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Production of quality planting material of Chandler strawberry by *in vitro* regeneration

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ABSTRACT

In order to explore the *in vitro* regeneration potential of strawberry, experiments were conducted on MS medium supplemented with different concentrations of cytokinins and auxins alone and in combination for establishment of aseptic cultures, shoot regeneration and multiplication, rooting and acclimatization of plantlets. Two type of explants viz., runner tip and nodal segment were used for the investigation. Establishment of aseptic cultures is a pre requisite for successful culture. Among several sterilizing agents ethanol (70%) for 30 sec followed by $HgCl_2$ (0.1 %) for 2 min was found to be the best treatment resulting in least contamination and maximum survival (53.3 ± 1.08 percent in runner tip and 53.1 ± 1.08 percent in nodal segments). Phenol exudation was the major problem during establishment which caused death of most of the explants. In our experiment, explants were inoculated in MS medium supplemented with ascorbic acid 200 mg per liter to control browning. Addition of BAP alone or in combination with IAA was found essential during shoot proliferation and multiplication. Among all the treatments, 3.0 mg/L BAP + 0.5 mg/L IAA was found most effective with regard to minimum number of days (16.1 ± 0.29) required for shoot induction and maximum number of shoots (12.8 ± 0.52). Half strength MS media with 1.5 mg/L IAA was found superior over other concentrations of IAA, IBA and NAA for various rooting parameters. During acclimatization, maximum percentage survival (45.2) was recorded when coco peat alone was used as primary hardening after 20 days and combination of coco peat with FYM (1:1 v/v) recorded maximum percent survival (65.0) after 30 days in secondary hardening.

Key words: *Fragaria* × *ananassa*, micropropagation, BAP, ascorbic acid, tissue culture.

INTRODUCTION

The cultivated strawberry (*Fragaria* × *ananassa* Duch.), a perennial, dicotyledonous low-growing herb belonging to family Rosaceae is produced in about 71 countries all over the world (Bhat *et al.,* 3). There are about 20 recognized species of strawberries having basic chromosome number 7 and cultivated strawberry is an octo-ploid (2n = 8x = 56). Strawberry fruits are rich source of vitamin C, B1, B2, protein, calcium, potassium, copper and iron (Anonymous, 1) and being rich in bioactive phyto-chemicals especially phenolic compounds with high antioxidant capacity are considered to be highly nutritious (Hannum, 9). It contains relatively high quantities of ellagic acid, which has a wide range of biological activity (Ashrafuzzaman, 2). The potential commercial value makes it highly desirable to develop methods for rapid, efficient and large scale multiplication of strawberry. It is usually propagated vegetatively using runners to obtain true-to-type plants. But the availability of large number of disease free planting materials is still a big challenge especially in tropical and subtropical areas where summer temperatures are very extreme and maintaining of planting material becomes very difficult. Micropropagation is considered as efficient method for implementing strawberry development as importing mother plants is not economically feasible for the farmers. *In vitro* propagation technique provides an alternative and novel approach of enhancing the production of planting materials, including virus-free plants for large-scale planting. Micro propagated strawberry plants are comparatively better in characteristics such as crown size, number of runners, flowering time and yield of berries than conventionally propagated runner plants (Kadhimi *et al*., 10). The main objective of this study was to standardize sterilization procedure and observe the effect of various growth regulators viz. IAA, NAA, BAP and IBA in different combinations on shooting and rooting of *in vitro* cultured nodal segment and runner tip explants.

MATERIALS AND METHODS

The experiment was conducted at Plant Tissue Culture Laboratory, Bihar Agricultural University (BAU), Sabour. Explants (runner tips and nodal segments) were collected from strawberry plants of var. Chandler growing at Horticulture garden, BAU Sabour. The explants were first washed thoroughly

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under tap water for half an hour followed by washing in solution containing 2-3 drops detergent (Tween 20) and 1-2 ml disinfectant liquid for about 10 minutes and subsequent washing with distilled water for 3-4 times. Furthermore, the washed nodal segment and leaf explant were dipped in 0.2% Bavistin solution for 30 minutes and again washed 3-4 times with distilled water to control the fungal contamination. Explants taken from field-grown plants are generally difficult to establish in *in vitro* conditions due to high degree of contamination and thus it is necessary to first standardize sterilizing procedure. For this, the washed explants were surface sterilized in laminar air flow with sterilizing agents viz., Mercuric Chloride (0.1%) and Sodium Hypochlorite (2%) each with four different time durations (1, 2, 3 and 4 minutes). Ethanol (70% for 30 seconds) followed by Mercuric Chloride (0.1%) was also used for time duration of 1, 2, 3 and 4 minutes. The treatments given were: SA0- Control, SA1-$HgCl_2$ (0.1 %) for 1 min, SA2-$HgCl_2$ (0.1 %) for 2 min, SA3- $HgCl_2$ (0.1 %) for 3 min, SA4-$HgCl_2$ (0.1 %) for 4 min, SA5- NaOCl (2 %) for 1 min, SA6- NaOCl (2 %) for 2 min, SA7- NaOCl (2 %) for 3 min, SA8- NaOCl (2 %) for 4 min, SA9- Ethanol (70%) for 30 sec + $HgCl_2$ (0.1 %) for 1 min, SA10-Ethanol (70%) for 30 sec + $HgCl_2$ (0.1 %) for 2 min, SA11- Ethanol (70%) for 30 sec + HgCl2 (0.1 %) for 3 min, SA12- Ethanol (70%) for 30 sec + HgCl2 (0.1 %) for 4 min. The explants were then washed 5-6 times with sterilized distilled water to remove traces of sterilizing agent.

To control phenolic exudation, the explants after surface sterilization procedure, were cultured in MS media containing ascorbic acid 200 mg/L (Mir *et al*., 11). Regenerated shoots were then taken for further investigation. The proliferated shoots were cultured in Murashige and Skoog media (Murashige and Skoog, 12) added with 3% sucrose and 0.8% agar for multiplication. The media was supplemented with various concentrations of BAP alone (1.5, 3.0 and 4.5 mg/litre) and in combination with IAA (0.5 and 1.0 mg/litre). pH was adjusted to 5.7 and the medium was autoclaved for 15 min at 121 degree C and 15 psi pressure. The inoculated cultures were kept at 25 ± 2 degree C in an air condition culture room with a light intensity of 2000-3000 lux by cool white fluorescent tubes. The light/dark cycles of photoperiod were maintained as 16/8 hours daily. Sub culturing was done in 4 weeks interval and was restricted up to seven cycles to avoid any kind of somaclonal variation. Multiplied shoots having shoot length of more than 5 cm were transferred to rooting media comprising of half strength MS media supplemented with different concentrations of IAA, IBA, and NAA each at 0.5, 1.5 and 3.0 mg per litre. The rooted plantlets were removed from the medium and their roots were gently washed with tap water to remove any adhering media. These regenerated plantlets were then transferred to pro trays containing various potting mixtures (cocopeat, sterilized sand and soil in various combinations) for primary hardening. Five hundred plantlets were taken in each substrate. After 20 days of primary hardening these plantlets were again transferred to different artificial substrates viz soil, cocopeat and FYM in different ratios for secondary hardening. For this 100 plantlets were taken in each treatment. The survival was recorded after 30 days of secondary hardening.

RESULTS AND DISCUSSION

Establishment of aseptic cultures is a pre requisite for successful culture. The plants when grown under field conditions often get contaminated with lot of soil and air borne pathogens. Therefore a thorough and effective sterilization procedure of the explants is necessary before culturing. All the cultures were contaminated in control when no sterilization treatment was given. Although increasing exposure time to the sterilants ($HgCl_2$ (0.1%), NaOCl (2%) and ethanol (70% for 30 seconds) + $HgCl_2$ (0.1%)) reduced contamination, the survival percent reduced after certain duration. Maximum survival of 53.3 ± 1.08 percent and 53.1 ± 1.08 percent in runner tip and nodal segment was recorded in treatment S10 where explants were treated with Ethanol (70%) for 30 sec + $HgCl_2$ (0.1 %) for 2 min (Fig. 1 and 2). Exposure of explants to $HgCl_2$ for longer durations damage and kills the plant tissue. So exposure at lower concentration for less time duration is recommended by many workers (Parveen *et al*., 13).

For shoot multiplication, different concentrations of BAP ranging from 1.5-4.5 mg/L (1.5, 3.0, 4.5 mg/L) singly as well as in combination with IAA (0.5 and 1.0 mg/L) were used, respectively. The explants inoculated in MS media supplemented with cytokinin (BAP) or in combination with auxin (IAA) responded well in general with respect to different shooting parameters. On the other hand control treatment (MS Media without having any PGR) failed to compete with other treatments and resulted in minimum explants regeneration (36.5 ± 0.96 percent), minimum number of regenerated shoots (2.1 ± 0.22) and leaves (3.0 ± 0.09), shorter length of regenerated shoots (2.3 ± 0.09 cm) requiring longest duration for regeneration(23.2 ± 1.06 days). The regeneration percentage of explants increased significantly with the increasing concentration of BAP (1.5mg/L to 4.5mg/L) giving regeneration of 56.9 ± 0.25 percent at 4.5 mg/L. It was also noted that IAA had a supplementary effect with BAP leading

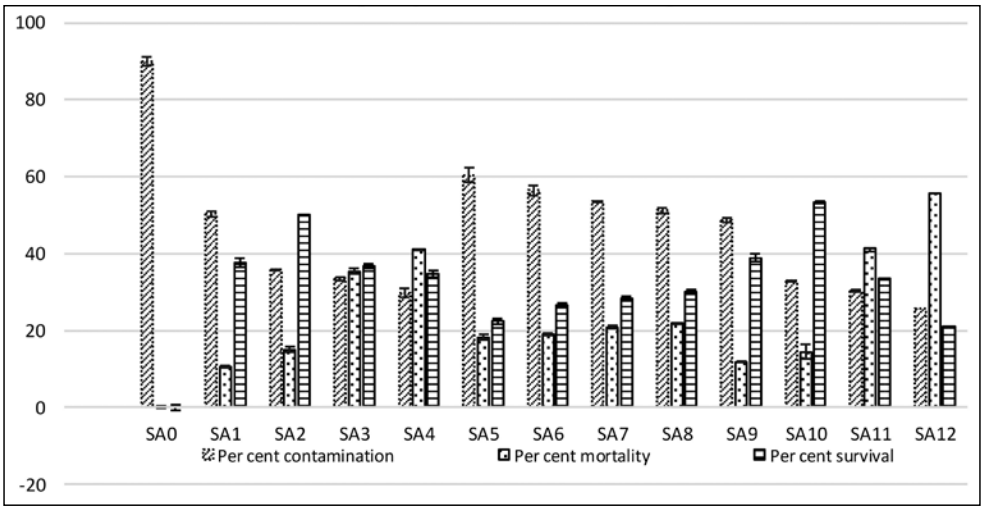

Fig. 1. Effect of different treatment duration of sterilizing agents on runner tip explants of Chandler strawberry.

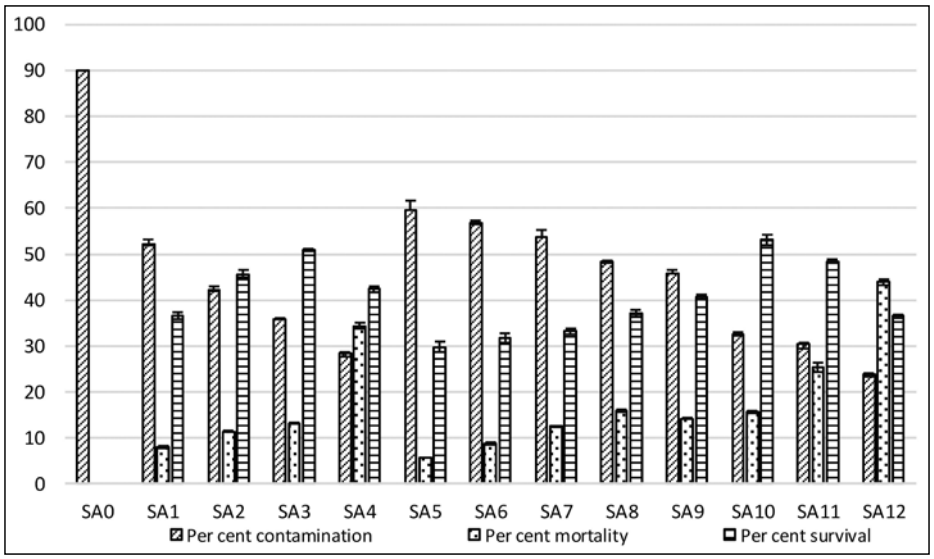

Fig. 2. Effect of different treatment duration of sterilizing agents on nodal segment explants of Chandler strawberry.

to further increase in regeneration percentage. Maximum percentage regeneration of shoots 65.3 ± 1.09 percent from cultured explants were obtained from MS media supplemented with 0.5mg/L IAA + 3.0mg/L BAP. MS media supplemented with various concentrations of BAP took significantly longer time for shoot multiplication than the treatments where BAP was combined with different concentrations of IAA. Minimum time for shoot multiplication (16.1 ± 0.29 days) was recorded in media containing 0.5 mg/L IAA + 3.0 mg/L BAP. Also this treatment resulted in maximum number of regenerated shoots per explants (12.8 ± 0.52 shoots). 4.5 mg/L BAP added in MS media resulted in longest shoots (7.8 ± 0.06 cm) while a combination of 1.0 mg/L IAA + 4.5 mg/L BAP resulted in maximum number of leaves per regenerated shoot. The shoot parameters viz., percentage of explants that regenerated shoots, number of shoots regenerated per explant, shoot length, and number of leaves per regenerated shoots increased with increasing concentration of BAP upto 4.5 mg/L (Table 1). This is in contrast with the reports of Diengngan and Murthy (6); Ashrafuzzaman (2); Haddadi (8) and Bhat *et al*. (3) who recorded better shoot parameters at relatively lower concentrations of BAP. A high ratio of auxin to cytokinin promotes rooting, while high concentration of cytokinin in the media as compared to auxin promotes shoot growth (Skoog and Miller, 15). The positive effects of combination of auxin and cytokinin have already

Table 1: Effect of different concentration of BAP alone and in combination with IAA in MS media on *in vitro* parameters of Chandler strawberry.

Media code	Hormonal composition (mg/L)	Percentage of explants that regenerated shoots	No. of days taken for multiplication	No. of shoots regenerated per explants	Shoot length	No. of leaves per regenerated shoots
T0	Control	35.4 (36.5 ± 0.96)	23.2 ± 1.06	2.1 ± 0.22	2.3 ± 0.09	3.0 ± 0.09
T1	1.5 BAP	49.6 (44.7 ± 1.26)	23.0 ± 1.03	4.4 ± 0.15	4.1 ± 0.18	4.4 ± 0.12
T2	3.0 BAP	68.8 (56.0 ± 1.00)	22.3 ± 0.52	5.4 ± 0.12	6.6 ± 0.17	4.8 ± 0.12
T3	4.5 BAP	70.2 (56.9 ± 0.25)	21.7 ± 0.12	6.6 ± 0.23	7.8 ± 0.06	5.1 ± 0.06
T4	0.5 IAA + 1.5 BAP	66.3 (54.5 ± 1.40)	17.3 ± 0.61	7.2 ± 0.09	5.5 ± 0.67	4.4 ± 0.18
T5	0.5 IAA + 3.0 BAP	82.5 (65.3 ± 1.09)	16.1 ± 0.29	12.8 ± 0.52	6.4 ± 0.58	5.7 ± 0.06
T6	0.5 IAA + 4.5 BAP	80.3 (63.7 ± 0.92)	17.0 ± 0.84	9.6 ± 0.29	6.1 ± 0.17	5.6 ± 0.17
T7	1.0 IAA + 1.5 BAP	70.4 (57.0 ± 0.22)	19.2 ± 0.29	6.4 ± 0.35	5.6 ± 0.29	4.9 ± 0.23
T8	1.0 IAA + 3.0 BAP	79.8 (63.3 ± 1.66)	17.8 ± 0.52	8.5 ± 0.30	5.9 ± 0.23	5.5 ± 0.18
T9	1.0 IAA + 4.5 BAP	75.9 (60.6 ± 1.72)	17.9 ± 0.60	7.6 ± 0.27	5.6 ± 0.18	6.0 ± 0.20
C.D. (0.05)		3.4	2.0	0.8	1.0	0.4

been established by various workers (Gantait *et al*., 7; Danial *et al.*, 4). Cytokinins are used to overcome apical dominance and enhance branching of lateral buds from the leaf axils (Danial *et al.,* 4). Additional shoots are produced through further axillary bud growth (Debnath, 5).

Regenerated shoots were cultured in half MS media fortified with various concentrations of IAA, IBA, and NAA for rooting. All the three hormones in different concentrations responded better than control. However, the extension of rooting varied according to auxin type and its concentration. Half MS media supplemented with 1.5mg/L IAA gave highest percent root regeneration, number of roots per regenerated shoot (10.6 ± 0.23) and earliest regeneration of roots (14.4 ± 0.35 days) while longest roots (5.8 ± 0.06 cm) were obtained with 3.0 mg/L IAA. IBA on the other hand resulted in production of a maximum of 8.2 ± 0.12 roots of an average of 5.3 ± 0.09 cm length with 57.3 ± 0.76 percent rooting at concentration of 1.5mg/L (Table 2). However Ashrafuzzaman *et al.* (2) reported generation of only 2 roots per culture at this concentration and found lower concentrations of IBA to be more effective in rooting leading to maximum of 6 roots per culture at 0.5mg/L IBA. 1.5 mg/L NAA was recorded to be

Table 2: Effect of different concentration of auxins in half MS basal medium on *in vitro* parameters of Chandler strawberry.

Media code	Harmonal composition (mg/L)	Percent root formation	Root length (cm)	Roots per regenerated shoot	No. of days taken for root initiation
T0	Control	10.2 (18.6 ± 0.44)	2.3 ± 0.09	2.9 ± 0.15	19.9 ± 0.91
T1	0.5 IAA	54.3 (47.5 ± 1.40)	4.1± 0.18	5.3 ± 0.23	15.3 ± 0.38
T2	1.5 IAA	96.4 (79.7 ± 2.61)	5.6 ± 0.12	10.6 ± 0.23	14.4 ± 0.35
T3	3.0 IAA	88.9 (70.5 ± 0.83)	5.8 ± 0.06	8.8 ± 0.06	14.6 ± 0.06
T4	0.5 IBA	45.0 (42.1 ± 0.90)	4.2 ± 0.17	5.2 ± 0.17	16.4 ± 0.58
T5	1.5 IBA	70.8 (57.3 ± 0.76)	5.3 ± 0.09	8.2 ± 0.12	15.0 ± 0.26
T6	3.0 IBA	65.6 (54.1 ± 0.96)	4.8 ± 0.07	7.7 ± 0.30	14.8 ± 0.19
T7	0.5 NAA	40.8 (39.7 ± 1.31)	4.5 ± 0.06	5.1 ± 0.09	17.2 ± 0.35
T8	1.5 NAA	62.8 (52.4 ± 1.06)	4.6 ± 0.12	8.0 ± 0.23	16.2 ± 0.46
T9	3.0 NAA	58.5 (49.9 ± 1.16)	4.6 ± 0.17	7.2 ± 0.19	15.5 ± 0.53
C.D. (0.05)		3.8	0.4	0.6	1.4

the best among various tested concentrations of NAA. Increasing the concentration did not have any significant effect on various rooting parameters. The results showed that IAA is most suitable for root induction in strawberry plants. Better performance of IAA than IBA for *in vitro* propagation of strawberry cv. Chandler has also been reported earlier (Gantait *et al*., 7; Danial *et al.,* 4) . While this result was not harmonious with those of Sakila *et al*. (14) who reported medium supplemented with IBA the best for root formation of strawberry.

Cocopeat was found to be best potting mixture for primary hardening giving a survival percentage of 45.2% after 20 days. While a mixture of FYM and coco peat in ratio of 1:1 was observed to be the most suitable potting mixture for secondary hardening resulting in 65% survival of explants after 30 days (Table 3 and 4).

The findings of this research overcome the major hurdles in establishment of cultures, phenolic exudation, shoot multiplication, rooting and acclimatization of *in vitro* regeneration of strawberry (Fig. 3a-f). The protocol is a low cost strategy for mass production of this potential cash crop of Bihar

Table: 3 Effect of different substrate on plant survival in primary hardening.

Treatments	Ratio	Plantlets transferred	Plant survival	Percentage Survival
Coco peat	-	500	226	45.2
Sand: Coco peat	1:2	500	185	37.0
Soil: Coco peat	1: 2	500	105	21.0
Soil: Sand: Coco peat	1: 1: 1	500	65	13.0

Table 4: Effect of artificial substrate on plant survival in Secondary hardening.

Treatments	Ratio	Plantlets transferred	Plant survival	Percentage Survival
Soil: Coco peat	1: 1	100	61	61.0
Soil: Coco peat	2 :1	100	42	42.0
FYM: Coco peat	1:1	100	65	65.0
FYM: Coco peat	2:1	100	48	48.0
Soil: FYM: Coco pit	1: 1: 1	100	52	52.0

(a) Inoculation of explants, ethanol (70%) for 30 sec + $HgCl_2$ (0.1%) for 2 min

(b) Shoot proliferation (4.5 mg/L BAP)

(c) Shoot multiplication (0.5 mg/L/AA)

(d) Rooting (Half MS media + 1.5mg/L IAA)

(e) *In vitro* generated plants after agar weaning ready for primary hardening

(f) Acclimatization (Coco peat)

Fig. 3 (a-f): Different stages of *in vitro* propagation of strawberry.

compared to high production cost and failure to survive under *ex vitro* conditions during hot and humid climate prevailing during summer months. The study is therefore a complete package for large scale production of strawberry runner plantlets that can be easily and equally adopted by both farmers and researchers. This protocol has a potential for allowing a large scale multiplication and area expansion of strawberry in Bihar.

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