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Contamination Free Callus Cultures in Strawberry (*Fragaria x ananassa* Dutchcv.)

Hatem K. Abbas[1]*, Ali A. Mahdi Al-Sallihy[2] and Kadhim M. Ibrahim[3]

[1]Horticulture office, Iraqi Agriculture Ministry/Iraq
[2]Institute of Genetic Engineering and Biotechnology, Baghdad University,Iraq
[3]Biotechnology College, Al-Nahrain University, Iraq.

Abstract : A successful callus initiation of *in vitro* culture depends on appropriate concentrations of plant growth regulators (PGRs) supplemented to the culture medium. Suitable protocol for surface serialization is crucial to overcome microbial contamination and may be browning problems to induce callus. This project was carried out to examine different concentrations ofα-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP), (M1= 4mg^{-1} NAA+1mg^{-1} BA, M2 =5mg^{-1} NAA+2mg^{-1} BA, M3= 0.3mg^{-1} NAA+1mg^{-1} BA or(M4= 0.5mg^{-1} NAA+2mg^{-1} BA)supplemented to MS medium culture. Two strawberry cultivars (Albion and festival) were chosen and leaf discs explants were dissected. Seven different sterilization materials were examined on both cultivars to determine the best one in reducing microbial contamination with minimum necrosis at relevant period of sterilization. Different levels of activated charcoal (0, 0.2, 0.3 and 0.4g^{-1}) were incorporated into culture medium to minimise browning. Results showed that M1 is the best PGRs combination for callus induction of festival leaf explants, while M2 was the best for Albion. Sodium hypochlorite at 4% for 20 min was the most efficient for causing 90% contamination free cultures for festival cultivar. The concentration 3% of sodium hypochlorite treated with Albion explants achieving 90% contamination free cultures as well. The concentration of 0.2g.l^{-1} of activated charcoal reduced browning in cultures permitting callus initiation. Developing an efficient protocol for callus initiation is important for strawberries propagation using *in vitro* approaches.
Key words : *Strawberry, PGRs, activated charcoal, surface sterilization, browning*.

Introduction

Strawberries have been grown widely in the world due to high levels of nutrition since fruit contains vitamin C, flavonoids, ellagic acid and autocianidin[1].Phytochemicals and antioxidants of strawberry lead to decrease the hazard of cardiovascular events disease and tumorogenesis[2].These healthy advantages of strawberry fruits have attracted researchers to develop new cultivars with desirable traits [3].

Food and Agriculture Organisation (FAO) [4] reported that the total yield of strawberry fruit in 2005 reached 3.9 tons. Strawberry in Iraq is cultivated in some provinces suchas Baghdad and Karbala, however the cost of production is high compared with neighbouring countries; one of the main reasons is an expensive price of imported plantlets [5]. Tissue culture technique can provide commercial advantages; it can regenerate large number of plantlets from one mother plant in a limited time at small space [6].*In vitro* strawberries have propagated since four decades [7]. Annually, millions of strawberry plants are propagated in European countries by using tissue culture[8].

Bacterial contamination in strawberry tissue cultivars is an important issue faces *in vitro* propagation after surface sterilization and culture on a suitable medium. Bacterial contamination can be detected during 48 hours[9]. Browning is another problem occurs after explants transfer onto a solid medium. Bacteria and browning prohibit callus induction. According to[10] and [11] browning at callus induction step of tissue culture method is an important reason for explants death.

Despite that many studies have been conducted to reduce microbial contamination and browning, none of these have neither eliminated contamination entirely nor removed browning *in vitro* strawberry cultures[12].

In addition, successful in*in vitro* callus induction depends on the concentration of plant growth regulators supplemented to the medium [13].Responding of callus induction is differed depending on plant species, type of explants or even between individual plants[14]. Also, the outcome of different quantity and quality of callus induction is associated with different combinations of PGRs supplemented to culture medium [15].

Although many studies in strawberry tissue culture were reported globally, still there are some drawbacks in the technique. Therefore, the current work is attempting to build up an efficient callus initiation protocol in two strawberry cultivars (Albion and festival) using commonly cultivated appropriate surface sterilization method and suitable concentration of activated charcoal to overcome browning. Thus, subsequent phases of plant micropropagation using indirect method can be carried out.

Materials and Methods

Two strawberry (*Fragaria*x *ananassa* Dutch.) cultivars'Albion' and 'Festival'were kindly supplied by Horticulture Research Det., Hort. Office, Ministry of Agric., Iraq. Plants were uniform in size, leaf explants were dissected from juvenile shoots.

Experimental conditions

For the establishment of culture, Leaves were collected from greenhouse grown strawberrycvs.(Albion and Festival). All tools such as glass wares and culture media were sterilized by autoclaving at1.16 kg-cm^{-2}pressures at 121 ºC temperature for 20 minutes to ensure aseptic conditions.

Surface sterilization

Dissected young leaves were rinsed with tap water for 5 times. Then the explants were transferred to laminar air flow cabinet. Leaves were cut to 1.0 cm^2 by surgical blade and forceps before inoculation. After that, leaf disks were subjected to different sterilization agents at various concentrations of aqueous solution of sodium hypochlorite(1.5, 2, 3, and 4%) individually or combined with (v/v) 70% ethanol. Sterilized timeswere15 or 20 min for sodium hypochlorite and30 sec using 70% alcohol as shown in Table 1.One drop of tween-20 was added to the solution .Leaf disks were washed three times in sterile distilled water. Ten replicates were used for each treatment. The percentage of contamination was calculated five weeks later.

Culture media

Standard MS medium [16] supplied by (Hi-media-India)was used, and then3% (w/v) sucrose was added. pH was adjusted to 5.7-5.8, then 0.7% (w/v) agar was added. Vials (10x2.5cm) were inoculated with 10 ml of prepared medium, and then sterilized by autoclaving at 1.16 kg-cm^{-2} pressures and 121 ºC for 20 min. Autoclaved vials were left at growth room tell use.

Activated charcoal and browning

Sterilized leaf disks were placed into sterile petri plates under the laminar flow cabinet then cut of four sides into 0.5cm2disks. Explants were cultured on MS medium supplemented with different concentrations of activated charcoal (0.2, 0.3 or 0.4g.l^{-1}), each treatment included ten replicates. Two weeks later the percentage of browning in explants and survival of leaf disks were recorded.

Callus initiation

MS medium supplemented with different combinations of BAP and NAA.PGRs were combined(M1= 4mg.l^{-1} NAA+1mg.l^{-1} BAP,M2 = 5mg.l^{-1} NAA + 2mg.l^{-1} BAP, M3=0.3mg.l^{-1} +NAA 1mg.l^{-1} BAP or M4= 0.5mg.l^{-1} NAA +2mg.l^{-1} BAP). Activated charcoal 0.2 % (w/v) and 3% (w/v) sucrose were added. The pH was adjusted to 5.7 - 5.8 before adding 0.7% (w/v) agar, then autoclaving were performed as mentioned above. Aseptically, leaf disks were cultured in vials containing 10 ml of dispensed medium. Ten replicates were used in this investigation for each PGR concentration. Vials were kept at a growth room under dark conditions at 25 ± 2°C. Aftera period of five weeks, fresh weigh of initiated callus was recorded using Digital scale sensitive balance (Sartorius TE214S- Germany).

Experimental design and statistical analysis

Experimental design and statistical analysis were conducted using a completely randomized design (CRD) with ten replicates. The Statistical Analysis System- SAS [17] program was used to analyse the differences between means in the studied parameters. Chi-square test was used to compare significance between the percentages. ANOVA was used to compare between means at $P \leq 0.05$.

Results and discussion

Surface sterilization and explants contamination

It was suggested that the best effective surface sterilization reagent was sodium hypochlorite [18]. Therefore, leaf disks of the two strawberry cultivars(Festival and Albion) were subjected to seven of different concentrations of sodium hypochlorite. Results showed that the percentage of contamination and necrosis in explants were affected significantly. The lowest percentage of contamination reached 10%of festival leaf discs using 4% sodium hypochlorite for 20 min and that was associated with 20% of necrosis (Table1).The same percentage of contamination 10% and 0% of necrosis of Albion leaf disks were achieved when explants treated with 3% of sodium hypochlorite for 15 min.

In addition, results showed that an interaction between 4% sodium hypochlorite and 70% alcohol led to removing the percentage of contamination completely; however, it caused a significant increase in necroses (70-100%). In contrast, when the percentage of sodium hypochlorite was decreased to 1.5% caused high microbial contamination in both cultivars even when it was combined with 70% alcohol.

This study was concurred with [19] who pointed out that an increase in the concentration of sodium hypochlorite and time of exposure for 15 min caused a decrease in contamination percentage, however, more than 2.5% led to a high percentage of browning. Present study disagreed with [20] which demonstrated that strawberry explants treated with1.5% sodium hypochlorite for 20 min + 70% ethanol caused80% combination free cultures and58% necrosis. Although sterilization with 2.5% of sodium hypochlorite of strawberry explants caused contamination free cultures, browning reached 100% and reduced the percentage of shoot survival [21]. Explants treated with 2.7% sodium hypochlorite demonstrated a disinfection of 46.8% [22].Sodium hypochlorite at 0.5% presented a good surface disinfected [18]. The current study exhibits a reasonable surface sterilization associated with a high removal of browning.

Table1. Percentage of contamination and necrosis as affected by different sterilization treatment and period.

Sterilization treatments	Period of treatment	%Albion cv. contamination	%Festival cv. contamination	%Albino cv. necrosis	%Festival cv. necrosis
NaOCl1.5 % + Alcohol 70%	15 min 30sec	100	100	50	10
NaOCl2% + Alcohol 70%	20 min 30 sec	80	70	60	15

NaOCl3% + Alcohol 70%	20 min 30sec	0	20	90	50
NaOCl4% + Alcohol 70%	15 min 30 sec	0	0	100	70
NaOCl2%	20 min	80	70	5	0
NaOCl3%	15 min	10	30	10	0
NaOCl4%	20 min	0	10	30	20
Chi-Square	---	12.759 **	12.644 **	11.752 **	9.261 **

Effect of activated charcoal

The main advantage of using activated charcoal *in vitro* cultures is to adsorb phenolic compounds from the culture media. Phenolics are exudates from wounded explants caused browning inhibit callus initiation [23].Results indicated that Albion cv. exhibited significantly higher browning than Festival cv. (fig.1).Adding of 0.4 $g.l^{-1}$ of charcoal was removed browning completely from both cultivars (Albion and Festival) but this percentage is significantly reduced the percentage of callus initiation 20% and 30% respectively(Table 2).The results explained that adding 0.2 $g.l^{-1}$ of charcoal was as an affective in inhibiting browning by 90 and 100% associated with initiating callus up to 80 and 90% for Albion and Festival cultivars respectively (Table2). High percentage of browning happened when leaf discs of greenhouse grown plants were used [24]. Browning appeared when young leaves of strawberry cultured on MS medium and caused inhibition of callus initiation [25]. Browning occurrence is generally attribute to oxidize phenolic substances and this phenomenon can be decreased by using activated charcoal [26].The successful technique of tissue culture depended on raised the ratio of survival cultured explants and decreased phenol stimulated browning in strawberry cultivar [27]. Therefore, activated charcoal was used to detect an efficient concentration that can overcome browning problem.

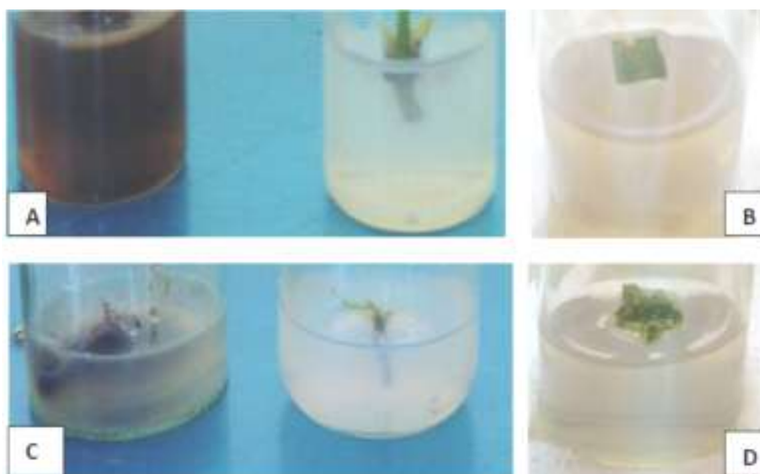

Fig. 1.The effect of activated charcoal on removal of browning in two strawberry cultivars (Albion and Festival).

The figure shows browning in explants of Albion (A) and Festival(C)when cultured on MS medium free of charcoal. Explants culture at first day presented to the right and explant cultures after two weeks resented to the left. B and D:show Albion and festival explants responds when adding 0.2%g of charcoal to removing browning after two weeks from culture.

Table2. Effect of charcoal treatments on browning and callus induction in two strawberry cultivars (Albion and Festival)

Activated Charcoal $g.l^{-1}$	Browning Albion %	Browning Festival %	Initiation Callus Albion %	Initiation Callus Festival %
0	100	20	0	50
0.4	0	0	20	30
0.3	20	0	40	50
0.2	10	0	80	90
Chi-Square** ($P\leq0.01$).	13.087 **	7.353 **	9.148 **	9.965 **

Callus induction

Figure 2 shows that both cultivars responded differently in callus initiation on MS medium supplemented with level of BAP and NAA. Festival cv. recorded higher callus fresh weight mean cultures in all combinations of PGRs achieving 169.11, 125.0, 155.55, and 117.00 mg for M1, M2,M3 and M4 respectively. Albion cv. recorded less fresh weight 78.33, 102.22, 81.11 and 73.33 mg for M1, M2, M3 and M4 respectively (Figure 3). It clear that MS media supplemented with $4mg.l^{-1}$ NAA and$1mg.l^{-1}$BAP (M1) achieved the highest callus fresh weigh of festival explants while the highest callus fresh weight of Albion explants achieved at $5mg.l^{-1}$NAA and $2mg.l^{-1}$ BPA (M2). The auxin NAA is commonly added to the culture media to induce cell division and cell elongation, while the cytokinen BAP is added to induce cell division. Optimum callus initiation can be achieved where appropriate combination of the auxin and cytokinen is supplemented to the media. The synergistic of both encouraged cell proliferation and subsequently gave high callus fresh weight. This conclusion is in accordance with [28]theory. The current results are concurred with [28] theory, also the result are in line with [13] they demonstrated that $4mg.l^{-1}$ NAA combined with $1.5mg.l^{-1}$ BA were formed the highest percentage of callus induction in all explants of strawberry cultivars. Moreover, it is agreed with [15]and [14] due to the illustrated that an increase in the percentage of auxin ten time over cytokinin caused the highest percentage of callus induction. In contrast, the present study disagrees with [29] and [30]as their results pointed out that $3mg.l^{-1}$ BA individually caused the highest percentage of callus induction.[31] reported that the best inducing of callus induction was when explants cultured on medium supplemented with $3mg.l^{-1}$ 2,4-dichlorophenoxy acetic acid (2,4-D).Many studies in strawberry propagation have used different dissected explants of various genotypes could induce high or low callus induction and that depends on type of explants or belong to genotype [13].

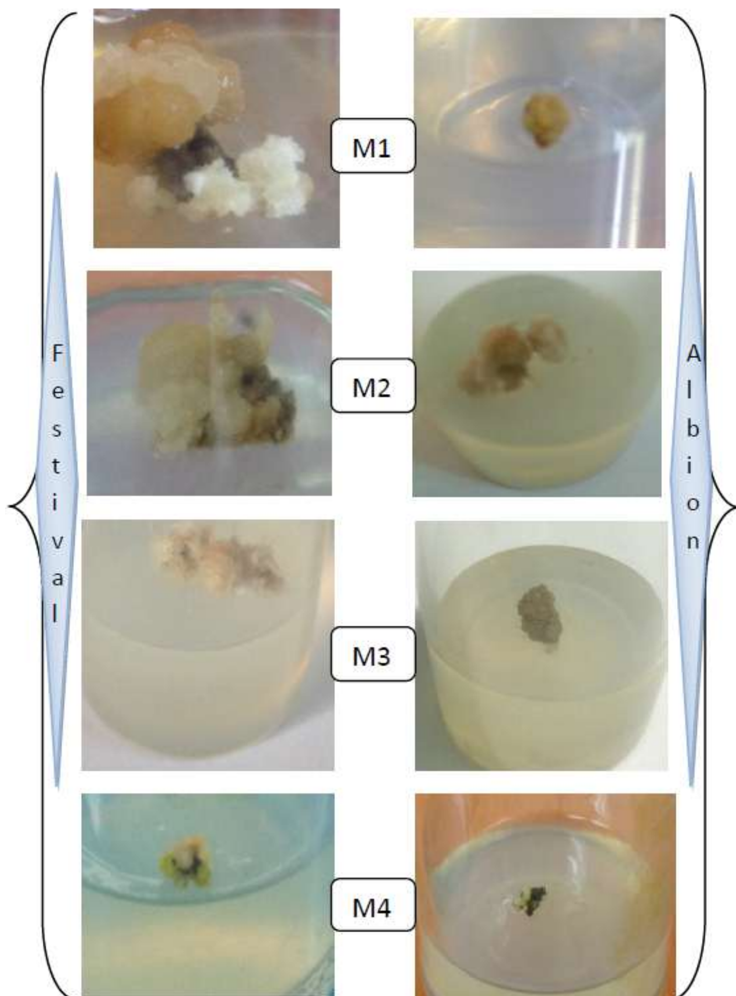

Fig.2Callus induction impacted by using different combination of PGRs in two strawberry cultivars.

The figure shows the left column is callus initiation of Festival explants. The right column is callus initiation of Albion explants. Leaf disks were cultured on MS media supplemented with different concentration of PGRs for five weeks, M1=4mg NAA+1mg BA, M2=5mg NAA+2mg BA, M3=0.3mg NAA+1mg BA and M4= 0.5mg NAA+2mg BA.

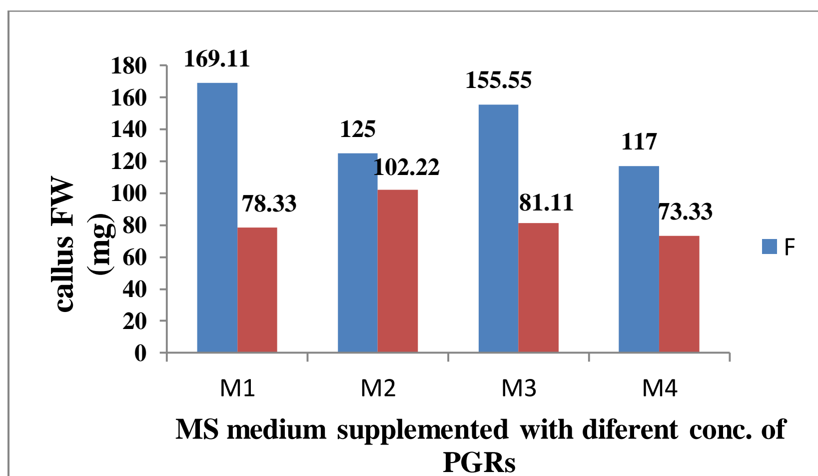

Fig.3. Callus fresh weight initiated after inclusion of different PGRs in media.

The figure shows the fresh weight (mg) of callus inititation. Explants were placed on different MS medium: M1= 4mg NAA+1mg BA, M2= 5mg NAA+2mg BA, M3= 0.3mg NAA+1mg BA and M4= 0.5mg NAA+2mg BA). The fresh weight value is reported as the mean weight of nine replicates treatment. Weights were measured after five weeks of callus initiation. Festival (F)fresh weight is represented by blue bars; Albion (A) fresh weight is represented by brown bars. The results of ANOVA statistical variance show significant differences at $P \leq 0.05$ as the table 4 below shown. The interaction was between cultivars (F and A) and PGRs treatments (M1, M2, M3 and M4).

Table 4.ANOVA Statistical variance fresh weight of callus induction on two strawberry cultivars

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	60378.13	1	60378.13	25.97056	3.3E-06	3.990924
Columns	8306.486	3	2768.829	1.190962	0.320298	2.748191
Interaction	12558.71	3	4186.236	1.800634	0.155978	2.748191
Within	48791.6	64	2324.868			
Total	230034.9	71				

Conclusion

Callus Initiation *in vitro* culture is responded differently depended on PGRs supplemented to medium culture and usually encounters contamination and lethal browning effects. The outcome of present study is concluded that Albion and festival strawberry cultivars can be surface sterilized effectively using leaf explants of Albion cultivar by the treatment with 3% sodium hypochlorite for 15 min and for festival leaf explants at 4% sodium hypochlorite for 20 min. Browning in cultures can be minimized after adding of 0.2%g of activated charcoal in both explants cultivar cultured medium. The level combination of PGRs relevant for maximum callus fresh weight was at 5mg.l NAA + 2mg.l for Albion cv. and at 4mg.l NAA+ 1mg.l for Festival cv.

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