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Full Length Research Paper

An effective disinfection protocol for plant regeneration from shoot tip cultures of strawberry

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An effective method of disinfection protocol and micropropagation with an enhanced survival rate of explants and reduced phenol induced browning in strawberry was developed. The survival rate of three genotypes was between 89.2 - 100%. Shoot tip were able to develop into plantlet on a hormone-free MS medium when cultured under dim light (500 lux). Two media, M1 and M2 were chosen to compare the effect on shoot multiplication of three genotypes. The shoot number per responding explants was between 5 - 8 and 11 - 14 after induction.

Key words: Strawberry, micropropagation, shoot tip.

INTRODUCTION

Strawberry belong to the genus *Fragaria* in the Rosaceae family is one of the most important fruit plants for both fresh consumption and food processing in the temperate and subtropical areas. According to Nutrient Database for Standard Reference (1999) the strawberry fruits are rich of vitamin C, B1, B2, protein, calcium, potassium, copper and iron, most of the nutritious elements essential for human being. Conventional method of strawberry cultivation is by use of vegetatively propagated plantlet for the field planting. Propagation of strawberry is achieved either by runners or by *in vitro* micropropagation. 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 al., 1987), vigor, yield per plant, the number of runners and leaves per plant (Zebrowska et al., 2003). Moreover, *in vitro* techniques are one of the reliable sources used for commercial plantlet production.

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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 (Pirttilla et al., 2008; Zaid, 1984). Thus, establishing a simple and fast disinfection protocol to increases survival of explants is very important for commercial production.

MATERIALS AND METHODS

Pot grown strawberry genotypes (Taoyuan No. 1, Taoyuan No. 3 and Chun-Hsiang) from Taiwan were maintained in a greenhouse at National Agriculture and Animal Resources Research Center, Ministry of Agriculture, Kingdom of Saudi Arabia. The offshoots of stock plants about 3-4 months old were used for initiation. After trimming the leaves, offshoots were cut into pieces (2.0 - 2.5 cm) each with a single segment. These explants were surface sterilized in sodium hypochlorite (0.5%) containing a few drops of Tween 20 for 7 min and rinsed 4 - 5 times with sterile water and subsequently outer leaves were separated from the dome in a circular fashion using a sterile surgical knife under a laminar flow. Shoot tip of about

2 – 3 mm were again surface sterilized in sodium hypochlorite (0.25%) containing Tween 20 for 1 min and rinsed 2 - 3 times with sterile water. The wound sites exposed to sterilization agent were trimmed and shoot tip with 1.5 - 2 mm base was used for explants and subsequently cultured onto MS medium (Murashige and Skoog, 1962) containing 1.5 g/l Phytagel (Sigama Chemical Co., USA) and pH adjusted to 5.6 prior to autoclaving at 121°C for 20 min. All chemicals used were of analytical grade (Sigma chemical co., USA; BDH Co., England).

To compare the survival rate the explants of three cultivars were cultured at 25 ± 2°C under 16 h photoperiod of 500 and 2000 lux by cool-white fluorescent lamps (GE lighting, F40D-EX). Nine shoot tips for each cultivar were placed in nine tubes each containing 10 ml of the media. Each treatment was replicated three times. The survival rate of explants was recorded 2 weeks after culture. The data were analyzed statistically according to a package by CoStat (Cohort Software, Minneapolis, MN). The developed plantlets from survived explants were cultured on MS medium supplemented with 0.5 mg/l 6-benzyladenine (BA) and 0.02 mg/l α-naphthalene acetic acid (NAA) for multiplying shoots and incubated in the culture room under cool-white fluorescent lamps (2000 - 2200 lux). To compare shoot multiplication rate three strawberry genotypes were cultured on two media M1 (MS+0.5 mg/l, BA+0.02 mg/l, NAA+7 g/l, Agar) and M2 (MS+1.0mg/l, BA+0.1mg/l, NAA+7g/l, Agar). Single shoot of about 0.5 cm long was placed in each tube containing 10 ml of the media. Each treatment was replicated ten times. The culture was held at 25 ± 2°C under light 16 h (day)/8 h (night) photoperiod with light source provided by irradiation intensity of 2000 - 2200 lux. The percentage of explants forming shoots was recorded 4 weeks after culture. The data were analyzed statistically according to a package by CoStat (Cohort Software, Minneapolis, MN). Regenerated shoots were cultured onto MS medium supplemented with 0.02 mg/l NAA, 0.1g/l activated charcoal and 30 g/l sucrose for the induction of roots.

RESULTS AND DISCUSSION

Shoots tips were able to develop into plantlets on a hormone-free MS medium when cultured under dim light (500 lux) after 2 weeks (Figure 1a). At this time, phenol secreted into the medium induced browning on most explants of the three genotypes and subsequent low recovery at initiation stage. The survival rate of three genotypes was between 89.2 - 100% (Table 1) when cultured under dim light (500 lux). The results showed that light intensity significantly affected survival rate of explants by induction of the phenolic. In the strawberry micropropagation process the browning at initial stage is the main cause the explants perished. In order to alleviate the browning of explants, methods like exposure to low temperature (Dhar and Upreti, 1999), PVP+sucrose (Anderson, 1975), or antioxidant (Ziv and Halvy, 1983) were adopted to address the browning issue. Bhatt and Dhar (2000) tested all the adopted methods in *Fragaria indica,* only to discover poor survival rate of explants ranging from 19 to 40% among the methods. Therefore, they developed an optimized protocol in dealing with browning phenomenon for initiation phase of *F. indica*. But the method is complicated both in sterilization and shortcomings of using chemicals like $HgCl_2$, bavestin, savlon and the time of reaction for 85 min was considered too long. Another drawback about this method is replacement of cultured explants every other 24 h until browning decreased, which will expose the explants to threat of contamination and incur extra labor cost. So the disinfection process we developed only uses bleaching agent like sodium hypochlorite and it takes merely 7 - 9 min. Furthermore, it is more suitable for the commercial mass propagation. Theoretically plant genotype varies in antioxidant capacity and phenolic productions during *in vitro* culture (Scalzo et al., 2005). When the cellular structure is damaged, phenolic compounds will be secreted to induce browning leading the explants to death. The disinfection process we developed can also reduce explant browning and thus increased significantly the survival rate of explants.

Table 1. The survival rate of meristem tips during initiation stage of three strawberry cultivars.

Light (Lux)	Cultivars	Survival explants (%)
500*	Taoyuan No. 1	89.2[a]
	Taoyuan No. 3	100.0[a]
	Chun-Hsiang	89.2[a]
2000*	Taoyuan No. 1	44.4[b]
	Taoyuan No. 3	55.5[b]
	Chun-Hsiang	44.4[b]

*MS medium + 1.5 g/l Phytagel.
Mean separation in columns by Duncan's multiple rang test at P > 0.05.

Two media, M1 and M2 were chosen to compare the effect on shoot multiplication of three genotypes. The results showed shoot multiplication occurred in all three genotypes (Figure 1b). The shoot number of per responding explants was between 5 - 8 and 11 - 14 (Table 2). Average shoots lengths was between 1.15 – 1.38 and 1.20 - 1.41 cm. Low concentration of BA increased shoot elongation rate but caused a low frequency of shoot formation. With an increase of BA concentration in the M2 medium, the number of shoots of the three genotypes also increased significantly. Significant variation in the number of shoots formed was observed in the three examined genotypes. The effect of genotype differences on the capacity of shoot multipli-cation was also evident with other research report (Passey et al., 2003).

The shoots from the seventh subculture was transferred onto an MS medium supplemented with 0.02 mg/l NAA, 0.1 g/l activated charcoal and 30 g/l sucrose for inducing adventitious roots (Figure 1c and d). Roots were initially induced on cutting surfaces of shoot base 5 - 7 days after culture. About 99% of shoots could be induced to form adventitious roots. The plantlets were maintained in the same medium and extra time of illumination so that their rooting systems may be developed properly. The *in vitro* grown plantlets were then planted in 1.5 inches plastic trays with peat moss, vermiculite and perlite mixture (1:1:1 ratio) and transferred to greenhouse under 70% shade for acclimation.

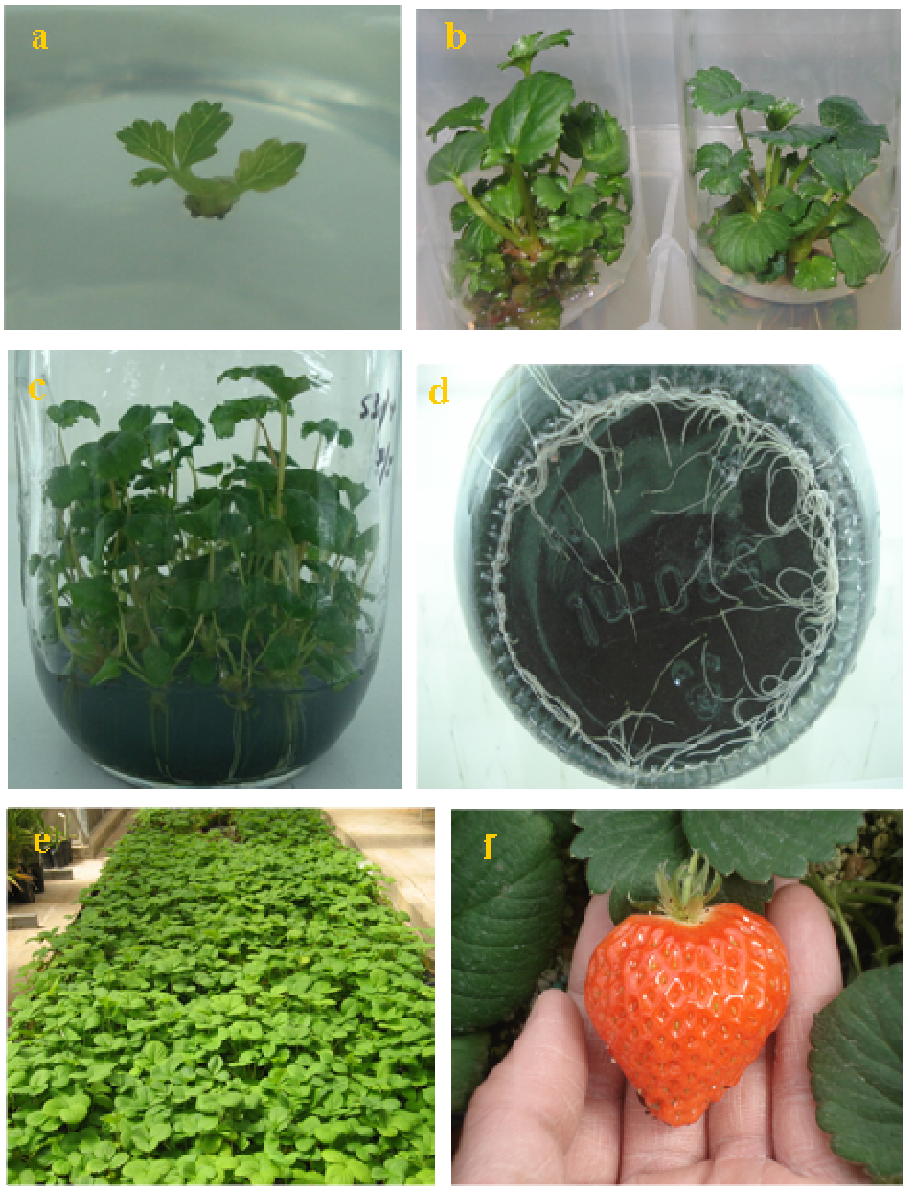

Figure 1. Different stages of *in vitro* micropropagation of strawberry. **a)** Shoot tip placed in medium which induced plantlet. **b)** After subculture, shoot multiplication was achieved. **c** and **d)** Rooting stage. **e)** Hardening process and acclimatized to new environment. **f)** Tissue culture plant bearing fruit.

Table 2. Shoots multiplication of three strawberry genotypes cultured on M1 and M2 medium.

Media	Cultivars	Shoots/responding explant	Shoot length (cm)
M1[a]	Taoyuan No. 1	6[b]	1.36[a b]
	Taoyuan No. 3	8[a]	1.38[a]
	Chun-Hsiang	5[c]	1.15[b]
M2[b]	Taoyuan No. 1	14[a]	1.27[a]
	Taoyuan No. 3	14[a]	1.41[a]
	Chun-Hsiang	11[b]	1.20[a]

[a]Ms + 0.5 mg/l BA+0.02 mg/l NAA.
[b]Ms + 1.0 mg/l BA+0.1 mg/l NAA.
Mean separation in columns by Duncan's multiple rang test at P > 0.05.

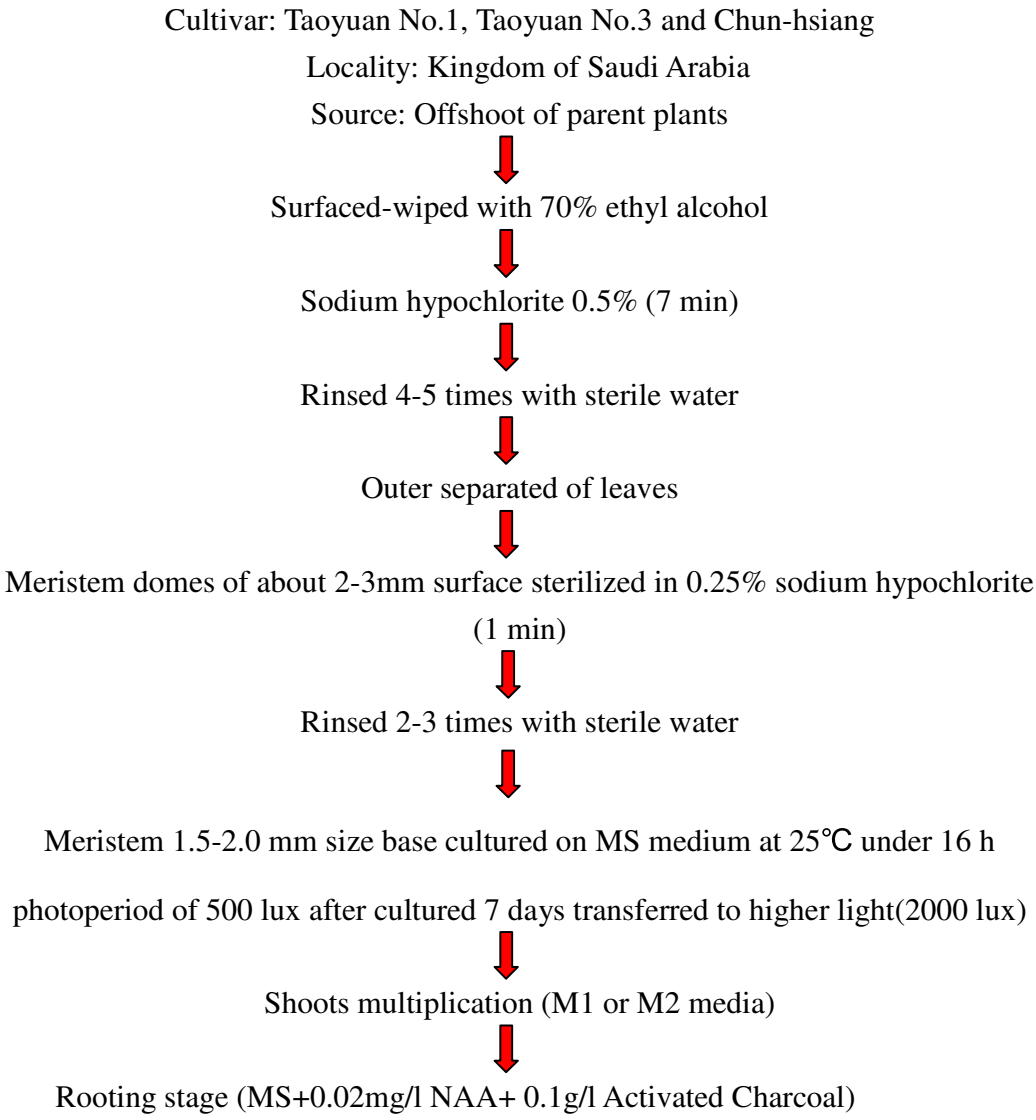

Figure 2. Schematic representation of disinfection protocol and meristem culture of three strawberry cultivars.

The survival rate was between 95 - 98% (Figure 1e). The regenerated plants started to produce fruits in 4 to 5 month after transplanting (Figure 1f). A summary of the optimized protocol of strawberry mass propagation is shown in Figure 2. In conclusion, we have developed the protocol of efficient and effective shoot multiplication and high survival rate of the shoot tip at initiation stage for commercial micropropagation in strawberry.

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