Journal of Scientific and Innovative Research 2018; 7(3): 70-74
Available online at: www.jsirjournal.com

Research Article

ISSN 2320-4818
JSIR 2018; 7(3): 70-74

Received: 10-08-2018
Accepted: 17-09-2018

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Establishment of Efficient Surface Sterilization Protocol on Different Types of Field Grown Strawberry Explants (*Fragaria x ananassa* Duch.)

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Abstract

The present study was conducted to develop the effective disinfection protocol for the in vitro micropropagation of strawberry (*Fragaria x ananassa* Duch) with the use of shoot tips, runner tips, nodal segments and leaf segments as explants. The explants used in this study were surface sterilized using antibiotics, fungicides and other sterilants for different time durations. Although using the same sterilants, the most effective and successive way of using sterilants is different upon the time duration for each sterilant. In this study, two sterilization protocols were used and each protocol included same fungicide and antibiotics concentrations for the same time durations but there were slightly different concentrations and time durations of other sterilants. The present investigation revealed that the most effective way of sterilization protocol which were observed on the nodal segments while treated with protocol II including (10ml/L) fungicide solution for 2 hours, (500mg/L) concentration of ciprofloxacin for 1 hour, (20%) chlorox solution with two drops of Tween 20 for 5 mins, (70%) ethanol solution for 5 mins and (0.1 %) mercuric chloride solution for 4mins. However the same sterilants using the same sterilization time did not give raise the survival rate for runner tip explants, because these treatments resulted in tissue necrosis and contamination and then finally the death of the explant materials. And also, the explants of shoot tips and leaf segments were not shown the effective result compared with using nodal segments. So, for the micropropagation of field grown strawberry, the sterilization protocol II was suite for the nodal segments used as explants for the culture initiation on MS basal medium.

Keywords: *strawberry, surface sterilization, nodal segment, micropropagation*.

INTRODUCTION

Strawberry (*Fragaria x ananassa* Duch) is a perennial crop comprising a crown from which shoot meristem, compressed stem and all other plant parts emerge and then grown as a complete plant. It is one of the crop including in the family Rosaceae. The genus of it is *Fragaria* and the strawberry plant is very important fresh fruit bearing crop because the fruits are very sweet and the colors are very bright. It is very useful in fresh fruit consumption and also useful in other bakery products [1].

Strawberry plants are growing in tropical and subtropical regions over the world. The leaves emerge from the crown spirally. The propagation of strawberry is mostly from the runners which arise from the leaf axils. After fertilization, strawberry flowers develop into a fleshy fruit and these fruits are bearing small seeds which are nearly two hundred seeds from one fruit. The plants are day length dependent types and there are three types of cultivars – long day, short day and day neutral crop types. The fruit of this is an achene type. The seeds are also joined around the over side of the fruit which is the receptacle.

The fruit is so delicious with just a little sour taste and sweet smell and sweet taste. It is also very popular fruit in the world because its fresh usefulness, freezing and processing as value added products are largely valuable in order to use as dessert fruit type. Strawberry fruit is so amazing fruits all over the world due to high aromatic properties and very much source of some vitamins and minerals and it also maintains very much source of dietary components and very high in vitamin C contents [2]. It also comprises so much significantly high levels of ellagic acid. This ellagic acid is thought as one of the anti-carcinogenic agents [3].

The Strawberry plants are highly cultivated in many countries around the world because the fruits from them are very important healthy properties such as digestive and tonic properties and also they have very high nutritional properties. The strawberry fruits are a rich source of phytochemical and antioxidant properties, vitamin C, fiber content, folic acid, potassium, flavonoids and anthocyanin.

Major problems encountered with micropropagation are the bacterial and fungal contaminations in the inoculation stage of the explant and multiplication stage of the culture. After initiation of the strawberry explants in the sterile culture medium, some whitish strain of bacteria or the spore bearing grayish fungal contamination was observed on the culture medium around the explants in the culture bottle after 2-4 days culture incubation. The problem is further exacerbated when the explant materials are directly taken from the sources of field grown plants [4].

Strawberries are generally cultivated by vegetative propagation means using runners produced from stolon of established plants. So the descendants of these plants are progenies of the characters of mother plant, viral diseases can then be frequently transmitted through the runners and the multiplication rate through the conventional way is too slow. The diseases acquired by means of vegetatively propagation are often infected by virus and mycoplasma [5]. These diseases cause significant reduction in yield. Healthy stock plants used for propagation through conventional ways are not available. Therefore there were so many attempts for the micropropagation of strawberry plants and these were introduced in 1974 [6].

Plant tissue culture techniques allow rapid multiplication of plantlets obtained from explants of different plant parts through direct or indirect organogenesis. Tissue culture plants are used by many researchers as the source plant material for plant regeneration and transformation studies. In some countries, the in vitro meristem culture has been successful in mass propagation of strawberry plants. Plant tissue culture can also be useful for the micropropagation and the storage of disease-free plant germplasm. There was estimation that several millions of plants can be prepared within one year from a few mother plants using plant tissue techniques [7].

Several studies have been tested that the tissue cultured plants were more advantageous than the plants achieved by using conventional propagation methods in terms of fruit yield, pest resistance, vigor, yield per plant, the number of runners and leaves per plant [8]. Callus culture can be obtained from any parts of the plant and regeneration from the callus tissue can play an important role in the propagation and improvement of crop plants [9] Plant regeneration from strawberry callus was reported by Jones and his colleagues in 1988 for the first time and Nehra and his colleagues showed that callus culture of *Fragaria x ananassa* Redcoat completely lost its regeneration capacity after 24 weeks of the culture period [10].

Micropropagation of strawberry from runner explants for the culture initiation has been reported and it may be applied to efficiently generate a large number of disease free plants. However there was a limitation to obtain runner explants in certain season because the strawberry only produces runners during vegetative developmental phase. If was can use the explant materials from offshoot, we can overcome this problem. But the offshoots are larger in size than the runners, so it is not easier for disinfection. In addition the browning at the establishing stage of in vitro culture is the major problem which leading to explant death.

Paredes and Lavin reported that explants of wild strawberry were surface sterilized using 70% ethanol plus the use of an antioxidant solution and 25% sodium hypochlorite solution for 15 mins and then rinsed with sterile distilled water [11]. Ko and his colleagues examined that an effective method of disinfection and micropropagation with enhanced survival rate of explants and reduced browning in strawberry in which the explants were surface sterilized using sodium hypochlorite (0.5%) with the use of a few drops of Tween 20 for 7 mins. Aarifa and his colleagues revealed that the effect of mercuric chloride (0.1%) and sodium hypochlorite (1.5%) alone and in combination with ethyl alcohol (70%) for varying duration on disinfecting the strawberry explants. To overcome the problems associated with the microbial contamination in in vitro cultures of strawberry, it becomes an imperative work to develop a sufficient protocol for disinfecting the field grown explant materials. Keeping in mind the problems of microbial contamination for the culture establishment, the present work was conducted to examine an efficient disinfection protocol to increase survival rate of explants.

MATERIALS AND METHODS

The experiment was performed at the Plant Tissue Culture Laboratory of the Biotechnology Research Department, Kyaukse Township, Mandalay Division under the Ministry of Education, Myanmar in 2017. The strawberry explant materials for use in in vitro culture were collected from the strawberry growers in Pyin Oo Lwin Township, Myanmar.

Runner tips, shoot tips, leaf segments and nodal segments were used as explant materials for culture initiation. The explants were excised into suitable segments using surgical blades and washed thoroughly under tap water. After that, the explants were gently rinsed with mild detergent for about 2 minutes and then washed with distilled water for 4 to 5 times. After rinsing the explants in distilled water, the explants were cut by reducing in size and removed excessive plant materials with surgical blade to achieve the size of 1.0 – 1.5 cm. The excised explants were soaked in 10 ml/L Homine (fungicide solution) for 2 hours and then rinsed with purified water for 3-4 times. After that, they were dipped in 500mg/l Ciprofloxacin tablet for 1 hour and then washed with purified water for 3-4 times.

After these steps, the explants were put into clean bench chamer and they were followed to the remaining sterilants such as 20% Chlorox (sodium hypochloride solution), 70% ethanol and 0.1% mercuric chloride for various time taken respectively as shown in Table 3, each followed by 3-4 times washed with sterilized distilled water. In this study, I used 2 protocols for surface sterilization of various strawberry explants. Each protocol is the same concentrations and time durations why using fungicide and antibiotics, but there were slightly different in concentrations used and time taken for chlorox, ethanol and mercuric chloride. After all surface sterilization procedures, the explants were put on to the MS medium supplemented with 30g/L sucrose, 6g/L agar and various concentrations of BAP for shoot regeneration. The concentrations and compositions of MS stock solutions for use as basal medium was given in Table 1 [12]. The culture bottles were maintained under continuous cool and white fluorescent tube light (1500 Lux) and 27±1˚C temperature. Observations were recorded on the survival %, contamination % and mortality % of the cultures after 4 weeks of culture periods.

Table 1: Compositions and Preparations of MS (Murashige and Skoog 1962) Stock Solution

Stock Group	Ingredients	Ingredient Weight (g/l)	Water used Volume (ml)	Stock Solutions (ml) taken for 1 L of Medium	Final Concentration of Ingredients in Medium (mg/l)
A	$NH_4 NO_3$	165.00	1000	10	1650
	KNO_3	190.00	1000	10	1900
B	$MgSO_4.7H_2O$	37.00	1000	10	370
	$MnSO_4.4H_2O$	2.23	1000	10	22.3
	$ZnSO_4$ $5H_2O$	0.86	1000	10	8.6
	$CuSO_4$ $.5H_2O$	0.0025	1000	10	0.025

C	$CaCl_2 .2H_2O$	44.00	1000	10	440
	KI	0.083	1000	10	0.83
	$CoCl_2 .6H_2O$	0.0025	1000	10	0.025
D	$KH_2 PO_4$	17.00	1000	10	170
	H_3BO_4	0.62	1000	10	6.2
	$Na_2MoO_4.2H_2O$	0.025	1000	10	0.25
E	$FeSO_4 .7H_2O$	2.784	1000	10	27.84
	$Na_2EDTA.2H_2O$	3.74	1000	10	37.4
F	Thiamine HCl	0.10	1000	10	1
	Nicotinic-acid	0.05	1000	10	0.5
	Pyridoxine-HCl	0.05	1000	10	0.5
	Glycine	0.20	1000	10	2
G	Myo-inositol	10.00	1000	10	100
	Sucrose				30000
	Agar				6000

Table 2: Different Concentrations of Sterilants and Time Durations

Sterilents	**Time Duration (mins)**
10ml/L Homine (Fungicide)	120
500mg/L Ciprofloxacin (Antibiotics)	60
10 %Chlorox with 2 drops of Tween 20	10
20 %Chlorox with 2 drops of Tween 20	5
70% Ethanol	10
70% Ethanol	5
0.1% Mercuric chloride	5
0.1% Mercuric chloride	4

RESULTS AND DISCUSSIONS

To optimize the efficient surface sterilization protocol, various sterilants were used for various time durations on the different strawberry explant types such as shoot tip, runner tip, nodal segment and leaf segment using MS medium as a basal medium Table (3). For the explant survival, treatment protocol II (25%, 55%, 20%) was better than protocol I (20%, 50%, 14%) on the shoot tip, nodal segment and leaf segment of strawberry explants (Figure 2).

But for the runner tip, protocol I (15%) was better treatment than protocol II (10%). The highest percentage of contamination was found in the treatment of protocol I (68%) on the explant type of leaf segment. The lowest contamination percentage rate were obtained when the nodal segment and shoot tip of strawberry explants were treated with protocol I (10% respectively). (Figure 3) The mortility percentage were significantly lower with the treatment of both protocol I and II (40%, 34%) on the strawberry nodal segment compared with other explant types. The highest mortality rate was found in the treatment of protocol II (75%) on the runner tip explants. (Figure 4)

The results in this study were similar with the work of Rattanpal and his colleagues. He studied strawberry by using tissue culture technique with the use of meristems as explant materials. He observed that the explant materials which were treated with mercuric chloride of the concentration of 0.1 percent for 4 minutes gave the best results with highest survival percentage along with very little tissue necrosis [13]. And also Gautam *et al*. reported that the strawberry explants were treated with the concentration of 0.1 percentage of mercuric chloride for 3 minutes gave the best for minimum contamination with maximum establishment of the culture [14].

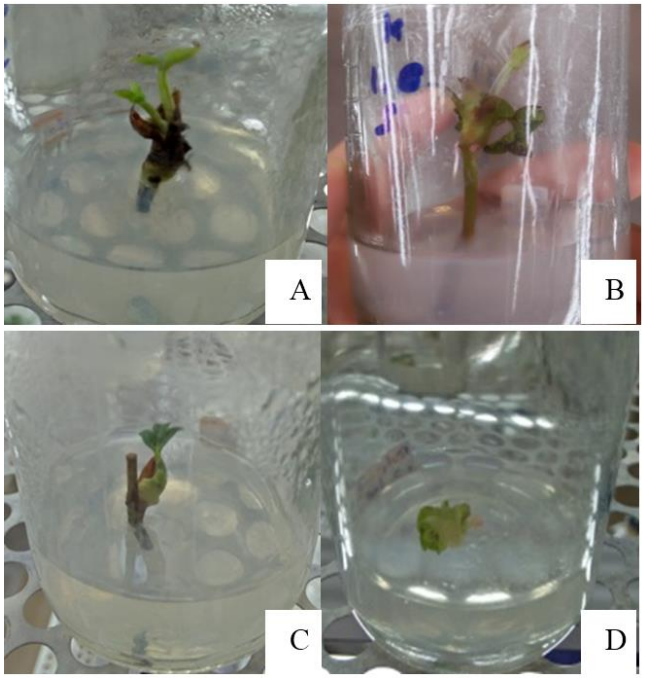

Figure 1: Different Types of Strawberry Explants on MS basal medium supplemented with 2mg/L BAP Incubated after 4 weeks of Culture Period. A. Shoot tip explant, B. Runner tip explant, C. Nodal Segment, D. Leaf Segment.

Table 3: Effects of Two Protocols Using Different Concentrations and Time Durations of Sterilants on Strawberry Explant Inoculation

Protocol	Sterilizing agent	Time duration (min)	Shoot tips			Runner tips			Nodal segments			Leaf segments		
			% sur	% con	% mor	% sur	% con	% mor	% sur	% con	% mor	% sur	% con	% mor
Protocol I	10ml/L Homine (Fungicide)	120	20	10	70	15	12	63	50	10	40	14	18	68
	500mg/L Ciprofloxacin (Antibiotics)	60												
	10%Chlorox	10												
	70% Ethanol	10												
	0.1%Mercuric chloride	5												
Protocol II	10ml/L Homine (Fungicide)	120	25	14	61	10	15	75	55	11	34	20	16	64
	500mg/L Ciprofloxacin (Antibiotics)	60												
	20%Chlorox	5												
	70% Ethanol	5												
	0.1%Mercuric chloride	4												

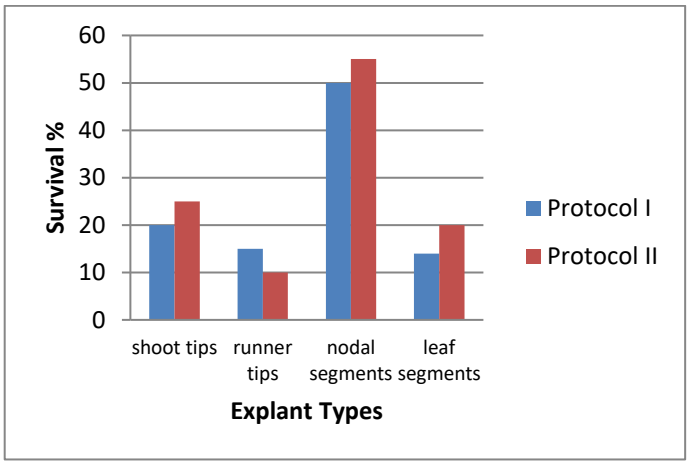

Figure 2: Effect of Two Surface Sterilization Protocols on Survival% of Various Strawberry Explants.

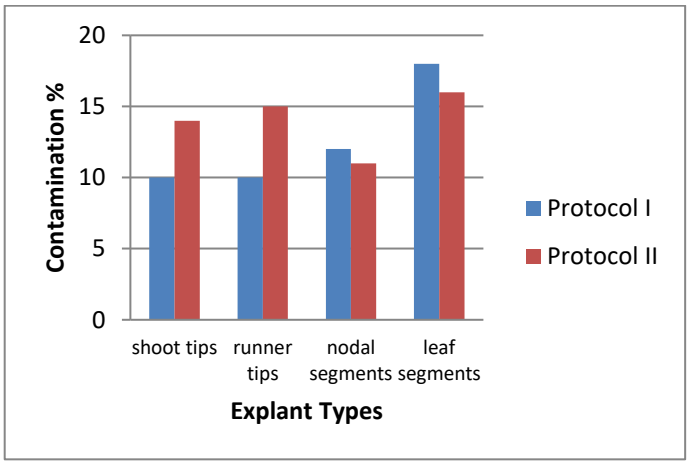

Figure 3: Effect of Two Surface Sterilization Protocols on Contamination % of Various Strawberry Explants

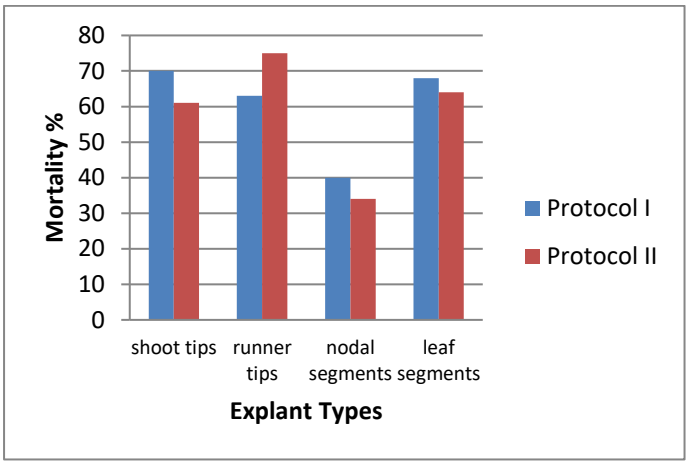

Figure 4: Effect of Two Surface Sterilization Protocols on Mortility % of Various Strawberry Explants

CONCLUSIONS

In this study, various sterilants were grouped as protocols for the surface sterilization of different types of field grown strawberry explants. The maximum survival rate with the minimum mortality rate and minimum contamination rate was achieved when the strawberry explants of nodal segment were treated with protocol II. The lowest survival percentage was found with the runner tip explants using protocol II. So, it can be concluded from the above study that the nodal segment was the best explant for strawberry tissue culture and the protocol II was the best sterilization protocol for surface sterilization of field grown strawberry explants.

Acknowledgment

I wish to express my deepest and heartfelt gratitude to our director, Dr. Aye Aye Khai, Director and Head, Biotechnology Research Department, Kyaukse Township, Mandalay Division, Myanmar, for her permission and encouragement throughout this study. I wish to convey special thanks to our colleagues, Dr. Kyaw Swar Oo and Dr. Yin Yin Mon and our lab team members from Plant Tissue Culture Laboratory, Biotechnology Research Department for their good wills and kind help during this research.

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