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Effect of surface sterilant for reducing microbial contamination of field grown strawberry explants intended for *in vitro* culture

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Abstract
An effective disinfection method for strawberry (*Fragaria x ananassa* Duch.) *cv.* Chandler micro propagation 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 minimum contamination percentage, maximum survival percentage, minimum nonresponsive explants and highest shoot length were obtained from explants runner tips and nodal segments when treated with 0.1% mercuric chloride for 7 min and 0.1% mercuric chloride for 7 min plus ethyl alcohol 70% for 30 s respectively. It was found that a single sterilant is more effective than the combination in case of runner tip but combination of mercuric choride and ethyl alcohol was found to be best when the explants nodal segment was used for *in vitro* culture.

Keywords: microbial contamination, strawberry explants, mercuric choride, ethyl alcohol

1. Introduction
Microbial contaminations is the 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) [6]. 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.

The majority of strawberry cultivars are generally propagated by runners (Gautam *et al*., 2001) [5]. 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) [2]. 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) [3]. 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 conven-tional propagation in terms of fruit yield (Moore *et al.*, 1991) [9], pest resistance (Rancillac *et al.*, 1987) [14], vigour, yield per plant, the number of runners and leaves per plant (Zebrowska *et al*., 2003) [17]. 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) [1, 3]. 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) [16, 13]. According to Paredes and Lavin (2005) [11], explants of wild strawberry were surface sterilized with 70% ethanol, use of an anti-oxidant 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) [7] 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.* Chandler planted at Department of Fruit Science and Horticulture Technology, College of Agriculture, OUAT, Odisha. 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. Then, 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 confirmed to the original polarity and exposed above the surface of medium. MS basal medium (Murashige and Skoog, 1962) [10] 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 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 UV light for 20 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 25±2°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 contamination, non-responsive explants and explants establishment were made within three weeks of inoculation. After success the shoot length was also measured. Each treatment combination was assigned to 20 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 transformations. The significant difference among treatments was compared by critical difference.

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

Treatments	Sterilants and their combination	Time duration
T_1	Mercuric chloride (0.1%)	3 min
T_2	Mercuric chloride (0.1%)	5 min
T_3	Mercuric chloride (0.1%)	7 min
T_4	Sodium hypochlorite (1.5%)	10 min
T_5	Sodium hypochlorite (1.5%)	15 min
T_6	Sodium hypochlorite (1.5%)	20 min
T_7	Mercuric chloride (0.1%) + ethyl alcohol (70%)	5 min + 30 s
T_8	Mercuric chloride (0.1%) + ethyl alcohol (70%)	7 min + 30 s
T_9	Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	15 min + 30 s
T_{10}	Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	20 min + 30 s

Table 2: Composition of basal MS medium

MACRO SALT	(mg/l)
KNO_3	1900
NH_4NO_3	1650
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170
MICRO SALTS	(mg/l)
$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025
Na_2Fe-EDTA	37.24
ADDITIVES	(mg/l)
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo-inositol	100
Sucrose	30000

Table 3: Amount of stock solutions added to the media

S no	Stock solution	Strength	Amount to be added (ml)
1.	Macronutrients	20X	50
2.	Micronutrients	1000X	1
3.	Iron source	200X	5
4.	Organic supplements	1000X	1

Agar (8g/l) and Myoinositol (100mg/l) were added separately

Result and Discussion

Strawberry explants (runner tips and nodal segments) were subjected to 10 different sterilization regimes using MS (Murashige and Skoog, 1962) [10] as the basal medium. The

effects of surface sterilizing treatments on culture establishment of explants are presented in Table 3 and 4. Runner tips explants gave significantly highest survival (80.67%) in treatment $HgCI_2$ (0.1%) for 7 min as it showed less contamination of culture (11.0%) and maximum length of shoot (4.1 cm) followed by $HgCI_2$ (0.1 %) for 7 minutes + ethyl alcohol 70% for 30 second. While, in case of nodal segment explants,$HgCl_2$ (0.1%) for 7 minutes + ethyl alcohol 70% for 30 second reported the highest survival (74.5%) with the less contamination (23.50%) and maximum length of shoot (3.99 cm) followed by $HgCI_2$ (0.1 %) for 7 minutes. Although, sodium hypochlorite (NaOCI 1.5 %) for 10 and 15 minute in both (runner tip and nodal segment) types of explants controlled 100 percent of contamination, there was no response for establishment of explants. Hence, $HgCI_2$ (0.1%) for 7 minutes and HgCI2 (0.1%) for 7 minutes + ethyl alcohol 70% for 30 second was found to be better surface sterilizing treatment for runner tips and nodalsegment respectively, with better culture establishment and growth of explants. Among different types of explants, nodal segment explants showed higher contamination, hence, it required more duration of treatment. Similar results were reported by Murkute et al. (2004). The response of runner tip and nodal segment explants of mature plant to surface sterilization treatments was different. The time needed to effective sterilization of nodal segment explants was more than runner tip explants. This may be due to difference in intensity of contaminants present in the explants.

Table 3: Effect of Surface sterilizing agents on contamination and establishment of runner tip explants of Strawberry cv. Chandler

Treatments	Contamination (%)	Non Responsive Explants (%)	Survival (%)	Length of Shoots (cm)
T_1	53.00 (46.91)	32.48 (35.00)	14.52 (23.88)	1.00
T_2	30.33 (33.6)	12.00 (20.26)	57.67 (46.20)	2.45
T_3	11.00 (19.36)	8.33 (16.77)	80.67 (63.10)	4.1
T_4	0.00 (0.00)	100.0 (90.00)	0.00 (0.00)	0.00
T_5	0.00(0.00)	100.0 (90.00)	0.00(0.00)	0.00
T_6	46.00 (42.9)	32.00 (34.44)	22.00 (27.86)	2.80
T_7	26.56 (31.05)	17.77 (30.89)	55.67 (45.33)	2.97
T_8	12.00 (20.10)	8.67 (17.12)	79.33 (61.89)	3.25
T_9	43.33 (41.16)	24.67 (29.78)	32.00 (34.44)	2.10
T_{10}	43.00 (40.16)	15.33 (23.05)	58.33 (50.10)	2.00
CD at 5%	0.85	0.45	1.77	0.22
CV %	1.58	1.82	3.80	5.93

Figure in the parenthesis are arcsine transformed values.

Table 4: Effect of Surface sterilizing agents on contamination and establishment of Nodal Segment explants of Strawberry cv. Chandler

Treatments	Contamination (%)	Non Responsive Explants (%)	Survival (%)	Length of Shoots (cm)
T_1	57.12 (49.09)	12.00 (20.26)	30.88 (30.11)	2.11
T_2	34.00 (35.67)	12.67 (20.84)	53.33 (45.84)	2.90
T_3	20.00(24.12)	9.67 (18.11)	70.33 (57.61)	3.50
T_4	0.00 (0.00)	100.0 (90.00)	0.00 (90.00)	0.00
T_5	0.00 (0.00)	100.0 (90.00)	0.00 (90.00)	0.00
T_6	62.00 (50.43)	22.67 (28.43)	15.33 (23.45)	2.67
T_7	30.30 (30.11)	12.67 (20.85)	57.00 (49.09)	2.81
T_8	23.50 (28.99)	2.00(11.15)	74.50 (60.31)	3.99
T_9	0.00 (0.00)	100.0 (90.00)	0.00 (90.00)	0.00
T_{10}	57.88 (50.11)	20.00 (24.12)	22.12 (26.88)	1.21
CD at 5%	2.81	1.21	0.85	0.31
CV %	3.7	1.93	1.60	5.89

Figure in the parenthesis are arcsine transformed values.

These results are in close conformity with those of Dalal et al. (1992) [4] in grape, Modgil et al. (1994) [8] in apple and Peer (2008) [12] in cherry. Our results are in line with those of Rattanpal et al. (2011) [15] who micropropaga-ted 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) [5] 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 survival cultures were achieved by treating the explants with mercuric chlorite (0.1%) for 7 min and mercuric chlorite (0.1%) for 7 min plus ethyl alcohol (70%) for 30 s when the runner tip and nodal segment were used as explants respectively. The maximum percentage of explant survival was achieved when explants were surface sterilized with 0.1% mercuric chloride for 7 min which yielded maximum length of shoot after successful establishment of explants.

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