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Standardization of an Efficient Protocol for Callus Induction and Regeneration of Strawberry (*Fragaria* × *ananassa* Duch.) cv. Winterdown

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A B S T R A C T

The present investigation was conducted at Biotechnology laboratory, Guru Kashi University Talwandi Sabo, Bathinda to develop and standardize an efficient protocol for callus induction of strawberry cv. Winterdown with the objective to study the effect of plant growth regulators on callus induction. Out of 11 different combinations of growth regulator, the maximum callus induction (90.90%) was observed in the callus establishment media (CEM_9), NAA (1 mg/l) + 2, 4-D (1 mg/l) + BA (0.1 mg/l) with minimum days taken (18) followed by CEM_4, NAA (2mg/l) + BA (2mg/l) showed 83.33% callus induction in 34 days. Highest sub-culturing up to 5 generations was recorded on CEM_9. Healthy green compact calli were transferred with a fresh weight of 100-150 mg on standardized direct establishment media (DEM), BA (2 mg/l) + IBA (0.1 mg/l) + GA_3(0.1 mg/l) for regeneration. After 20-25 days, the established plantlets were hardened in the standardized potting mixture containing coco-peat, vermiculite, perlite and riverbed soil in the ratio 3:1:1:1.

Key words: Callus, *Fragaria,* Growth regulator, Standardization, Strawberry

Strawberry (*Fragaria* × *ananassa* Duch.), member of Rosaeceae family is a natural hybrid of two diocecious octaploid species, *Fragaria chiloensis* L. P Mill and *Fragaria virginiana* Duch (Bowling 2000). It contains ellagic acid, a potential anti-cancerous compound, which had a wide range of biological activity (Sakila *et al.* 2007). It is good source of natural antioxidants including carotenoids, vitamins, phenols, dietary glutathionine metabolites and exhibits a high level of antioxidant capacity against free radicals (Kresty *et al.* 2001). It is native of temperate regions, but varieties are available which can be cultivated in subtropical climate (Rahman 2011). China leads the world (2.0 million MT) in fruit production (Zhang *et al.* 2014) followed by USA (1.31 million MT). India produced 5 thousand Million tonnes strawberry during the year 2017-2018 and was cultivated on 1000 ha area (Anonymous 2018). In India, Maharashtra is the leading state in strawberry production. It is conventionally propagated by runners (Biswas *et al.* 2008), although plants retain all the characters of the parent but results in transmission of over 30 virus and phytoplasmic diseases (Gautam *et al.* 2001, Martin and Tzanetakis 2006) and yield reduction up to 80 per cent (Thompson and Jelkman 2003). Although on a commercial scale, *in-vitro* raised strawberry plants are estimated to cost four to five time more than by conventional propagation but micro-propagation has several advantages i.e. produces large number of virus free plants in a limited time at small place, which makes it economically viable (Chawla *et al.* 2002). Successful *in-vitro* callus induction depends on type and concentration of plant growth regulators and different combinations of PGRs (Karim *et al.* 2011). Although many studies in strawberry tissue culture were reported globally, still there are some drawbacks in the technique. Hence, the current work was attempted to standardize an efficient protocol for callus induction and regeneration of strawberry cv. Winterdown.

MATERIALS AND METHODS

The experiment was conducted in the Biotechnology laboratory and experimental field of Department of

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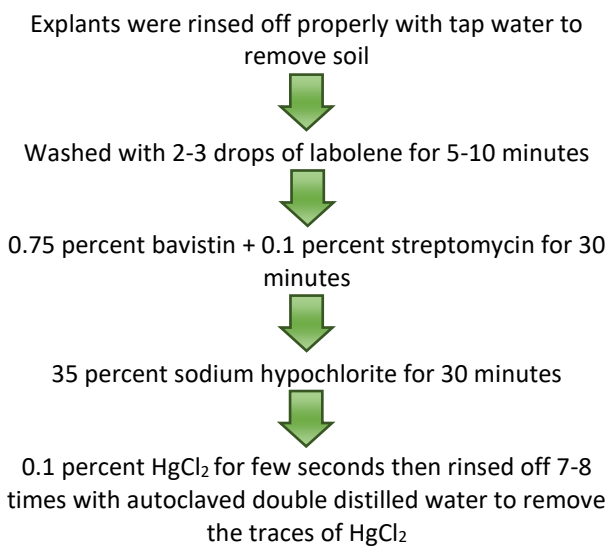
Experimental conditions: Healthy and disease-free plant material (meristems, shoot tips and nodal segments) of strawberry cv. Winterdown were collected. All the tools like glasswares and culture media were sterilized by autoclaving at 15 psi pressure and 121°C temperature for 20-30 minutes. Then these were dried in hot air oven at 80-100°C for 2-4 hours.

Surface sterilisation of explants: Surface sterilization is the most important step in preparation of explants because controlling fungal and bacterial contamination of plant from field sources is very difficult. The contamination usually decreases the propagation rate in *in-vitro* cultures or rooting is seriously diminished so the plant material used for the culture was treated with an appropriate sterilizing agent to inactivate the microbes present on the surface. Standardized sterilization procedure and duration of sterilizers used, is given below in the flow chart:



All the sterilization treatments were practised under the hood of laminar air flow and after sterilization (Kaur, 2018).

Inoculation and incubation: The surface sterilized explants were inoculated on MS media supplemented with various concentrations of BA, IAA, IBA, NAA and 2,4-D under the hood of laminar air flow cabinet. The brown cut ends of explants were removed before inoculation to avoid toxic effects of sterilants and browning. After inoculation, the culture jars and test tubes were incubated in incubation room at 25±2°C temperature in dark (24 hours) for 30-40 days and were subsequently exposed to 16 hours photoperiod for regeneration.

Activated charcoal and browning: Explants were cultured on MS media + 0.5 mg/l activated charcoal with various concentrations of plant growth regulators to control browning and to study the effect of activated charcoal on callus initiation.

Callus initiation: To study the effect of plant growth regulators (BA, IBA, IAA, 2, 4-D and NAA) on callus induction of strawberry cv. Winterdown, the explants were properly inoculated in the 11 different callus establishment media (CEM) supplemented with various concentrations and combinations of plant growth regulators (Table 1). After 25-30 days, healthy, compact, preferably green coloured actively growing callus from initial explants was separated and used for further multiplication by cutting them into small sections and sub-culturing them onto same concentration fresh media. Visual observations with respect to growth, texture, days for callusing and colour of callus were recorded.

Table 1 Different treatments used for callus formation in strawberry cultivar Winterdown

Treatments	Concentration of growth regulators (mg/l)
CEM_1	MS media + IAA (5)
CEM_2	MS media + NAA (2)
CEM_3	MS media + IBA (2) + BA (2)
CEM_4	MS media + NAA (2) + BA (2)
CEM_5	MS media + NAA (1) + BA (2)
CEM_6	MS media + NAA (2.5) + BA (0.1)
CEM_7	MS media + 2,4-D (2) + BA (2)
CEM_8	MS media + 2,4-D (4) + BA (1)
CEM_9	MS media + NAA (1) + 2,4-D (1) + BA (0.1)
CEM_{10}	MS media + 2, 4-D (2)
CEM_{11}	MS media + NAA (1.5)

Sub culturing of callus: After 25-30 days of callus induction, healthy and preferably green coloured callus is taken from the cultures, under the hood of laminar air flow, is used for further multiplication by cutting them into small sections and sub culturing them on same concentration media.

Regeneration: On the basis of the observations, the healthy, green and compact calli pieces were transferred to the standardised direct establishment media (DEM) supplemented with BA (2 mg/l) + IBA (0.1 mg/l) + GA_3 (0.1 mg/l) + activated charcoal (0.5 mg/l) for shoot and root induction.

Hardening and Acclimatization of rooted plantlets: After 20-25 days the *in-vitro* raised plantlets having three to four leaves and three to four roots were taken out from culture bottle. The roots were washed thoroughly in tap water to remove adhering agar and then treated with 0.75% Bavistin for 3-5 minutes were transplanted in thermocol cup containing standardized potting mixture of Coco-peat, vermiculite, perlite and riverbed soil in the ratio 3:1:1:1 and after 60 days, established plantlets were acclimatized in the field for production and runner multiplication.

Data analysis: The data was analyzed using the software OPSTAT developed by CCSHAU, Hissar and Critical difference (CD) values at 5% level of significance

were used for checking the significance of effect of different factors on different parameters.

RESULTS AND DISCUSSION

Explants of strawberry cv. Winterdown showed the highest response to callus formation on MS medium supplemented with combination of NAA, 2, 4-D and BA. The strawberry nodal explants induced to show callus development in few culture media formulations, however, the effect of different PGR formulations on the degree and callus induction percentage were different (Karim *et al.* 2011).

Effect of activated charcoal

The efforts were made to induce callus from strawberry cv. Winterdown using meristems as explants. In the trials conducted during session 2017-2018, MS Media fortified with different concentration of growth regulators and charcoal (0.5 mg/l) did not show any results in response to callus induction. The effect of charcoal in media is known for inhibiting browning due to phenolics exudates from wounded explants but results revealed that it has inhibitory effect on callus induction when used in MS media for callus induction (Plate 1). Similar results were found by Hatem *et al.* (2017) in strawberry cv. Albion and Festival who recorded significantly reduced callus percentage when media were supplemented with (0.2gm/L) of activated charcoal.

Plate 1 Inhibitory effect of charcoal on callus induction

Callus induction percentage

The results (Table 2) revealed that media CEM_9, NAA (1 mg/l), 2, 4-D (1 mg/l) and BA (0.1 mg/l) yielded the significant highest callus induction (90.90%) (Plate 2). The calli were creamy green in colour and mostly compact in nature but few were loosely compact in texture. Akter *et al.* (2008) found similar results. Notable amount of callus induction (83.33%) was observed in media CEM_4, NAA (2 mg/l) + BA (2 mg/l) from meristem which was statistically at par with treatment CEM_6 (80%), NAA (2.5 mg/l) + BA (0.1 mg/l). No callus induction percentage was observed in CEM_1, IAA (5 mg/l) and CEM_7 2, 4-D (2 mg/l) + BA (2 mg/l).

Table 2 Effect of different concentrations and combinations of growth regulators on callus induction, days for callus initiation and contamination percentage

Treatments		Callus induction (%)	Days for callus initiation	Contamination (%)
MS media + growth regulators (mg/l)		Mean ± SE	Mean ± SE	Mean ± SE
CEM_1	IAA (5)	0.00 ± 0.00	-	-
CEM_2	NAA (2)	66.67 ± 2.05	25 ± 1.44	33.31 ± 2.30
CEM_3	IBA (2) + BA (2)	15.38 ± 0.88	40 ± 2.77	84.62 ± 3.41
CEM_4	NAA (2) + BA (2)	83.33 ± 1.80	34 ± 1.61	16.71 ± 0.88
CEM_5	NAA (1) + BA (2)	70.00 ± 2.76	36 ± 1.24	30.08 ± 0.28
CEM_6	NAA (2.5) + BA (0.1)	80.00 ± 3.10	22.03 ± 0.60	20.00 ± 1.27
CEM_7	2,4-D (2) + BA (2)	0.00 ± 0.00	-	-
CEM_8	2,4-D (4) + BA (1)	56.25 ± 4.03	38.03 ± 2.62	43.73 ± 2.03
CEM_9	NAA (1) + 2,4-D (1) + BA (0.1)	90.90 ± 0.57	18 ± 0.93	3.63 ± 2.72
CEM_{10}	2, 4-D (2)	70.23 ± 2.22	21.03 ± 1.18	29.98 ± 0.26
CEM_{11}	NAA (1.5)	40.00 ± 0.81	30 ± 1.93	60.00 ± 2.76
	C.D (at 5%)	6.152	4.64	5.54

Number of days taken for callus induction

The data presented in (Table 2) reveals that minimum number of days taken for callus induction (18.00) was recorded on CEM_9, NAA (1 mg/l) + 2, 4-D (1 mg/l) + BA (0.1mg/l), which was at par with CEM_{10}, 2, 4-D(2 mg/l) but significantly better from rest of the treatments. The maximum number of days (40.00) taken for callus induction was observed in CEM_3, IBA (2mg/l) + BA (2mg/l), which was statistically at par with CEM_8, 2,4-D (4 mg/l) + BA (1 mg/l) treatment and significantly different than rest of the treatments. Alizadeh (2011) stated that the kind of explants and medium optimization are the main factors in successful callus induction. Mousavizadeh *et al*. (2010) investigated callus induction of cv. Camarosa using leaf and meristem and concluded that only the meristem is capable of inducing callus with combination of BA (10 mg/l) + NAA (0.01 mg/l) while on other treatments, the meristem entered direct regeneration phase i.e. they were not capable of producing callus but in the present investigation the results were only found in the meristem explants. In the present study the best results were recorded by using NAA, BA and 2,4-D at very low concentrations because at the higher concentration of BA (≥ 5.0 mg/l) whole of the callus turned brown and finally died. When IBA and IAA were used as auxins instead of NAA, after 4-5 days of inoculation, the calli turned brown and finally died. Auxin (NAA and 2,4-D) is commonly added to the callus media to induce cell division and cell elongation while the cytokinin (BA) is added to induce cell division. Optimum callus initiation can be achieved where appropriate combination of auxin and

cytokinin is supplemented to the callus media. The synergistic effect of both encouraged cell proliferation and subsequently gave high callus induction. IBA had no significant effects in combination with BA in terms of increasing callus rate. In contrast, NAA and 2.4- D had a positive effect in combination with BA and significantly increasing callusing rate. The current results are concurred with Skoog *et al.* (1957) theory and also the results are in agreement with Biswas *et al.* (2010). Explants using CEM_1, IAA (5 mg/l) and CEM_7, 2, 4-D (2 mg/l) + BA (2 mg/l) showed no callus induction (0%). In contrast the present studies disagree with Sorvari *et al.* (1993), Skoog *et al.* (1957) pointed out that 3mg/l BA individually caused the highest percentage of callus induction.

Plate 2 Callus induction on MS medium supplemented with 2, 4-D (2 mg/l) + NAA (2 mg/l) + BA (0.1 mg/l) of strawberry cv. Winterdown

Plate 3 Callus induction on MS medium supplemented with NAA (2 mg/l) of strawberry cv. Winterdown

Table 3 Effect of different concentrations and combinations of growth regulators on colour, texture and degree of callusing

Treatments		Degree of callusing	Colour	Texture
MS media + growth regulators (mg/l)				
CEM_1	IAA (5)	+	-	-
CEM_2	NAA (2)	+++	Creamy	Loosely compact
CEM_3	IBA (2) + BA (2)	+	Creamy	Friable
CEM_4	NAA (2) + BA (2)	+++	Creamy green	Compact
CEM_5	NAA (1) + BA (2)	+++	Creamy green	Loosely compact
CEM_6	NAA (2.5) + BA (0.1)	+++	Creamy brown	Friable
CEM_7	2,4-D (2) + BA (2)	+	-	-
CEM_8	2,4-D (4) + BA (1)	++	Creamy light green	Loosely compact
CEM_9	NAA (1) + 2,4-D (1) + BA (0.1)	++++	Creamy green	Compact and loosely compact
CEM_{10}	2, 4-D (2)	+++	Creamy light green	Compact
CEM_{11}	NAA (1.5)	++	Creamy	Friable

++++ = Highly callusing (81-100%) ++ = Poor callusing (31-60%)

+++= Moderate callusing (61-80%) + = Very poor callusing (0-30%)

Sub culturing of callus

Callus formation in strawberry is controlled by different levels of plant growth regulators (auxins and cytokinins) in culture media. The callus establishment media which resulted in the best callus growth were further used for sub culturing of callus up to five generations in CEM_9, NAA (1 mg/l) + 2, 4-D (1 mg/l) + BA (0.1 mg/l) (Plate 4) and for 3 times in CEM_4, NAA (2 mg/l) + BA (2 mg/l). It was found that after 5^{th} sub culturing, the degeneration of callus was observed as the callus section sub cultured for 5^{th} time showed browning and very poor growth (Plate 4E). However, when same were grown on direct establishment media, they showed pronounced growth in terms of shooting from callus. Similar observations have been made by Muhammad *et al.* (2004) who reported that all the explants of Banana spp. do not behave similarly *in-vitro* in terms of

multiplication and sub culturing of callus. It was also noted that the 2nd generation was most productive while 5th generation was found to be least productive. It was also concluded that the cultures showing higher rate of multiplication in the first two or three subcultures continue this behaviour in the next sub culturing. Cote (1993) recommended that the number of subculture cycles should be limited to ten. Economically, the division within isolated calli is not very easy; it takes a lot of time.

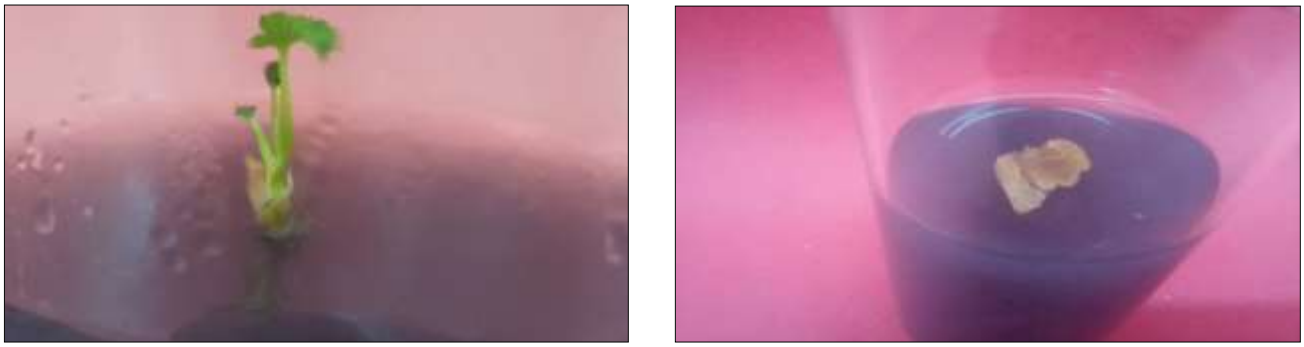
(A) 1st generation

(B) 2ndgeneration

(C) 3rd generation

(D) 4th generation

(E) 5th generation



(F)

Plate 4 Subcultured callus generations (A) 1st generation, (B) 2nd generation, (C) 3rd generation, (D) 4th generation, (E) 5th generation cultured on CEM_9, 2, 4-D (1 mg/l) + NAA (1 mg/l) + BA (0.1 mg/l), (F) cultured on standardized diect establishment media (DEM)

Regeneration

Healthy, green friable and compact calli were sub-cultured to the standardized direct establishment media (DEM) supplemented with as BA (2.0 mg/l) + IBA (0.1 mg/l) + GA_3 (0.1 mg/l) + Activated charcoal (0.5 mg/l) (Plate 4F). Activated charcoal was added to leach out the excess of hormones, eliminates light, prevents undesirable callusing and helps in rooting. Regeneration started when some of the calli started browning. After 7 days, the regeneration percentage (18.32%), average number of leaves (1.98), shoots (2.06) and length of shoots (2.04 cm) were observed. At 14 days, regeneration percentage (29.54%), average number of leaves (2.87), average number of shoots (3.67) and length of shoots (3.70 cm) were recorded and at 21st day, the regeneration percentage increased to 69.32%, average number of leaves per calli (5.98), average number of shoots (6.84) and length of shoots (6.20 cm). Rooting percentage (80%), average number of roots (8) and average length of roots (10.5 cm) were also recorded after 21 days.

Cytokinins are known to play a major role in shoot multiplication. BA is the cytokinin that is mostly used for in vitro shoot proliferation of strawberry (Haddadi *et al*. 2010). BA along with GA provided synergistic effect on regeneration. Moradi *et al.*, (2011) reported that the maximum number of roots were obtained in MS medium combined with cytokinin BA and auxin IBA. Borkowska (2001) who studied the micropropagation of strawberry cultivars Senga sengana, Kent and Kama used a medium supplemented with 1.0 mg/L IBA. The calli from *in-vitro* strawberry cultivars had very poor shoot regeneration because the hormonal imbalance maintained the explant on a high cytokinin medium prior to explant preparation.

Plate 5 Rengerated plantlets after 21 days

Table 4 Effect of direct establishment media (DEM) on regeneration

Hormonal composition of Direct establishment media (DEM) BA (2.0mg/l) + IBA (0.1mg/l) + GA_3 (0.1mg/l) + Activated charcoal (0.5 mg/l)	Regeneration percentage (%)	Average number of leaves per calli	Average number of shoots	Length of shoots (cm)
Effect on 7th day	18.32	1.98	2.06	2.04
Effect on 14th day	29.54	2.87	3.67	3.70
Effect on 21st day	69.32	5.98	6.84	6.20

Hardening and acclimatization of rooted plantlets

The *in-vitro* raised plantlets having three to four leaves and three to four roots were taken out from culture bottle (Plate 5). The roots were washed thoroughly in tap water to remove adhering agar and then treated with 0.75% bavistin for 3-5 minutes. Then the plantlets were transplanted in thermocol cup containing potting mixture of Coco-peat, vermiculite, perlite and riverbed soil in the ratio 3:1:1:1 (Plate 6). After three weeks these were brought under green shade net for *ex-vitro* hardening and then to the field after 20 days for acclimatization.

Plate 6 Hardening and acclimatization of *In-vitro* raised plantlets

Callus initiation *in-vitro* culture responded differently depending on plant growth regulators supplemented to MS medium cultures and usually encountered contamination and lethal browning effects. The outcome of present investigation concluded that MS media fortified with different conc. of growth regulators and charcoal (0.5mg/l) did not show any results in response to callus induction. The effect of charcoal in media is known for inhibiting browning due to phenolics exudates from wounded explants but results revealed that it has inhibitory effects on callus induction when used in MS media for callus induction. Explants (meristem and nodal segments) of strawberry cv. Winterdown showed the highest response to callus formation on MS medium supplemented with combination of NAA, 2, 4-D and BA. The level combination of plant growth regulators supplemented to MS media relevant for maximum callus induction percentage (90.09%) in minimum number of days (18) at NAA (1 mg/l), 2, 4-D (1 mg/l) and BA (0.1 mg/l). The calli were creamy green in colour and mostly compact in nature but few were loosely compact in texture at NAA (1 mg/l), 2, 4-D (1 mg/l) and BA (0.1 mg/l). Regeneration was recorded on standardized direct establishment media (DEM), BA (2 mg/l) + IBA (0.1 mg/l) + GA_3 (0.1 mg/l) + activated charcoal (0.5 mg/l) and hardened on standardized potting mixture of coco- peat, vermiculite, perlite and riverbed soil at the ratio 3:1:1:1.

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