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Surface sterilization method for reducing microbial contamination of field grown strawberry explants intended for *in vitro* culture

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An effective disinfection method for strawberry (*Fragaria x ananassa* Duch.) *cv.* Senga Sengana micropropagation using runner tips and nodal segments as explants was developed. The explants were surface sterilized with different sterilants for different durations. The present studies on the effect of different regimes of sterilization revealed that maximum aseptic cultures were obtained from both explants runner tips and nodal segments when treated with 1.5% sodium hypochlorite for 20 min plus ethyl alcohol 70% for 30 s, but the surviving percentage was less because this treatment resulted in necrosis and tissue injury of explants. However, mercuric chloride (0.1%) for 4 min resulted in less percentage of aseptic cultures but gave highest percentage of surviving explants as most of researchers have found that a single sterilant is more effective than the combination. Surface sterilization with mercuric chloride (0.1%) for 4 min was the optimum duration which resulted in highest percentage of explant survival.

Key words: *In vitro*, senga sengana, strawberry, sterilization.

INTRODUCTION

Microbial contaminations present a major challenge to the initiation and maintenance of viable *in vitro* cultures. These contaminants are particularly dangerous when they are plant pathogens. The problem is further exacerbated when explants material is sourced directly from field grown plants. Contamination in this paper refers to fungi or bacteria naturally present on the surface and natural openings on the explants material, which become manifested after initiation and can either, be overt or covert. Overt refers to contamination that can be identified by visible inspection, whereas covert refers to latent contamination, which requires special indexing and/or assaying techniques for identification. The cultivated strawberry (*Fragaria x ananassa* Duch.) a member of Rosaceae is the most important soft fruit worldwide (Hancock, 1990). They are valued for delicious flavour and fragrance and for health resorting qualities. These qualities have ensured that the economic importance of this crop has increased throughout the world and nowadays, it remains a crop of primary interest for both research and crop production. It offers quicker return on capital investment than any other fruit crop. Since, under special methods of cultivation, a crop can be picked as early as first summer after planting.

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Table 1. Different sterilents and their combination for varying time duration.

Sterilants and their combination	Time duration
Mercuric chloride (0.1%)	2 min
Mercuric chloride (0.1%)	3 min
Mercuric chloride (0.1%)	4 min
Sodium hypochlorite (1.5%)	10 min
Sodium hypochlorite (1.5%)	15 min
Sodium hypochlorite (1.5%)	20 min
Mercuric chloride (0.1%) + ethyl alcohol (70%)	2 min + 30 s
Mercuric chloride (0.1%) + ethyl alcohol (70%)	3 min + 30 s
Mercuric chloride (0.1%) + ethyl alcohol (70%)	4 min + 30 s
Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	10 min + 30 s
Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	15 min + 30 s
Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	20 min + 30 s

The majority of strawberry cultivars are generally propagated by runners (Gautam et al., 2001). Vegetative propagation by runners produced from stolons of established plants, though perpetuates all the characters of mother plant, viral diseases can be frequently transmitted through the runners and the rate of multiplication through conventional method is too slow. The strawberry plants propagated vegetatively are often infected by virus and mycoplasma diseases (Biswas et al., 2007). These diseases result in significant reduction in yield. Healthy stocks used for propagation through conventional methods are not available. Micropropagation of strawberry plants were introduced in 1974 (Boxus, 1974). Tissue culture techniques allow rapid multiplication of plantlets obtained from different explants through direct or indirect morphogenesis. The division of offshoots and runners of strawberry are not always suitable for this type of cultivation due to their vulnerability and susceptibility to pathological agents. Several studies have attested the tissue cultured plants being more advantageous than those by conventional propagation in terms of fruit yield (Moore et al., 1991), pest resistance (Rancillac et et al*.,* 1987), vigor, yield per plant, the number of runners and leaves per plant (Zebrowska et al., 2003). Micropropagation of strawberry from runners for initiation has been reported and may be applied to efficiently generate a large number of disease free plants (Adams, 1972; Boxus, 1974). However, they are often limited in certain season because the strawberry only produces runners during the vegetative development phase. If we can obtain explant materials from offshoot, this problem will be overcome. But the offshoot larger than runner size is also more difficult for disinfection. In addition the browning at initial establishing stage of *in vitro* culture is the main cause leading to explant death (Zaid, 1984; Pirtilla, 2008). According to Paredes and Lavin (2005), explants of wild strawberry were surface sterilized with 70% ethanol, use of an antioxidant and sodium hypochlorite (25%) for 15 min and rinsed in sterile and distilled water. An effective method of disinfection and micropropagation with enhanced survival rate of explants and reduced phenol induced browning in strawberry was developed (Ko et al., 2009) in which the surface sterilization of the explants was done in sodium hypochlorite (0.5%) containing a few drops of Tween 20 for 7 min. However, in the present study, effect of mercuric chloride (0.1%) and sodium hypochlorite (1.5%) alone and in combination with ethyl alcohol (70%) for varying time duration was studied on disinfecting the explants. To avoid the problems of microbial contamination in *in vitro* cultures, it becomes imperative to develop a protocol for disinfecting the field grown explants intended for *in vitro* culture. Keeping in view the problems of microbial contamination in *in vitro* cultures, an efficient and simple disinfection protocol to increase survival of explants was developed in this study.

MATERIALS AND METHODS

Runner tips and nodal segments were used as explant for *in vitro* culture. They were collected from field grown strawberry plants *cv.* Senga Sengana planted at Division of Fruit Science Farm Sher-e-Kashmir University of Agricultural Science and Technology Kashmir (J&K). The explants were washed with tween 20 detergent for 5 min then rinsed with water for 4-5 times. After washing, the explants were reduced in size by removing tissues of size (0.5-1.0 cm) with the help of surgical blade and forceps before inoculation. After washing the explants, they were brought to laminar flow cabinet and were subjected to surface sterilization. The explants were subjected to different sterilants and their combinations for varying time durations as shown in Table 1, followed by a 5 min rinse in sterile distilled water under aseptic conditions in the laminar flow chamber. The explants were put on medium in such a manner that conformed to the original polarity and exposed above the surface of medium. MS basal medium (Murashige and Skoog, 1962) was used during the study. The composition and preparation of stock solutions for MS (1962) medium is given in Table 2. Appropriate quantities of various stock solutions and plant growth regulators were pipetted out and stirred with 400 ml distilled water. After adding sucrose at a concentration of 3%, pH was adjusted to 5.7 with 0.1 N NaOH and 0.1 HCl. Lastly agar agar at concentration of 0.7% was added and the final volume was made to 1 L with distilled water. The medium was sterilized in an autoclave at 15 psi (121°C for 15 min). The table surface of laminar flow cabinet was first swabbed with 95% ethanol and all the required materials except

Table 2. Composition and preparation of stock solutions for MS (1962) medium.

Stock solution designation	Ingredient	Weight of ingredient (mg)	Volume of water used (ml)	Volume of stock solution taken for making 1 lt of medium (ml)	Final conc. of the ingredient in the medium (mg l^{-1})
I	**Macronutrient**				
A	$NH_4 NO_3$	16500	500	50	1650
	KNO_3	19000			1900
B	$MgSO_4 7H_2O$	3700	500	50	370
	$KH_2 PO_4$	1700			170
C	$CaCl_2 2H_2O$	4400	500	50	440
II	**Micronutrient**				
	$H_3 BO_3$	620			6.20
	KI	83			0.83
D	$Na_2 MoO_4 2H_2O$	25	500	50	0.25
	$CoCl_2 6 H_2O$	2.5			0.025
	$CuSO_4$	2.5			0.025
E	$ZnSO_4$	860	500	50	8.6
	$MnSO_4$	2230			22.30
F	Na_2 EDTA $2H_2O$	373	200	20	37.3
	$FeSO_4 7H_2O$	278			27.8
	Glycine	40			2.0
	Nicotinic acid	10			0.5
G	Thiamine HCL	2	200	20	0.1
	Pyridoxine HCL	10			0.5
	Myo-inositol	2000			100
H	Sucrose	30000			30000
	Agar				8000

living plant tissues were kept inside the chamber and exposed to UV light for 60 min. The laminar flow was switched on 10 min prior to inoculation or sub culturing. The culture room used for incubating the culture was maintained at temperature of 24±1°C by regulating the room air conditioner or thermostatically controlled heater as per requirement. For maintaining light, flourecent light tubes of 3000-3200 lux were fixed to maintain 16 h photoperiod.

Observations were percentages (%) of aseptic cultures, necrotic cultures and explant survival and were made within three weeks of inoculation. Each treatment combination was assigned to 10 explants with one explant per test tube and replicated three times. The data generated was subjected to ANOVA in complete randomized design using R- software at 5% level of significance. To satisfy model, assumptions of experiments were subjected to arc sine and square root transformations. The significant difference among treatments was compared by critical difference.

RESULT AND DISCUSSION

Strawberry explants (runner tips and nodal segments) were subjected to 12 different sterilization regimes using MS (Murashige and Skoog, 1962) as the basal medium. The effect of various sterilization regimes and explants on culture asepsis, necrosis and explant survival (Table 3) was highly significant. The highest percentage of aseptic cultures (78.33%) was obtained by treating the explants with 1.5% sodium hypochlorite for 20 min + 70 % ethyl alcohol for 30 s. The aseptic frequency of runner tips explants was significantly higher (53.05 %) than the nodal segments (47.22 %). Interaction studies showed that the maximum culture asepsis was 80.00% when the runner tips were treated with 1.5% sodium hypochlorite 20 min + 70 % ethyl alcohol for 30 s. The highest necrotic cultures (58.32%) was obtained when explants were surface sterilized with 1.5% sodium hypochlorite 20 min + 70 % ethyl alcohol for 30 s .The lowest mean (8.88%) of necrotic cultures was obtained by treating the explants with mercuric chloride 0.1% for 4 min sterilization regime. The percentage of necrotic cultures of runner tip explants was lower (28.69%) than the nodal segments (31.64%).

Table 3. Influence of different sterilants on per cent aseptic cultures, explant survival and necrotic culture in strawberry *cv.* Senga Sengana.

Sterilants (time duration)	*Aseptic cultures (%)			**Explant survival (%)			**Necrotic cultures (%)		
	Runner tip	Nodal segment	Mean	Runner tip	Nodal segment	Mean	Runner tip	Nodal segment	Mean
Mercuric chloride (0.1%) (2 min)	30.00 (33.20)	26.66 (31.08)	28.33 (32.14)	21.11 (4.69)	17.77 (4.32)	19.44 (4.51)	11.11 (3.47)	14.44 (3.92)	12.77 (3.69)
Mercuric chloride (0.1%) (3 min)	36.66 (37.25)	33.33 (35.25)	35.00 (36.25)	24.44 (5.04)	17.77 (4.32)	21.10 (4.68)	14.44 (3.92)	17.77 (4.32)	16.01 (4.12)
Mercuric chloride (0.1%) (4 min)	43.33 (41.16)	40.00 (39.23)	41.67 (40.20)	37.77 (6.22)	31.11 (5.66)	34.44 (5.94)	6.66 (2.76)	11.11 (3.47)	8.88 (3.12)
Sodium hypochlorite (1.5%) (10 min)	43.33 (41.16)	36.66 (37.26)	40.00 (39.21)	26.66 (5.26)	17.77 (4.32)	22.21 (4.79)	17.77 (4.32)	20.00 (4.58)	18.88 (4.45)
Sodium hypochlorite (1.5%) (15 min)	50.00 (45.00)	40.00 (39.16)	45.00 (42.08)	23.33 (4.93)	11.00 (3.47)	17.16 (4.20)	27.77 (5.36)	31.11 (5.66)	29.44 (5.51)
Sodium hypochlorite (1.5%) (20 min)	53.33 (46.91)	43.33 (41.16)	48.33 (44.04)	20.00 (4.58)	7.77 (2.95)	13.88 (3.76)	34.44 (5.95)	37.77 (6.22)	36.10 (6.08)
Mercuric chloride (0.1%) + ethyl alcohol (70%) (2 min + 30 s)	50.00 (45.00)	43.33 (41.16)	46.67 (43.08)	27.77 (5.36)	18.88 (4.45)	23.32 (4.90)	23.33 (4.93)	26.66 (5.26)	24.99 (5.09)
Mercuric chloride (0.1%) + ethyl alcohol (70%) (3 min + 30 s)	53.33 (46.91)	46.66 (43.08)	50.00 (45.00)	31.11 (5.66)	18.88 (4.45)	24.99 (5.06)	24.44 (5.04)	34.44 (5.95)	29.44 (5.49)
Mercuric chloride (0.1%) + ethyl alcohol (70%) (4 min + 30 s)	60.00 (50.79)	56.66 (48.83)	58.33 (49.81)	34.44 (5.95)	28.88 (5.46)	31.66 (5.70)	27.77 (5.36)	31.11 (5.66)	29.44 (5.51)
Sodium hypochlorite (1.5%) + ethyl alcohol (70%) (10 min + 30 s)	66.66 (54.73)	56.66 (48.83)	61.66 (51.78)	17.77 (4.32)	14.44 (3.92)	16.10 (4.12)	51.11 (7.21)	45.33 (6.65)	48.22 (6.93)
Sodium hypochlorite (1.5%) + ethyl alcohol (70%) (15 min + 30 s)	70.00 (56.80)	66.66 (54.73)	68.33 (55.77)	23.33 (4.93)	17.77 (4.32)	20.55 (4.63)	47.77 (6.98)	51.11 (7.21)	49.44 (7.10)
Sodium hypochlorite (1.5%) + ethyl alcohol (70%) (20 min + 30 s)	80.00 (63.44)	76.66 (61.12)	78.33 (62.28)	22.22 (4.81)	21.11 (4.79)	21.66 (4.75)	57.77 (7.66)	58.88 (7.73)	58.32 (7.70)
Mean±SD	53.05±14.31 (46.86±8.53)	47.22±14.4 (43.41±8.54)		25.82±6.0 (5.15±0.56)	18.59±6.49 (4.36±0.74)		28.69±16.26 (5.25±1.51)	31.64±14.84 (5.55±1.30)	

Values in the parenthesis are *arc sine and **square root transformed.

Interaction studies show maximum percentage of necrotic cultures (58.88%) when the runner tips and nodal segments were treated with 1.5% sodium hypochlorite for 20 min + 70% ethyl alcohol for 30 s, while the lowest necrotic percentage (6.66%) was obtained when explants were surface sterilized with mercuric chloride 0.1% for 4 min.

The highest percentage of surviving cultures (34.44%) was obtained by treating the explants with mercuric chloride (0.1%) for 4 min. The response of runner tips explants was significantly higher (25.82%) than the nodal segments (18.59%). Interaction studies showed that maximum percentage of surviving explants was (37.77%) when the runner tips were treated with mercuric chloride (0.1%) for 4 min. Surfaces of plant carry wide range of microbial contaminants. To avoid these sources of infection, the tissues must be surface sterilized before planting on nutrient medium. The present studies on the effect of different regimes

of sterilization revealed that maximum aseptic cultures were obtained from both the explants (runner tips and nodal segments) when treated with 1.5% sodium hypochlorite for 20 min plus ethyl alcohol 70% for 30 s, but the surviving percentage was less because this treatment resulted in necrosis and tissue injury of explants. However, mercuric chloride (0.1%) for 4 min resulted in less percentage of aseptic cultures but gave highest percentage of surviving explants as most researchers have found that a single sterilant is more effective than the combination. These results are in close conformity with those of Dalal et al. (1992) in grape, Modgil et al. (1994) in apple and Peer (2008) in cherry. Our results are in line with those of Rattanpal et al. (2011) who micropropagated strawberry through meristem culture and found that treating explants with mercuric chloride (0.1%) for 4 min was the most effective surface sterilization procedure for maximum survival of explants with minimum tissue injury. Likewise, Gautam et al. (2001) also found that treating the explants of strawberry with 0.1% mercuric chloride for 3 min gave minimum contamination with maximum culture establishment.

Conclusion

Various sterilization treatments yielded aseptic cultures but the highest percentage of aseptic cultures were achieved by treating the explants with sodium hypochlorite (1.5%) for 20 min plus ethyl alcohol (70%) for 30 s. The maximum percentage of explant survival was achieved when explants were surface sterilized with 0.1% mercuric chloride for 4 min. The use of sodium hypochlorite (1.5%) for 20 min plus ethyl alcohol (70%) for 30 s gave the highest aseptic cultures but resulted in higher necrotic cultures. So it is concluded from the above study that sterilization treatment of 0.1% mercuric chloride for 4 min is effective for disinfecting the field grown strawberry explants intended for *in vitro* culture. This treatment resulted in maximum percentage of explant survival.

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