Applied Biological Research 14 (2): 193-197, 2012 **Short communication**

STERILIZATION STRATEGIES FOR ASEPTIC *in vitro* ESTABLISHMENT AND SURVIVAL OF SHOOT TIP EXPLANTS IN *Gerbera jamesonii* Bolus

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ABSTRACT

The study involved two experiments aimed at comparing post-sterilization *in vitro* asepsis and subsequent survival of gerbera shoot tip explants sourced from either field-grown or pot-raised plants under pro-phylactic fungicide cover and shoot tip explants with or without leaves. Six sterilant-combinations used were S_1 [carbendazim 50 WP @ 200 ppm for 30 min. + 0.1% mercuric chloride ($HgCl_2$) for 7.5 min.]; S_2 (carbendazim 200 ppm for 30 min. + 0.1% $HgCl_2$ for 10 min.); S_3 (0.05% Megapin + S_1); S_4 (0.05% Megapin + S_2); S_5 (S_3 + 70% ethyl alcohol dip for 10 sec.); S_6 (S_4 + 70% ethyl alcohol dip for 10 sec.). Post-sterilization culture asepsis in shoot tips sourced from fungicide-treated plants and shoot tips without appended leaves was significantly high as compared to those sourced from field-grown plants or shoot tips with leaves intact. Amongst the sterilants, treatment-combinations of explant dip for 10 sec. in 70% ethyl alcohol proved most effective in reducing infection *in vitro*. Survival of shoot tips with leaves intact was higher in comparison to those without leaves. Treatments involving 70% ethyl alcohol dip of explants improved post-sterilization survival significantly as compared to the treatment-combinations wherein ethyl alcohol was not used.

Keywords: Asepsis, gerbera, *in vitro*, sterilization, survival

INTRODUCTION

Gerbera (*Gerbera jamesonii* Bolus.) figures among the top five selling cut flowers in world flower markets. Thousands of gerbera cultivars have been bred with incredible variations in shape, size and colour. However, hardly 300 cultivars are grown for marketing purpose. Gerbera is propagated by vegetative and sexual methods, and among the vegetative means multiplication through division of clumps is the most common. However, the method is very slow and propagation coefficient is also very low. More importantly systemic disease and virus load carried through successive divisions does not allow full realization of potential yield of a given cultivar. There are significant advantages for growers in opting for tissue cultured gerbera in terms of low costs on disease management and high flower yield (Jothi *et al.*, 2003). Establishing gerbera *in vitro* is the first vital step for initiating a tissue culture programme. In gerbera different types of explants have been tried during the course of its 40 year history of *in vitro* propagation (Kanwar and Kumar, 2008). These include leaf and petiole sections, ray and disc florets, ovules and intact capitula and capitular sections. The most important are shoot tips as they allow an easy route to mass multiplication through axillary branching with high degree of clonal fidelity. However, even recent workers concede establishing shoot tips *in vitro* remains a formidable challenge (Nafees *et al.*, 2009). Gerbera have a sympodial rhizome called crown with

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closely placed nodes that bear leaves. Shoot tips are usually formed underground tightly packaged in young leaves. The tips and young leaves are extremely hairy and are situated in the crotches of older leaves - an ideal part of plant anatomy for harbouring moisture and disease spores. Problem is further compounded in field-grown plants where inoculum level is relatively high. Further, the extraction of shoot tips is a destructive method and hence needs to be employed with discretion particularly where mother stock is limited. Use of high concentration of sterilants often leads to the death of shoot tips even if they remain aseptic under *in vitro* conditions. There is a need to strike a balance between culture asepsis and subsequent survival of explants by employing a right strength and duration of exposure to chemicals during sterilization process. This is even more important in shoot tips where asepsis level and subsequent survival is relatively less in comparison to other explants. Therefore, the present study was aimed at to devise a protocol which may help in achieving sufficient degree of culture asepsis in gerbera shoot tip explants and also ensure a reasonable survival *in vitro.*

MATERIALS AND METHODS

The present study involved two cut flower cultivars of gerbera viz., 'Rejiko' (a medium sized maroon red cultivar) and 'South Pacific' (a large yellow flowered cultivar). In one experiment culture asepsis and survival of shoot tips extracted from field-grown and pot-raised gerbera plants was compared after treatment with 6 sterilant formulations. To raise pot-bound plants, 10 cm plastic pots were filled with vermiculite coco-peat mix [1:1]. Healthy plants of both the cultivars were dug out from the field and stripped off of all the outer leaves. Plants were thoroughly washed, manually divided, allowed to dry in shade and later planted in pots with growing points above media surface. Pots were maintained in a polyhouse. A spray schedule of various systemic and non-systemic fungicides was employed to keep the pot-bound plants free of disease. In second experiment asepsis and subsequent survival of shoot tips with and without leaves under six different sterilant regimes was investigated. In both experiments 10 test tubes were used per treatment with each treatment replicated thrice in a completely randomized design. The gerbera plants were brought to the laboratory after shaking off soil and media, older leaves removed and roots separated with a sharp scalpel. Shoot tips in crown were separated manually and subsequently outer leaves removed to reduce shoot tips to 1-2 cm size. The tips were cleaned individually under running tap water and then vigorously shaken for 30 min. in a few drops of Tween-20 fortified with either carbendazim 50WP or Megapin or both. The surfactant and fungicide/antibiotic solution was later removed by placing shoots under running tap water followed by 2-3 rinses with single distilled water. Six different chemical formulations tried were S_1 (carbendazim 200 ppm for 30 min. + 0.1% $HgCl_2$ for 7.5 min.); S_2 (carbendazim 200 ppm for 30 min. + $HgCl_2$ 0.1% for 10 min.); S_3 (0.05% Megapin + S_1); S_4 (0.05% Megapin + S_2); S_5 (S_3 + 70% ethyl alcohol dip for 10 sec.); S_6 (S_4 + 70% ethyl alcohol dip for 10 sec.). The final preparation of shoot tips including removal of leaves and hairy exoderm, application of $HgCl_2$ and ethyl alcohol was done under laminar flow hood. The shoot tips were washed 5-6 times with sterile water before placing on establishment media. Cultures were incubated at 24±1°C at 16/8 h light/dark regime and a light intensity of 3500 lux. Data for culture asepsis was recorded after 3 weeks incubation and survival recorded at the end of 5 weeks. The data was analyzed using Minitab 16 software package (Minitab Inc).

RESULTS AND DISCUSSION

In the present investigation shoot tips extracted from pot-raised plants had higher culture asepsis than tips extracted from field-grown plants (Table 1). This might be due to lower inoculum carried by shoot tips extracted from fungicide-treated plants raised in vermiculite coco-peat mix. Roalte (1978) while attempting *in vitro* propagation in gerbera removed all leaves from mother plants and planted them in green houses before extraction of shoots and tissues.

Ethyl alcohol proved most effective sterilant whether used alone or in combination with Megapin (Table 1). Significantly higher culture asepsis was observed in sterilant-treatments involving 10 sec. dip in ethyl alcohol. Increase in culture asepsis in explants where only carbendazim was used as compared to the explants treated with Megapin + carbendazim was statistically marginal. However, ethyl alcohol dip for 10 sec. at the end of sterilization procedure under laminar air flow hood significantly increased culture asepsis. Concentrated ethyl alcohol is a powerful disinfectant that kills majority of fungal and bacterial spores through instant dehydration. Ethyl alcohol has been used as a reliable disinfectant of gerbera shoot tips by several workers (Aswath and Nazneen, 2004). Parathasarathy *et al*. (1990) used 10 sec. dip in 90% ethanol for surface sterilization of chrysanthemum nodal and shoot tip explants. In another experimnt the shoot tips with leaves removed showed significantly higher asepsis as compared to the shoot tips with appended leaves (Table 2). Young leaves in gerbera are extremely hairy which have potential to trap disease spores. Jerzy and Lubomski (1991) recommended the removal of attached leaves of shoot tips before sterilization. Aswath and Choudhary (2001, 2002) reduced shoot tips to 0.5 cm size before surface sterilization. Moreover, the chances of contamination are directly proportional to size or surface area of explants. Hence, smaller size of shoot tips without leaves may be a contributing factor to higher culture asepsis.

The shoot tips extracted from field-grown plants or fungicide-treated container-grown plants exhibited uniform survival after sterilant treatment (Table 2). Gerbera shoot tips are hairy and protected by tightly packed young leaves that make them less vulnerable to toxic side effects of sterilants. These features make their sterilization difficult and necessitate exposure to $HgCl_2$ for longer duration as compared to explants like leaves, ray and disc florets. $HgCl_2$ treatment for 7.5 min. in both the cultivars resulted in significantly higher survival as compared to the treatments involving 10 min. exposure. Ethyl alcohol dip for 10 sec. significantly improved the survival of shoot tip explants. Concentrated ethyl alcohol is a

Table 1: Influence of sterilants and shoot tip explant source on culture asepsis and survival (%) of two cultivars of *Gerbera jamesonii* Bolus. cv. 'South Pacific' and 'Rejiko'

	Rejiko						South Pacific					
	Culture asepsis			Survival			Culture asepsis			Survival		
Steri-lants	Field grown	Pot raised	Mean	Field grown	Pot raised	Mean	Field grown	Pot raised	Mean	Field grown	Pot raised	Mean
S_1	41.48	43.33	42.40	21.33 (27.51)	22.33 (28.20)	21.83 (27.85)	48.14	46.66	47.40	24.33 (29.55)	25.66 (30.43)	25.00 (29.99)
S_2	42.96	43.33	43.14	17.67 (24.86)	19.33 (26.08)	18.50 (25.47)	46.66	51.85	49.25	22.33 (28.20)	22.00 (27.97)	22.16 (28.08)
S_3	44.81	48.14	46.48	22.00 (27.97)	23.33 (28.88)	22.66 (28.42)	48.14	51.85	49.99	24.66 (29.77)	26.33 (30.87)	25.50 (30.32)
S_4	46.66	48.14	47.40	18.33 (25.35)	20.66 (27.03)	19.50 (26.19)	50.00	53.33	51.66	21.33 (27.50)	23.66 (29.10)	22.49 (28.30)
S_5	55.88	68.55	62.22	32.000 (34.450)	33.33 (35.26)	32.66 (34.85)	65.55	69.77	67.66	34.66 (36.07)	32.33 (34.65)	33.50 (35.36)
S_6	60.22	72.88	65.55	33.66 (35.46)	33.66 (35.46)	33.66 (35.46)	68.88	72.00	70.44	34.66 (36.07)	36.33 (37.06)	35.50 (36.56)
Mean	48.66	54.06		24.16 (29.36)	25.44 (30.15)		54.32	57.82		27.00 (31.19)	27.72 (31.68)	
$CD_{0.05}$												
Sterilants (S)			5.68			1.85			4.54			1.54
Explant source (E)			3.28			Ns			2.62			ns
S x E			ns			Ns			ns			ns

S_1: Carbendazim 200 ppm for 30 min. + 0.1% $HgCl_2$ for 7.5 min.; S_2: carbendazim 200 ppm for 30 min. + 0.1% $HgCl_2$ for 10 min.; S_3: 0.05% Megapin + S_1; S_4: 0.05% Megapin + S_2; S_5: S_3 + 70% ethyl alcohol for 10 sec.; S_6: S_4 + 70% ethyl alcohol for 10 sec.; Data in parenthesis are arcsine transformed values.

Table 2: Influence of sterilants and shoot tip explant type on culture asepsis and survival (%) of *Gerbera jamesonii* Bolus. cv. 'South Pacific' and 'Rejiko'

Steri-lants	Rejiko						South Pacific					
	Culture asepsis			Survival			Culture asepsis			Survival		
	With leaves	Without leaves	Mean	With leaves	Without leaves	Mean	With leaves	Without leaves	Mean	With leaves	Without leaves	Mean
S_1	40.00	58.87	49.44	23.33	21.66	22.50	46.66	62.22	54.44	24.33	21.66	23.00
	(39.23)	(50.16)	(44.69)	(28.87)	(27.73)	(28.30)	(43.07)	(52.09)	(47.58)	(29.54)	(27.73)	(28.64)
S_2	44.81	62.22	53.51	21.00	15.83	18.41	46.66	64.44	55.55	20.50	17.50	19.00
	(42.01)	(52.09)	(47.05)	(27.24)	(23.43)	(25.34)	(43.07)	(53.41)	(48.24)	(26.86)	(24.69)	(25.78)
S_3	51.85	65.55	58.70	24.16	20.83	22.50	48.14	71.66	59.90	24.16	19.50	21.83
	(46.06)	(54.09)	(50.07)	(29.43)	(27.14)	(28.29)	(43.93)	(57.86)	(50.89)	(29.43)	(26.20)	(27.82)
S_4	51.85	65.55	58.70	20.66	19.00	19.83	51.85	68.88	60.36	20.66	19.00	19.83
	(46.06)	(54.09)	(50.07)	(27.03)	(25.83)	(26.43)	(46.06)	(56.10)	(51.08)	(27.03)	(25.83)	(26.43)
S_5	62.22	80.00	71.11	33.66	29.16	31.41	65.55	72.59	69.07	32.66	28.33	30.50
	(52.09)	(63.93)	(58.01)	(35.45)	(32.68)	(34.06)	(54.09)	(58.48)	(56.29)	(34.84)	(32.15)	(33.50)
S_6	63.33	82.59	72.96	39.00	30.50	34.75	67.77	75.92	71.84	35.66	29.83	32.75
	(52.77)	(65.62)	(59.19)	(38.64)	(33.50)	(36.07)	(55.41)	(60.69)	(58.05)	(36.66)	(33.09)	34.88
Mean	52.34 (46.37)	69.13 (56.66)		26.97 31.11	22.83 28.39		54.44 (47.61)	69.28 (56.44)		26.33 (30.73)	22.63 (28.28)	
C.D $_{0.05}$												
Sterilants (S)			4.38			1.45			2.99			1.56
Explant source (E)			2.52			0.84			1.72			0.90
S x E			ns			ns			4.23			ns

S_1: Carbendazim 50 WP @ 200 ppm for 30 min. + 0.1% $HgCl_2$ for 7.5 min.; S_2: Carbendazim 200 ppm for 30 min. + 0.1% $HgCl_2$ for 10 min.; S_3: 0.05% Megapin + S_1; S_4: 0.05% Megapin + S_2; S_5: S_3 + 70%ethyl alcohol for 10 sec.; S_6: S_4 + 70%ethyl alcohol for 10 sec.; Data in parenthesis are arcsine transformed values of percentages

powerful sterilant that kills bacterial and fungal spores by instantly drawing water out of them. Along with water, ethyl alcohol removes any traces of Hg^{2+} ions from the explant tissue. This might be the reason for significantly higher survival of shoot tips in ethyl alcohol dip treatment for 10 sec.

Significantly higher number of shoot tips with appended green leaves survived sterilant treatments than the shoot tips stripped off the protection of leaves (Table 2). Shoot tips exposed to 10 min. $HgCl_2$ exhibited significantly lower survival than those where 7.5 min. treatment was employed. Longer ethyl alcohol dip again aleviated the toxic side effects of $HgCl_2$. This was reflected in terms of increase in survival by almost 10% in shoot tips treated with ethyl alcohol. Posada *et al.* (1999), Aswath and Choudhary (2001, 2002) and Aswath and Nazneen (2004) have reported excellent results with the use of ethyl alcohol as a sterilant in gerbera. In present investigation the lower culture asepsis was observed in cv. 'Rejiko' which may be attributed to more hairy leaves/shoot tips in cv. 'Rejiko' that rendered sterilant treatments less effective. However, survival of explants in both the cultivars was comparable.

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