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An improvised low cost hardening protocol for *in vitro* raised plantlets of *Gerbera jamesonii*

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ABSTRACT

The present investigation comprised two experiments comparing media and containers for optimization of hardening protocol of *in vitro* raised plantlets of *Gerbera jamesonii* Bolus. cultivars Rejiko and South Pacific. In one experiment six different media formulations, viz. cocopeat, cocopeat + perlite (1:1), cocopeat + perlite + vermiculite (1:1:1), perlite, perlite + vermiculite (1:1) and vermiculite were tried in six replication completely randomized design. Days to establishment (13.60 and 12.97 in cultivars Rejiko and South Pacific respectively) was significantly lower in *in vitro* raised plantlets hardened in perlite + vermiculite (1:1) media formulation. Hundred percent (100%) survival of *in vitro* raised plantlets was achieved in the same media. In the second experiment traditional 500 ml wide mouthed glass bottle was compared with an improvised module devised out of two 250 ml polypropylene glasses-one inverted over the other and joint sealed with a strip of para film. Mean survival rate of 90% *in vitro* raised plantlets was achieved in polypropylene hardening module as against 70 % in glass bottle containers. Field survival of polypropylene module hardened gerbera plants after 4 and 8 weeks was significantly higher (above 95%) compared to around 70% in plants previously hardened in glass bottles. Vegetative growth in terms of leaf area and leaf number/plant was significantly higher in plants derived from polypropylene hardening modules. The hardening module developed in our study is low cost and can be employed in a variety of crops in low budget tissue culture units that cannot afford expensive hardening facilities.

Key words: Gerbera, Hardening, *In vitro*, Media

Gerbera (*Gerbera jamesonii* Bolus.) has consistently figured among the top five selling cut flowers in the world as they can be grown under a wide variety of climates. Thousands of cultivars exist which vary in shape, size and colour. However, around 300 different varieties are being grown for florist markets. In several trials researchers over the years have demonstrated significant improvements in flower yield in tissue culture raised gerberas over those multiplied through cuttings (Buisman 1984, Osiecki 1988, Jothi *et al*. 2003). However, significant technical hurdles have to be overcome before the aforementioned advantages can be realized. Most formidable challenge is to accomplish low mortality of *in vitro* raised plantlets during the hardening phase.

In vitro raised plants are very tender and delicate owing to high humidity in culture vessels (80-100%), controlled temperatures (typically 25 ± 2°C), low light intensity and hetero or mixo-trophic mode of nutrition (Kozai *et al*. 1997). Owing to these factors *in vitro* grown plants develop a peculiar external morphology characterized by underdeveloped stomata and absence of waxy cuticle. These features make *in vitro* plants vulnerable to wilting under ambient conditions and there is a need to run them gradually through a series of more demanding environments in a process called hardening or acclimatization. This process allows *in vitro* raised plants to get equipped for more exacting conditions outside a tissue culture laboratory. Traditionally wide mouthed glass bottles (usually Jam bottles) with air tight lids are used in Indian tissue culture laboratories for hardening of *in vitro* raised plantlets. Media for hardening vary from various kinds of peats to inert materials like sand, charcoal, and mica based minerals like perlite and vermiculite. Hardening in traditional jam bottles is cumbersome and costly as it involves much effort and expense on autoclaving and handling. Moreover owing to their wide mouth maintaining humidity in these containers is difficult. Disposable transparent polypropylene glasses provide an excellent low cost alternative as they are light weight and sterile and hence do not need autoclaving. They usually come in stacks of 100 and occupy negligible cupboard space in the laboratory. Plants can be gradually

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Table 1 Influence of hardening media on survival of *in vitro* raised plantlets in *Gerbera jamesonii* cv. Rejiko and South Pacific

Media	Rejiko		South Pacific	
	Days to establishment*	Survival (%)	Days to establishment*	Survival (%)
Coco peat	15.13	61.11 (51.44)	13.46	63.88 (53.08)
Coco peat + Perlite (1:1)	14.86	66.66 (54.84)	13.93	69.44 (56.59)
Cocopeat+ Perlite+ Vermiculite (1:1:1)	13.73	72.22 (58.24)	13.73	69.44 (56.48)
Perlite	15.86	97.22 (84.4)	13.53	100 (90)
Perlite + Vermiculite (1:1)	13.6	100 (90.00)	12.93	100 (90.00)
Vermiculite	14.73	100 (90.00)	13.6	100 (90.00)
CD (P=0.05)	0.96	8.85	0.44	4.45

*Calculated as the number of days to the appearance of first leaf. Data in the parenthesis are arcsine transformed values of the original percentages

exposed to ambient conditions by punching small holes into the container top which can be later widened as the hardening progresses. Therefore, present investigation was carried out to optimise media and container requirements for low cost hardening protocol for *in vitro* raised plantlets of gerbera

MATERIALS AND METHODS

In the current study two experiments involving different types of media, and containers were conducted to standardize optimal passage of ex-agar rooted plantlets of two gerbera cultivars Rejiko and South Pacific through the hardening process. In one experiment, six different media formulations were compared for *ex-vitro* hardening of rooted plantlets of gerbera. The formulations were cocopeat, cocopeat + perlite (1:1), cocopeat + perlite + vermiculite (1:1:1), perlite, perlite + vermiculite (1:1) and vermiculite. In the second experiment two types of containers were compared. One was a wide mouthed 500 ml glass jam bottle with air tight lid, whereas, second container was fashioned out of two 250 ml polypropylene glasses one inverted on the other and the joint sealed with a strip of para-film.

Media formulations were prepared by mixing components in oven dried containers. Approximately 200 ml of media was dispensed into jam bottles followed by 20 ml of sterile liquid MS nutrient solution without vitamins and organic supplements. The bottles were then sealed with air tight lids and autoclaved at 121°C under 1.05 kg/cm^2 for 1 hour. Media for polypropylene glass containers was made wet with sterile nutrient solution at the same rate as used in the bottle containers. Media formulations were later placed in white cotton cloth bags wrapped in aluminium foil sheets and autoclaved at 121°C under 1.05 kg/cm^2 pressure for 1 hour, allowed to cool and stored until use. Media was dispensed into polypropylene glasses under sterile conditions of laminar flow hood cabinet. Rooted ex-agar plantlets of gerbera were transferred into hardening containers under sterile conditions of a laminar flow hood cabinet. Both bottles and polypropylene containers were placed in culture room conditions (25±2°C) under 16/8 hours of light/dark regime. Light intensity was maintained at 3500 lux. After the plants showed signs of establishment (growth of new leaf), lids of the bottles were removed for a few hours every day and sprayed with water. Lids were completely removed when plantlets became established - a stage indicated by absence of signs of wilting on removal of lids. For polypropylene glasses small holes were made into the top of the container after 12 days of transfer. The holes were made larger after signs of establishment of the plant. Later on the upper inverted glass was permanently removed. Hardened plantlets were transferred to polyhouse in 10 cm pots containing growing media mixture of sand, soil and

Table 2 Influence of hardening containers on survival of *in vitro* raised plantlets of *Gerbera jamesonii* cv. Rejiko and South Pacific

Containers	Days to establishment			Survival (%)		
	Rejiko	South Pacific	Mean	Rejiko	South Pacific	Mean
Glass bottle	17.833	15.333	16.830	72.000 (58.369)	68.000 (55.835)	70.000 (57.102)
Polypropylene glasses	17.000	14.467	15.733	96.000 (84.687)	96.000 (84.687)	96.000 (84.687)
Mean	17.416	14.900		84.000 (71.528)	82.000 (70.261)	
	CD(P=0.05)					
		Container	1.012			9.220
		Variety	1.012			NS
		Container × Variety	NS			NS

*Calculated as the number of days to the appearance of first leaf. Data in the parenthesis are arcsine transformed values of the original percentages

Table 3 Influence of hardening containers on field survival of *in vitro* raised plants of *Gerbera jamesonii* cv. Rejiko and South Pacific

Containers	Survival (%)					
	Rejiko			South Pacific		
	4th week	8th week	Mean	4th week	8th week	Mean
Glass bottle	70.268 (57.110)	70.268 (57.110)	70.268 (57.110)	76.56 (61.129)	71.988 (58.263)	74.274 (59.696)
Polypropylene glasses	97.14 (85.556)	93.14 (80.243)	95.14 (82.900)	97.14 (85.556)	97.14 (85.556)	97.14 (85.556)
Mean	83.704 (71.333)	81.704 (68.677)		86.85 (73.343)	84.564 (71.910)	
CD (P = 0.05)						
		Container	8.637			7.374
		Time	NS			NS
		Container × Time	NS			NS

Data in parenthesis are arcsine transformed values of the original percentages

sheep manure (1:1:1). In both the experiments data recorded from four replicates of each treatment were analyzed in a completely randomized design. Data on days to establishment and survival during hardening, field survival, leaf area and number/plant were recorded in both cultivars.

RESULTS AND DISCUSSION

Main goal of a hardening system is to maintain an atmosphere with low evaporative demand around tissue cultured plant freshly extricated from a sequestered system. This helps the plant to avoid substantial tissue water deficits before roots start functioning in the hardening module. Towards this end, hardening media plays an important role in *ex-vitro* establishment of tissue cultured plants. Data reveal that hardening media wielded a significant influence on days to establishment and percent survival in both cultivars. Least number of days to establishment was recorded in 1:1 perlite/vermiculite mix followed by 1:1:1 cocopeat/perlite/vermiculite formulation. Perlite is a chemically inert material with a bulk density of 95.37 kg/ m^3. However, it has no cation exchange capacity and comparatively lower water holding capacity than vermiculite which has a bulk density of 105-140 kg/m^3 and a cation exchange capacity of 19-22 meq^{-100g}. Vermiculite and perlite mixes form excellent media with optimum water holding capacity, aeration and drainage. The formulation also holds nutrients in an easily exchangeable form thus allowing the roots to resume normal function significantly earlier than other media. This could be the reason for early establishment of tissue cultured plants in perlite and vermiculite containing formulations. Only addition of cocopeat in 1: 1: 1 cocopeat/ perlite/vermiculite mixes are comparable but survival in cocopeat containing formulations is significantly lower. This is because cocopeat contains latent fungal infection which is difficult to eradicate even with long duration autoclaving. Laliberte *et al*. (1985) used 1:1 perlite: Sphagnum moss jiffy-7 pellets and encountered only 5% loss through the hardening process. Petru and Matous (1984) used peat: perlite mix (1:1) successfully. Pytlewski and Martyn (1986) reported a significant effect of water/air content on survival of plants and best quality plants were obtained on substrate of 1: 1 peat/perlite.

There were no significant differences between the containers in terms of days to establishment. However, plant survival was significantly higher in polypropylene containers. This may be due to option of a gradual exposure

Table 4 Influence of hardening containers on field leaf area and leaf number of *Gerbera jamesonii* Bolus. cv Rejiko and South Pacific

Container	Rejiko						South Pacific					
	Leaf area/plant (cm^2)			Leaf number/plant			Leaf area/plant (cm^2)			Leaf number/plant		
	4th week	8th week	Mean	4th week	8th week	Mean	4th week	8th week	Mean	4th week	8th week	Mean
Glass bottle	19.540	42.640	31.090	8.176	6.166	7.171	20.940	45.040	32.990	7.976	5.938	6.957
Polypropylene glasses	25.880	47.400	36.640	8.640	6.652	7.646	27.840	48.780	38.310	8.136	6.508	7.322
Mean	22.710	45.020		8.408	6.409		24.390	46.910		8.056	6.223	
CD (P=0.05)												
Container			1.636			0.372			2.008			0.452
Variety			1.636			0.372			2.008			0.452
Container × Variety			NS			NS			NS			NS

of the plants to lower humidity in polypropylene containers in comparison to bottles where the transition is comparatively abrupt. In polypropylene containers small holes can be made in the top of the containers by poking with a hot needle. The holes can later be widened as the plants become more accustomed to lower humidity. Several workers (Miller 1983, Dunstan and Turner 1984, Preece and Sutter 1991, Lavanya *et al.* 2009) have stressed upon the importance of the gradual weaning of tissue cultured plants off high humidity conditions. Nagae *et al*. (1995) used a disposable box shaped culture vessel made of fluorocarbon polymer film. He observed no microbial contamination on the plantlets even when planted under non sterile conditions.

An optimum environment and a gradual exposure to ambient conditions during hardening are vital in ensuring success of the hardened plants under field conditions. Our study reveals significantly high field survival of plantlets hardened in polypropylene containers in comparison to those hardened in glass bottles. More control over the process of exposure to ambient conditions in polypropylene containers allows better overall development of the tissue cultured plants during the hardening process. This is reflected in significantly higher survival of plants of both the varieties in the field. Superior quality of root and shoot development during hardening in the polypropylene containers may also be responsible for better growth in the field measured in terms of leaf area and leaf number after 4 and 8 weeks. Moreover, plantlets hardened in polypropylene containers experience no transplanting shock at the time of transfer to the field. This is possible because the plants can be freed easily by cutting off the container without any disturbance to the root ball. Plants in the bottle containers have to be tapped out during which loose perlite/vermiculite root ball gets disturbed and the roots are exposed and as a result shock experienced at the time of transplanting significantly delayed plant development in the field. This is reflected in significantly less leaf area and leaf number after 4 and 8 weeks in the field in bottle hardened plants of both the cultivars. Pockock (1983) also emphasized the importance of maintaining integrity of root ball at the time of transfer of hardened tissue cultured plants to the field.

The hardening module developed in our study is low cost and can be employed in a variety of crops in low budget tissue culture units that cannot afford expensive hardening facilities. Hardening can be achieved in one step in the module itself and there is no need for maintaining an expensive separate high humidity chamber for the purpose.

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