International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2020; 8(1): 726-729

Received: 01-11-2019
Accepted: 03-12-2019

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Standardization of surface sterilization protocol for *in vitro* mass multiplication of grape rootstock dog ridge (*Vitis champini* P.)

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DOI: https://doi.org/10.22271/chemi.2020.v8.i1k.8349

Abstract
To standardize surface sterilization protocol in grape Dog Ridge rootstock, two types of explants *viz*., axillary buds and shoot tips were subjected to surface sterilization with sodium hypochlorite (0.25% and 0.5%) for three different duration (5,10 & 15 minutes). Prior to surface sterilization, the explants were pretreated with Carbendazim (0.3% and 0.5%) for 30 min. Pre-treatment of the axillary buds with 0.5% Carbendazim for 30 minutes and surface sterilization with 70% ethanol for 30 seconds followed by 0.5% sodium hypochlorite for 10 minutes under laminar air flow chamber resulted in the highest survival rate (68.33%) with least contamination (15.00%) and mortality (16.67%). However, in shoot tips, the highest survival rate (56.67%) with least contamination (30.00%) and mortality (13.33%) was recorded by pre-treating with 0.3% Carbendazim for 30 minutes and surface sterilization with 70% ethanol for 30 seconds followed by 0.25% sodium hypochlorite for 10 minutes.

Keywords: Grape rootstock, dog ridge, tissue culture, surface sterilization

Introduction
Grape is one among the important commercial fruit crops in the word, mostly cultivated for its fresh fruits and also for processing into different value added products like raisins and wine. Though it originated in temperate region *viz*., Armenia near the Black and Caspian seas in Russia, its high genetic plasticity enabled its adaptation to temperate, sub tropical and tropical regions of the world (Sajid *et al*., 2006) [17]. Grape cultivation expanded after the introduction of rootstock for commercial cultivation. Among the different rootstocks used for grape propagation, 'Dog Ridge' is widely used in India because of its tolerance towards nematode, salinity and drought. In addition, it imparts great vigour in the grafted vine and absorbs more potassium ions which led to production of quality fruits. Conventional multiplication method *i.e.,* propagation through semi hardwood cuttings is difficult in Dog Ridge because of the presence of phenolic compounds that hinder rooting ability (Mhatre and Bapat, 2007 and Wong, 2009) [11, 23]. In such situation micropropagation is an important alternative through which large number of disease free uniform planting materials independent of season can be produced (Thorpe, 2007) [20]. Identification of suitable explant and standardization of effective disinfection protocol are essential for successful micropropagation technique. The type, size, age of explant and method of culture determine the success of micropropagation (Murashige, 1974) [14]. In grape, shoot tip and axillary bud explants are vital and mostly used for direct organogenesis due to their operational feasibility and genotype stability (Torregrosa *et al*., 2001; Mhatre *et al*., 2000 and Ikten and Read, 2010; Krizan *et al*., 2012; Ruma, 2014 and Wafa, 2015) [21, 12, 7, 10, 16, 22].

The surface sterilization of explants is essential to remove contaminants with minimal damage to plant cells. The selection of chemicals for sterilization depends on the type of explant to be used for micropropagation. The most commonly used disinfectants in tissue culture are calcium hypochlorite, sodium hypochlorite, ethanol and mercuric chloride. Use of 0.1 per cent mercuric chloride for surface sterilization of grape explants is reported by earlier workers (Mhatre *et al*., 2000; Singh *et al*., 2004; Zhang *et al*., 2010; Krizan *et al*., 2012 and Jamwal *et al*., 2013) [12, 18, 24, 10, 8]. Combined use of 0.5 per cent Carbendazim with 70 per cent ethanol followed by 0.1 per cent mercuric chloride for sterilization was also reported in grape (Ruma,

2014 and Wafa, 2015) [16, 22]. Though mercuric chloride is highly effective, it is extremely toxic to both plants and animal tissues and must be disposed of with care. Owing to its phytotoxic effect, many rinses are required to remove all traces of the element from the explants before inoculation. Hence, sodium hypochlorite which has similar function and comparatively less toxic was tried for surface sterilization in the present study.

Materials and Methods

An investigation to standardize the surface sterilization protocol for *in vitro* mass multiplication of grape rootstock Dog Ridge (*Vitis champini* P.)' was carried out at the Tissue Culture Laboratory, Horticultural College and Research Institute, TNAU, Coimbatore. The explants for the present study were collected from the mother vines of grape rootstock Dog Ridge, maintained in the propagation chamber at College Orchard. The explants were collected from pest and disease free elite mother plants by excising (using sterile surgical blade) newly emerged 3 to 6 week old shoots containing dormant buds. The collected shoots were taken to the tissue culture laboratory in clean polyethylene bags within 15 minutes of collection, then immediately washed under running tap water until all the dirt was removed. The leaves and other unwanted parts in the collected shoots were removed and the single nodal axillary bud (2 cm) and shoot tip (1 cm) explants were prepared by cutting with sterile blades. Then, the explants were washed under running tap water thoroughly and treated with two to three ml of Teepol® in 100 ml of distilled water by constant stirring for 10 minutes followed by rinsing thrice in distilled water. The explants were then pretreated with Carbendazim (0.3 or 0.5 per cent) for 30 minutes and were thoroughly rinsed with distilled water. The explants were surface sterilized with 70 per cent ethanol for 30 seconds, followed by NaOCl (0.25 or 0.5 per cent) for different durations (5, 10 and 15 minutes) and finally washed in sterile distilled water (2-3 times) and then inoculated in the basal MS medium.

The experiment was conducted in CRD with 2 factors and the data interpretation was done as per the standard procedures of Panse and Sukhatme (1985) [15]. Observations recorded as percentage were subjected to angular transformation. The CD values were worked out for five per cent probability and the results were interpreted. Analysis was carried out with AGRES software package and MS Excel® spreadsheet.

Result and Discussion

Microbial contaminations present a major challenge in the initiation and maintenance of viable *in vitro* cultures (Jan *et al*., 2013) [9] and it hinders the growth and multiplication of *in vitro* cultures because microbes use nutrients from the culture media as their energy source for their growth. Hence the tissue culture techniques usually involve growing mother plants in protected environment without infestation of pest and diseases to minimize infection during culture establishment, treating the explants with disinfecting chemicals and sterilizing the tools used for dissection and media in which cultures are grown (Bausher and Niedz, 1998) [4]. The most effective way of preventing microbial contamination in *in vitro* culture is elimination of microbes from the explants that are inoculated into the culture **(**Mihaljevic *et al*., 2013) [13].

The chemicals used for sterilization of explants in tissue culture should be effective, cheap, easily available and non toxic. An effective chemical should also be strong enough to inhibit the growth of disease causing microorganisms by treating at suitable concentration for a specified duration and during sterilization, the plant materials should not lose their biological activity (Singh *et al*., 2011) [19]. Carbendazim is a broad spectrum systemic fungicide and it is effective to control *in vitro* fungal contaminants. Ethanol is a powerful sterilizing agent but also extremely phytotoxic and therefore, the explants are exposed to ethanol for only few seconds. Ethanol kills organisms by denaturing their proteins and dissolving their lipids and is effective against most bacteria and fungi and many viruses (Ali *et al*., 2001 and Singh *et al*., 2011) [3, 19]. Hypochlorite is known to be a very effective killer of bacteria and even micro molar concentrations are enough to reduce bacterial populations significantly (Singh *et al*., 2011) [19].

In the present study, data on percentage survival, contamination and mortality of explants revealed significant influence of the sterilants on explants. Among the explants, the axillary buds recorded higher survival percentage (41.92 per cent), with contamination and mortality percentage of 38.72 per cent and 18.85 per cent respectively. However, the shoot tips recorded lower survival percentage (23.59 per cent), lower contamination (28.85 per cent) and high mortality (47.56 percent) (Table 1). Among the sterilization treatments, axillary buds pretreated with 0.5 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.5 per cent NaOCl for 10 minutes (S11), recorded the highest survival percentage (68.33 per cent), lowest contamination percentage (15.00 per cent) and lowest mortality percentage (16.67per cent). Similarly shoot tips pretreated with 0.3 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.25 per cent NaOCl for 10 minutes (S4), recorded the highest survival percentage (56.67 per cent), lowest contamination percentage (30.00 per cent) and mortality percentage (13.33 per cent) (Table1). The result is in parallel with the findings of Gray and Benton (1991) [6], Aazami (2010) [1], Diab *et al*. (2011) [5] and Abido *et al*. (2013) [2] in other grape rootstocks.

The interaction between the explants and sterilization treatments was found to be significant. The axillary buds registered the highest survival percentage (68.33 per cent) in S11 (0.5 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.5 per cent NaOCl for 10 minutes), whereas the shoot tips registered the lowest

Table 1: Effect of sterilization treatments on survival, contamination and mortality percentage of explants in grape rootstock Dog Ridge

Sterilization treatment combinations	Survival percentage		Contamination percentage		Mortality percentage	
	Axillary bud	Shoot tip	Axillary bud	Shoot tip	Axillary bud	Shoot tip
S_1 - Washing with sterile distilled water (Control)	5.00 (12.92)	6.67 (14.76)	90.00 (71.95)	86.67 (68.67)	5.00 (12.92)	6.67 (14.76)
S_2 - 0.3% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.25% (5 min)	13.33 (21.34)	23.33 (28.67)	75.00 (60.08)	68.33 (55.98)	11.67 (19.89)	8.33 (16.21)
S_3 - 0.3% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.5% (5 min)	21.67 (27.71)	40.00 (39.21)	61.67 (51.78)	48.33 (44.04)	16.67 (24.05)	11.67 (19.31)
S_4 - 0.3% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.25% (10 min)	33.33 (35.22)	56.67 (48.84)	51.67 (45.96)	30.00 (33.21)	15.00 (22.60)	13.33 (21.34)
S_5 - 0.3% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.5% (10 min)	45.00 (42.12)	46.67 (43.09)	33.33 (35.22)	31.67 (34.23)	21.67 (27.71)	21.67 (27.71)
S_6 - 0.3% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.25% (15 min)	43.33 (41.17)	23.33 (28.86)	40.00 (39.21)	28.33 (32.14)	16.67 (24.05)	48.33 (44.04)
S_7 - 0.3% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.5% (15 min)	60.00 (50.79)	18.33 (25.31)	16.67 (23.74)	15.00 (22.60)	23.33 (28.86)	66.67 (54.75)
S_8 - 0.5% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.25% (5 min)	53.33 (46.92)	31.67 (34.23)	40.00 (39.18)	16.67 (24.05)	6.67 (14.76)	51.67 (45.96)
S_9 - 0.5% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.5% (5 min)	53.33 (46.95)	20.00 (26.45)	33.33 (35.17)	13.33 (21.15)	13.33 (21.34)	66.67 (54.89)
S_{10} - 0.5% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.25% (10 min)	58.33 (49.83)	15.00 (22.60)	25.00 (29.93)	13.33 (21.34)	16.67 (24.05)	71.67 (57.99)
S_{11} - 0.5% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.5% (10 min)	68.33 (55.77)	11.67 (19.89)	15.00 (16.60)	11.67 (19.89)	16.67 (24.05)	76.67 (61.15)
S_{12} - 0.5% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.25% (15 min)	48.33 (44.04)	8.33 (16.60)	13.33 (21.34)	6.67 (14.76)	38.33 (38.19)	85.00 (67.41)
S_{13} - 0.5% Carbendazim (30 min) + Ethanol 70 % (30 sec) + NaOCl 0.5% (15 min)	41.67 (40.18)	5.00 (12.92)	8.33 (22.60)	5.00 (12.92)	43.33 (41.17)	90.00 (71.57)
Mean	41.92 (39.44)	23.59 (27.80)	38.72 (37.90)	28.85 (31.15)	18.85 (24.72)	47.56 (42.85)
	Survival percentage		**Contamination percentage**		**Mortality percentage**	
Source of variation	**SEd**	**CD (P=0.05)**	**SEd**	**CD (P=0.05)**	**SEd**	**CD (P=0.05)**
Explants	0.81	1.64	0.80	1.60	0.85	1.70
Treatments	2.08	4.17	2.03	4.08	2.17	4.35
Explants X Treatments	2.94	5.90	2.87	5.76	3.06	6.15

Note 1. Numbers in parentheses are arcsine transformed values, **Note 2**. sec - Seconds, min – Minutes

contamination percentage (5.00 per cent) in S13 (0.5 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.5 per cent NaOCl for 15 minutes) which was on par with S12 treatment in shoot tips and also S13 treatment in axillary buds. The shoot tips registered the highest mortality percentage (90.00 per cent) in S13 (0.5 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.5 per cent NaOCl for 15 minutes), which was on par with S12 (0.5 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.25 per cent NaOCl for 15 minutes) with 85.00 per cent for shoot tips (Table 1).

Effective surface sterilization should be cost effective, safe to use, eliminate microorganisms at lower concentration and at the same time it should not kill the explants. The results of the present investigation fulfilled these requirements and hence, can be recommended for surface sterilization to eliminate microbes during *in vitro* culture in grape rootstock Dog ridge.

Conclusion

From this experiment, it was concluded that pre treatment with 0.3 per cent Carbendazim for 30 minutes and surface sterilization with 70 per cent ethanol for 30 seconds followed by 0.25 per cent NaOCl for 10 minutes duration was ideal for shoot tips. For 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 0.5 per cent NaOCl for 10 minutes increased the survival per cent and reduced the contamination percentage in Dog Ridge.

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