supplemented with 2.0 mg/l BAP and 1.5mg/l IBA. In case of root induction, MS medium supplemented with 1 mg/l IBA was found to be most effective. Following the development of roots, the *in vitro* regenerated plantlets were successfully transplanted into soil.

Introduction

In vitro plant regeneration methods are significantly used in modern plant development programs for introducing new traits, to grow appropriate cultivars in the minimum procedure, to produce different types of disease free plants and also use in genetic transformation protocols (Zulkarnain et al. 2015). Plant biotechnology and tissue culture techniques facilitate the production of genetically engineered plants, soma clonal variants, and the rapid multiplication of difficult-to-propagate species (Zakaria et al. 2014). The propagation of high quality strawberry plants has special importance as importing mother plants are not economically logical. Besides growing demands of strawberry as fresh fruit or processed product, application of plant tissue culture and genetic engineering in strawberry cultivation is of special value to obtain improved or desirable traits. In this connection, establishment of an efficient regeneration system in strawberry, is considered as a first and a prerequisite step for both approaches.

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Strawberry has a great economic importance, due to the high-quality characteristics of the fruits and their nutritional properties, highly appreciated by consumers, especially for the presence of polyphenols, ellagic acid, anthocyanins, and vitamin C. The demand for strawberries is increasing across the country, especially in urban areas. It was about a decade before Bangladeshi farmers cultivate strawberries. The climate in the northern region of Bangladesh provides the optimum conditions for strawberry cultivation during November to December (Yaseen et al. 2016). In our country, importing mother plants is expensive and healthy stocks used for propagation through conventional methods are not always available.

Large quantity of plants can be produced within a year from a few mother plants by deploying tissue culture techniques (Boxus et al. 1983; Nehra, et al. 1994). This technique is also useful for the introduction of new cultivars. Propagation of strawberry by runners is not always suitable because of their sensitivity to pathogens (Moradi et al. 2011). Therefore, micropropagation is a unique technique to efficiently generate a large number of disease free plants as well as to use for genetic transformation. To date, adventitious buds and shoots regeneration has been previously reported from different explants such as leaves (Chung et al. 2021, Zakaria et al. 2014, Omar et al. 2013, Husaini et al. 2011, Mohamed et al. 2007, Debnath et al. 2006 and Hanhineva et al., 2005) node (Biswas et al. 2010 and Moradi et al. 2011), petioles (Husaini et al. 2011) (Omar et al. 2013) (Suta et al. 2010) (Passey et al. 2003) (Debnath et al. 2006) stipules (Ghasemi et al. 2015) (Sönmez et al. 2012), runner tissue (El Kichaoui et al. 2014) (Ashrafuzzaman et al. 2013) (Biswas et al. 2008), root (Youssef et al. 2016) (Passey et al. 2003) and stem tissue (Ramesh et al. 2006). In the present experiment, a simple, effective and reproducible protocol has been developed to propagate strawberry from leaf explants through callus induction.

Materials and Methods

The leaf explants were collected from the field grown strawberry plants which were maintained in the experimental field of Bangladesh Council of Scientific and Industrial Research (BCSIR). The leaf explants were subjected to different sterilization regimes, such as placed in normal detergent solution, spray with alcohol, placed in $HgCl_2$, washed with Tween20 for varying time duration. After treated with each regime, explants were cultured on MS medium supplemented with different combinations and concentrations of hormone; these included BAP, IBA and Kn to induce callus and consequent development of shoots. Sub-cultured was carried out regularly to fresh medium at an interval of 15- 20 days for further multiplication. All *in vitro* grown cultures were maintained under illumination of a 16 hrs photoperiod at $25 \pm 2^\circ C$. Fully elongated shoots were treated with three different auxins, namely, IBA, IAA and NAA used for induction of roots. Following sufficient development of roots, plantlets were taken out and transplanted into small plastic pots and kept in room temperature for 4-5 days. Finally plants were transferred to field.

Results and Discussion

Surface sterilization is an initial and a prerequisite step towards the establishment of an *in vitro* regeneration protocol. Previous study showed that 3-15% losses occurred due to contamination in the majority of commercial and scientific plant tissue culture laboratories, caused by bacterial, fungal and yeast contaminants (FAO, 2004). Tissue culture contamination ultimately results in an abundant waste of effort, time and material. The sterilization procedure described in this paper was effective in reducing both bacterial and fungal contamination of leaf explants of strawberry. Jan et al. (2013), demonstrated that the effect of different sterilants on runner tips and nodal segment explants of strawberry. They showed that maximum aseptic cultures with lower surviving percentage were obtained from both explants when treated with 1.5% sodium hypochlorite for 20 min + ethyl alcohol 70% for 30s, while 0.1% HgCl₂ treatment for 4 min resulted in less percentage of aseptic cultures but higher percentage of surviving explants. In our study, high percentage of aseptic culture and surviving rate obtained by treating the explants with detergent for 10 min + tween20 for 5 min + 70% alcohol for 30s + 0.1% HgCl₂ for 2 min. The lowest necrotic percentage was also obtained in this regime (Table1).

Table1. Influence of different sterilants on aseptic cultures, explant survival and necrotic culture in strawberry leaf.

Sterilants and their combination	Time duration in different sterilants condition	Aseptic cultures (%)	Necrotic cultures (%)	Explant survival (%)
Detergent + 70% Alcohol + 0.1% HgCl ₂	10min+30sec+1min	40	-	-
Detergent + 70% Alcohol + 0.1% HgCl ₂	10min+30sec+2min	43	-	-
Detergent + 70% Alcohol + 0.1% HgCl ₂	10min+30sec+2.30min	76	64.28	66
Detergent + Tween20 + 70% Alcohol + 0.1% HgCl ₂	10min+5min+30sec+1min	88	16.67	83.33
Detergent + Tween20 + 70% Alcohol + 0.1% HgCl₂	10min+5min+30sec+2min	95	10.5	90
Detergent + Tween20 + 70% Alcohol + 0.1% HgCl ₂	10min+5min+30sec+2.30min	97.5	50	75

Regulation of auxin and cytokinin balance has long been recognized as a key factor in the control of cell division and organogenesis in most dicot plants and this might be the reason for the complete absence of organogenesis and scarce callus formation of strawberry leaf explants on different basal media in absence or improper balance of plant growth regulators. In this study, obtained callus were green and nodular in most cases which is in conformity with the reports of Youssef et al. (2006), while on media containing BA and Kn, the calli were creamish brown and friable.

George (1993) explained that *in vitro* regeneration and developments of plants is regulated by interaction and balance between the growth hormone supplied in the medium. Adventitious shoot and root formation often required a balance between auxin and cytokinin (Kumar et al. 2011). Taiz et al. (1991) demonstrated that high levels of cytokinin compare to auxin led to the formation of shoots, whereas high levels of auxin stimulated the formation of roots, which is in close conformity with our findings.

Most of the leaf explants were produced callus when they were cultured on MS medium supplemented with BAP and IBA. The margin of the leaf became curl either away or adjacent to the medium. Creamish small calli were formed from the edge of the leaf (Fig. 1A,B,C). Leaf explant margin curling from the medium is in harmony with the results reported by Nehra et al. (1989). Husaini and Srivastava (2011) reported that the edges of leaf discs can produced pink calli on TDZ supplemented MS media.

In our study, after 15-20days of culture, best response towards callus formation (77.14 %) was observed when leaflet explants were cultured on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IBA(Fig. 1A). Similar results were reported by Husaini and Srivastava (2011). Highest shoot regeneration response was observed from the callus on MS media with 2.0 mg/l BAP and 1.5 mg/l IBA (Fig. 1D). MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l IBA also showed the good response both in callus initiation and shoots formation.

Table 2. Effect of different concentration of BAP, IBA and Kn towards regeneration of shoots from leaf explants of strawberry.

Hormonal supplements			% of responsive explants towards callus formation	Days required for initiation of callus	% of callus showed shoot initiation	Mean no. of shoots/ explant after 3 months of culture	Mean length (cm)
BAP	IBA	Kn					
1	0.5	-	31.40	22-23	-	-	-
1	1.0	-	34.28	22-23	-	-	-
1	1.5	-	57.14	15-20	35	4.28	0.32
1	-	1	56.67	15-20	52.63	4.11	0.43
1	-	1.5	63.33	15-20	58.82	5.26	0.52
2	0.5	-	77.14	15-20	77.78	6.67	0.40
2	1.0	-	74.28	15-20	80.76	7.14	0.57
2	1.5	-	71.42	15-20	88	8.80	0.78
2	-	1	73.33	15-17	54.54	4.09	0.53
2	-	1.5	66.7	15-17	65	5.25	0.61

Some previous observation also revealed the effective response of BAP and IBA combination on strawberry regeneration from leaf (Barceló et al. 1998), nodal segments (Moradi et al. 2011) and stipules (Rugini et al. 1992).

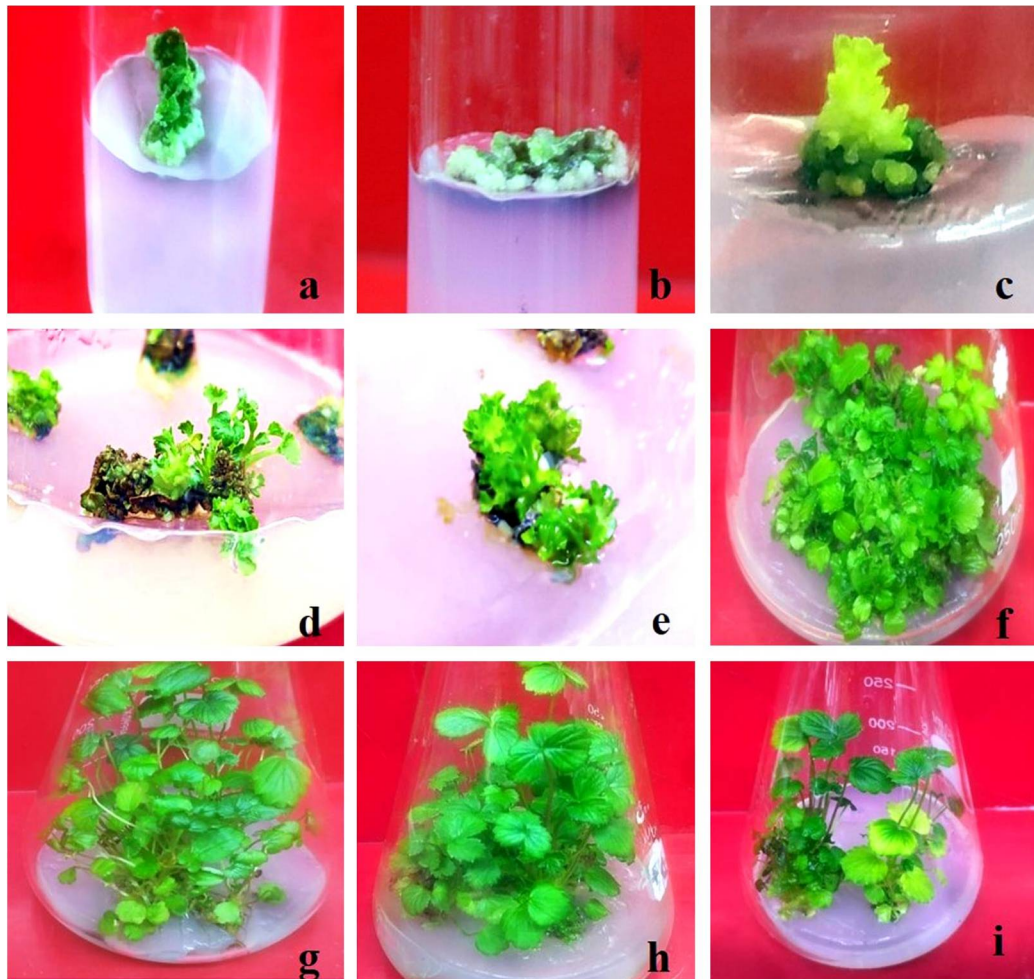


Fig. 1a-i: Different stages of shoot regeneration from leaf explant in strawberry. (a) Callus induction from leaflet after 15-20 days of culture on MS with 2.0 mg/l BAP and 0.5 mg/l IBA. (b) Same as fig. a but on MS medium with 2.0 mg/l BAP and 1.5 mg/l IBA. (c) Direct shoot initiation on MS medium with 3.0 mg/l BAP and 0.5 mg/l IBA. (d) Multiple shoots formation from the callus on MS with 2.0 mg/l BAP and 1.5 mg/l IBA. (e) Formation of multiple shoots on MS medium with 2.0 mg/l BAP and 1.5 mg/l Kn. (f) Compact numerous stunted growth of multiple shoots on MS medium with 2.0 mg/l BAP and 1.5 mg/l IBA after 60 days of culture. (g-h) Elongated and developed multiple shoots on MS with 0.05 mg/l BAP and 0.05 mg/l IBA supplemented media after 50-60 days. (i) Elongated shoots on hormone free MS media after 50-60 days of culture.

Among the various combinations tested, maximum response towards shoot regeneration was observed on MS medium supplemented with 2.0 mg/l BAP with 1.5 mg/l Kn (Fig. 1C,E). In the present experiment, it was noticed that percentage of shoot formation was low in all the combinations of BAP and Kn compared to BAP and IBA (Table-2). Previously shoot formation by using different concentration and combinations of BAP and Kn from runner tips (Roy et al. 2018), nodes (Sakila et al. 2007) and leaf explants (Zakaria et al. 2014) were reported. Shoots were induced in clusters (Fig 1f), and

were transferred to three different media for elongation. MS media containing 0.5 mg/l BAP and 0.05 mg/l IBA showed highest shoot elongation efficiency (Fig. 1g, 1h). A study showed that media containing 1.5 mg/ l BAP and 0.5 mg/l Kn was the best for elongation of shoots in both Festival and Sweet Charlie, while 1.5 mg/l BAP and 0.1 mg/l Kn showed the best shoot elongation in Florida (Zakaria et al. 2014).

Table 3. Effect of different growth regulators on elongation of regenerated shoots.

Combination of media	No. of compact shoots inoculated for elongation	% of elongated shoots	Mean length (cm) of per shoots after 45 days
MS without hormone	50	87	2.3
MS+ 0.5mg/l BAP	50	60	1.1
MS+0.5mg/l BAP+0.05mg/l IBA	50	88	2.5



Fig. 2: Formation of roots and transplantation of regenerated plantlets of strawberry in soil. (a) Formation of roots from regenerated shoots of strawberry on MS within 1.0 mg/l IBA. (b) Formation of roots on MS with 1.0 mg/l IAA, (c) The regenerated plantlets of strawberry transferred to coco dust containing small pots, (d) Young regenerated plant with flower in large plastic pot. (e) *In vitro* raised plantlets of strawberry growing in the field.

Table 4. Effect of different growth regulators on induction of roots from regenerated shoots.

Name of Hormone	Conc. of hormone (mg/l)	No. of shoot inoculated for root induction	% of shoots forming roots	Days required to initiate roots	Mean no. of roots/ shoot
IBA	0.2	20	67	20-22	5.5
	0.5		74	15-20	7.8
	1.0		91	10-15	8.8
IAA	0.2	20	56	20-25	5.8
	0.5		69	15-20	7.7
	1.0		88	15-20	7.8
NAA	0.2	20	-	-	-
	0.5		58	22-25	4.5
	1.0		61	22-25	5.0

Out of different concentrations of IBA, NAA and IAA tested, 1.0 mg/l IBA (Fig. 2A) and 1.0 mg/l IAA (Fig. 2b) both proved to be most suitable for root induction within 15-20 days. Similar effect also found in previous study (Zakaria et al. 2014, Husaini and Srivastava 2011, Sakila et al. 2007, Akter et al. 2013). On the other hand, Zakaria et al. reported MS medium containing 1.5 mg/l NAA revealed the highest percentage of forming root. Ko et al. reported that roots were successfully formed at low concentrations of NAA (0.02 mg/l). Following sufficient development of roots, plantlets of strawberry were successfully transplanted into two types small plastic pots one contain a mixer of soil and cow dung and another contain coco dust (Fig. 2C) alone. Findings showed that, coco dust alone resulted in high survival rate than the mixed one. For their further growth and establishment, the survived plantlets were transferred to larger pots (Fig. 2D) and finally in the field (Fig. 2E).

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