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EFFICIENT STERILIZATION PROTOCOL FOR MICRO PROPAGATION OF GRAPEVINE CVS. RED GLOBE AND CRIMSON SEEDLESS

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ABSTRACT

Optimizing an efficient standard protocol for disinfection of field grown explants is an important task to mimic the contamination level for any perennial crops. Field grown explants collected from plants grown in open field condition harbour large consortia of microbial load which are to be removed without disturbing the potency of the explant.Two types of explants *viz*. axillary buds and shoot tips were studied and compared. Of these, axillary buds registered higher response (86.00% shoot proliferation in Red Globe and 75.00% in Crimson Seedless) compared to shoot tips (56.66% shoot proliferation in Red Globe and 47.77% in Crimson Seedless) and were found to be the most suitable explants for micropropagation. Treating the explants with 0.5% Carbendazim solution for 30 minutes and 70% ethanol followed by 0.1% $HgCl_2$ sterilization for 10 minutes under laminar air floor chamber gave the least contamination with the highest survival rates for axillary buds and shoot tips.

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INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most important perennial deciduous woody vine and delicious refreshing sub-tropical fruits of the world. It is a much preferred fruit and is a good source of the minerals calcium, phosphorous and iron, antioxidants and the vitamins B_1 and B (Khasif *et al*., 2005) . Grapes are mainly used for wine production, fresh fruit, dried fruit, and juice production. The major grape growing state in India are Maharashtra, Karnataka, Andhra Pradesh, Tamil Nadu and Mizoram under the area of 117.60 thousand hectares with a production of 2483.10 thousand MT and productivity of 21.1 MT per ha (NHB Database, 2013-14). Grapes are affected by many diseases caused by fungi, bacteria, viruses and nematodes (Krongjai, 2005). Further, in recent past, it has been observed that non-availability of adequate number of true to type and disease free planting materials has been the major constraint for the establishment of ideal vineyards.In vitro technique is an important alternative to conventional methods of plant propagation. The most important step for aseptic culture establishment is sterilization of explants. Successful tissue culture of all plant species depends on the removal of exogenous and endogenous contaminating microorganisms (Constantine, 1986; Buckley and Reed, 1994). For surface sterilization, the nodal segment explants were agitated in a solution of $HgCl_2$ (0.1% v/v) containing few drops of Tween-20 for seven minutes followed by three to four rinsing with double-distilled water (Singh *et al.,*2004). A number of accessions were used in combination with varying durations of the treatments with 0.1 per cent mercuric chloride (Khasif *et al.,* 2005). The most commonly used sterilizing agents for obtaining aseptic tissues are sodium hypochlorite and calcium hypochlorite. Sterilizing the explants with 1 per cent NaOCl for 7 minutes was found to be optimum. This sterilization should be preceded by 70 per cent alcohol for 30 seconds (Kinfe, 2010). Determination of the duration of sterilization of explants is also very essential to avoid the problem of contamination during *in vitro* culture (Loyala and Vazques, 2006). It was observed that the viability of culture increased with the increased duration of treatment with the disinfection (Muhammad Ali *et al*., 2014). Indeed, the order of magnitude in demand for planting materials indicates that micropropagation will inevitably be necessary for mass propagation and disease free plating material in fruit crops. Thus the aim of this study to the development of an efficient sterilization protocol for regenerated disease free planting materials under in vitro has been studied.

MATERIALS AND METHODS

The present study was carried out during 2013 to 2014 at the Tissue Culture Laboratory, Department of Fruit Crops,Horticultural College and Research Institute, TNAU, Coimbatore. Nodal cuttings at semi hard wood stage (two cm long) and young shoots (one to two cm long) were collected from field growing vines maintained at the College Orchard of TNAU for the preparation of explants

(axillary buds and shoot tips respectively). The explants were thoroughly washed with constant stirring in the solution containing two to three ml of Teepol per 100 ml of distilled water for 10 minutes in cleaned bottle followed by rinsing with distilled water completely and then treated with a fungicide solution (0.1 to 0.5 % Carbendazim) for 30 minutes. Then the explants were thoroughly washed with distilled water. The washed explants were taken to the laminar air flow chamber and sterilized with 0.1% $HgCl_2$ for different durations as listed in Table 1.

Details of the observations recorded and the criteria considered for the various observations are the survival percentage of explants, contamination percentage of explants, mortality percentage of explants were counted and calculated. And their regeneration ability was studied by culturing in various levels of BAP. The experiment was laid out in Factorial Completely randomized design (CRD). The data generated were subjected to statistical analysis as per the standard procedures of Panse and Sukhatme (1985). Observations recorded as percentage were subjected to angular transformation (Snedecor and Cochran, 1980).The CD values were worked out for five per cent (0.05) probability and the results were interpreted. Analysis was carried out with AGRES software package and MS Excel® spreadsheet.

RESULTS AND DISCUSSION

Survival percentage of explants

The data on percentage survival of explants revealed significant influence of the sterilant on the varieties (Table 2). Among the two varieties, Red Globe recorded the highest survival percentage (31.79) and Crimson Seedless registered the lowest percentage (29.86) of survival. Among the explants, the axillary buds of cv. Red Globe recorded the highest survival percentage (38.88) and the lowest survival percentage (22.82) was recorded in shoot tips of cv. Crimson Seedless. Among the sterilization treatments, pre-treatment with 0.5 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.1 per cent $HgCl_2$ for 10 minutes (S_9) recorded the highest survival percentage (58.90). The least survival percentage (3.50) was recorded in control (washing with sterile distilled water).There were no significant differences between the interactions of varieties with explants, varieties with sterilization treatments and varieties with explants and sterilization treatments.The interaction between the explants and sterilization treatments was found to be highly significant. The axillary bud explants registered the highest survival percentage (93.50) in S_9 (0.5 per cent of Carbendazim for 30 minutes and surface sterilized with 70 per cent ethanol for 30 seconds followed by 0.1 per cent $HgCl_2$ for 10 minutes) and shoot tips registered the lowest survival percentage (2.00) in control (washing with sterile distilled water). The favourable effect of using combination of Carbendazim and 0.1 per cent mercuric chloride as surface sterilant has also been reported earlier by Jamwal*et al.*(2013). The effectiveness of mercuric chloride as surface sterilant was earlier reported by many workers (Barreto *et al.,* 2006); Nookaraju*et al.,* 2008; Alizadeh*et al.,* 2010; Hasimi, 2011) in grapes. Also reported the favorable effect of $Hgcl_2$ by Kansara, 2014.

Contamination percentage of explants

Data on percentage contamination of explants indicated a significant influence of the sterilant on the two varieties (Table 3.) Among the two varieties, Crimson Seedless recorded the highest contamination percentage (36.25) followed by Red Globe (33.83). There was no significant difference between the two explants for contamination percentage. Among the sterilization treatments, pre-treatment with 0.5 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.1 per cent $HgCl_2$ for 10 minutes (S_9) recorded the lowest contamination percentage (12.12). The highest contamination percentage (93.25) was recorded in control (washing with sterile distilled water). There were no significant differences between the interactions of varieties with explants, varieties with sterilization treatments and varieties with explants and sterilization treatments.The interaction between explants and sterilization treatments was found to be highly significant. The axillary buds recorded the least contamination (2.50%) in S_9(0.5 per cent Carbendazim for 30 minutes + 70 per cent ethanol for 30 seconds + 0.1 per cent $HgCl_2$ for 10 minutes). The highest contamination (96.00%) was recorded in shoot tips washed with sterile distilled water.

Mortality percentage of explants

The data on percentage mortality of explants did not influence the varieties and were found to be non-significant (Table 3). Among the explants, the axillary buds recorded the least mortality percentage (25.90) and the highest mortality percentage (41.84) was recorded in shoot tips in cv. Crimson Seedless.Among the sterilization treatments, pre-treatment with control recorded the lowest mortality percentage (3.00). The highest mortality percentage (74.35) was registered in S_{10} (0.5

Table 1: Details of sterilization treatments

Treatment No.	Concentration of Carbendazim(30 min)	Ethanol (70%)	Duration of $HgCl_2$ (0.1%)
S_1 (Control)	Washing with sterile distilled water		
S_2	0.1%	30 sec.	5 min
S_3	0.1%		10 min
S_4	0.1%		15 min
S_5	0.3%		5 min
S_6	0.3%		10 min
S_7	0.3%		15 min
S_8	0.5%		5 min
S_9	0.5%		10 min
S_{10}	0.5%		15 min

Table 2: Effect of sterilization treatments on survival percentage of explants in Red Globe and Crimson Seedless cultivars of grapes

Sterilization treatments	Survival percentage Red Globe			Crimson Seedless			Mean Survival Percentage
	Axillary buds	Shoot tips	Mean	Axillary buds	Shoot tips	Mean	
S_1	5.00 (12.92)	3.00 (9.97)	4.00 (11.54)	4.00 (11.54)	2.00 (8.13)	3.0 (9.97)	3.50 (10.78)
S_2	35.33 (36.47)	33.33 (35.26)	34.33 (35.87)	33.33 (35.26)	31.50 (34.14)	32.41 (34.70)	33.37 (35.29)
S_3	60.06 (50.80)	22.21 (28.11)	41.13 (39.90)	58.67 (49.99)	21.98 (27.96)	40.32 (39.42)	40.73 (39.66)
S_4	25.55 (30.36)	20.66 (27.03)	23.10 (28.72)	23.34 (28.89)	18.77 (25.68)	21.05 (27.31)	22.08 (28.02)
S_5	20.33 (26.80)	39.98 (39.30)	30.15 (33.30)	19.66 (26.32)	38.78 (38.52)	29.22 (32.72)	29.68 (33.01)
S_6	77.77 (61.87)	26.54 (31.01)	52.15 (46.23)	73.33 (58.91)	25.50 (30.32)	49.41 (44.67)	50.78 (45.45)
S_7	20.05 (26.60)	17.06 (24.40)	18.55 (25.51)	18.87 (25.75)	15.00 (22.79)	16.93 (24.30)	17.74 (24.91)
S_8	35.56 (36.61)	45.66 (42.51)	40.61 (39.59)	34.50 (35.97)	43.33 (41.16)	38.91 (38.60)	39.76 (39.09)
S_9	93.50 (75.23)	28.67 (32.37)	61.08 (51.40)	89.00 (70.63)	24.43 (29.62)	56.71 (48.86)	58.90 (50.13)
S_{10}	15.66 (23.31)	10.00 (18.43)	12.83 (20.99)	14.43 (22.33)	7.00 (15.34)	10.71(19.10)	11.77 (20.06)
Mean	38.88 (38.58)	24.71 (28.81)	31.79(34.32)	36.91 (37.41)	22.82 (28.54)	29.86 (33.12)	30.83 (33.73)

Sources of variation	SEd	CD (P=0.05)
Varieties	0.347	0.690**
Explants	0.347	0.690**
Treatments	0.775	1.542**
Varieties X Explants	0.490	0.976 ^{NS}
Explants X Treatments	1.096	2.182**
Varieties X Treatments	1.096	2.182 ^{NS}
Varieties X Explants X Treatments	1.550	3.086 ^{NS}

NOTE 1: Numbers in parentheses are arcsine transformed values; NOTE 2: *, ** Significant at 5 % level and 1% level, respectively; NS-Non significant; NOTE 3: Carb.-Carbendazim, EtOH - Ethanol, sec - Seconds, min - minutes

Table 3: Effect of sterilization treatments on contamination percentage of explants in Red Globe and Crimson Seedless cultivars of grape

Sterilization treatments	Contamination percentage Red Globe			Crimson Seedless			Mean Contamination percentage
	Axillary buds	Shoot tips	Mean	Axillary buds	Shoot tips	Mean	
S_1	90.00 (71.57)	94.00(75.82)	92.00 (75.57)75.4639 75.4639	93.00(74.66)	96.00 (78.46)	94.50 (76.44)	93.25 (76.94)
S_2	45.66 (42.51)	56.62 (48.00)	51.14 (45.66)	46.67 (43.09)	57.50 (49.31)	52.08 (46.19)	51.61 (45.92)
S_3	30.00 (33.21)	35.56 (36.61)	32.78 (34.93)	34.00 (35.67)	36.67 (37.27)	35.33 (36.47)	34.05 (35.70)
S_4	25.00 (30.00)	20.00 (26.57)	22.5 (28.32)	31.11 (33.90)	22.00 (27.97)	26.55 (31.01)	24.52 (29.68)
S_5	57.77 (49.47)	49.53 (44.73)	53.65 (47.09)	59.77 (50.63)	50.54 (45.31)	55.15 (47.96)	54.40 (47.52)
S_6	10.03 (18.47)	17.91 (25.04)	13.97 (21.95)	16.60 (24.04)	18.00 (25.10)	17.30 (24.58)	15.63 (23.29)
S_7	14.77 (22.60)	12.94 (21.08)	13.85 (21.85)	16.00 (23.58)	14.00 (21.97)	15.00 (22.79)	14.42 (22.32)
S_8	50.00 (45.00)	21.01 (27.28)	35.50 (36.57)	52.00 (46.15)	23.03 (28.68)	37.51 (37.77)	36.51 (37.17)
S_9	2.50 (9.10)	18.00 (25.10)	10.25 (18.67)	5.00 (12.92)	23.00 (28.66)	14.00 (21.97)	12.12 (20.37)
S_{10}	15.34 (23.06)	10.00 (18.43)	12.67 (20.85)	17.67 (24.86)	12.50 (20.70)	15.05 (22.83)	13.87 (21.87)
Mean	34.10(35.73)	33.55 (35.36)	33.83 (35.57)	37.18 (37.57)	35.32 (36.46)	36.25 (37.02)	35.04 (36.30)

Sources of variation	SEd	CD (P=0.05)
Varieties	0.474	0.943**
Explants	0.474	0.943^{NS}
Treatments	1.059	2.108**
Varieties X Explants	0.670	1.333^{NS}
Explants X Treatments	1.498	2.981**
Varieties X Treatments	1.498	2.981^{NS}
Varieties X Explants X Treatments	2.118	4.215 ^{NS}

NOTE 1:*Numbers in parentheses are arcsine transformed values;* NOTE 2: *, ** Significant at 5 % level and 1% level, respectively; NS-Non significant;NOTE 3: Carb.-Carbendazim, EtOH - Ethanol, sec - Seconds, min - minutes

per cent Carbendazim for 30 minutes + 0 per cent ethanol for 30 seconds $_{+}$ 0.1 per cent $HgCl_2$ for 15 minutes). The interaction effect between varieties and explants was significant. The axillary buds of Crimson Seedless recorded the lowest mortality percentage (25.90). The shoot tips recorded the highest mortality percentage (41.63 and 41.84 respectively in Red Globe and Crimson Seedless), which were on par with each other.

The interaction effect between the explants and sterilization treatments was found to be highly significant. The shoot tips registered the highest mortality percentage (80.00, 80.50) in S_{10} (0.5 per cent Carbendazim for 30 minutes + 70 per cent

ethanol for 30 seconds + 0.1 per cent $HgCl_2$ for 15 minutes), which were on par. The least mortality percentage (2.00) was recorded in control for shoot tips. There were no significant differences between the interactions of varieties with sterilization treatments and varieties with explants and sterilization treatments.The basic aim of sterilization is improving the success of cultures by reducing the loss of explants due to contaminations. Disinfection requires the use of chemicals that are toxic to microorganisms but non-toxic to plant material. Tissue culture has become possible with the use of convenient and effective disinfectants such as ethanol, mercuric chloride and sodium hypochlorite (Krikorian, 1962). Though mercuric chloride is an effective sterilant, it is extremely poisonous due to high bleaching action of two chloride atoms and also ions that combine strongly with proteins causing the death of organisms as reported by Pauling (1955). Therefore, in this study, low concentration (0.1 per cent) of mercuric chloride has been used for sterilization of explants without killing or damaging the young tissues of explants.

In this study, among the ten sterilization treatments used for sterilizing axillary buds, pre-treatment with 0.5 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.1 per cent mercuric chloride for 10 minutes recorded the highest survival and the lowest contamination percentage. For shoot tips, sterilization with 0.1 per cent mercuric chloride for five minutes recorded the maximum survival percentage. Thus it was observed that the survival and contamination rates of explants varied with the types of explant and duration of sterilization. The above results are in line with the findings by Mhatre *et al.* (2000) in the grapes cultivars Thompson Seedless, Sonaka and Tas-e-Ganesh. The favourable effect of using combination of Carbendazim and 0.1 per cent mercuric chloride as surface sterilant has also been reported earlier by Jamwal*et al.* (2013).The effectiveness of mercuric chloride as surface sterilant was earlier reported by many workers (Khasif *et al.,* 2005; Barreto *et al.,* 2006; Nookaraju *et al.,* 2008; Alizadeh*et al.,* 2010; Hasimi, 2011) in grapes.

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