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Standardization of *in vitro* Hardening Strategies for Tissue Cultured Wine Grape (*Vitis vinifera* L) Genotypes

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A B S T R A C T

A study was carried out to establish efficient hardening strategy for *in vitro* raised plantlets of four grape genotypes. *In vitro* grown plantlets of Pusa Navrang, Hybrid 76-1 (Hur x Cardinal), Pearl of Csaba and Julesky Muscat were hardened *in vitro* using hardening medium consisting coco peat + vermiculite + perlite (2:1:1) ratio and two different types of hardening pots *viz.* glass jars with polypropylene cap (GJPP) and plastic pots with polyethylene cover (PPPC). The glass jar containing coco peat + vermiculite + perlite (2:1:1) was found the most effective strategy for hardening *in vitro* raised plantlets which gave the highest survival (85.97%) and earliest duration for acclimatization of plantlets (23.56 days). Plastic pot with the coco peat + vermiculite + perlite (2:1:1) (T_2) was found non-significant since it required a longer duration for acclimatization (27.93) and also giving lower survival rate (63.46%).

Introduction

Grapevines (*Vitis* sp.) are one of the major horticultural crops grown throughout the world and it has emerged as an important fruit crop of India too. At present, it covers an area of about 1.36 million ha with the production of 2.68 million MT (NHB, 2017). During the last two decades, grape cultivation has gained popularity among fruit growers of north India. In recent past, it has been observed that non-availability of an adequate number of true to type, disease-free planting material has been the major constraint for the establishment of ideal vineyards (Jamwal *et al.*, 2013). Grape is traditionally propagated through woody cuttings, suffered many limitations. Micropropagation offers another method for the rapid multiplication of many plant species. *In vitro* micropropagation has been standardized for several horticultural crops. However, its more widespread use is

restricted by the often high percentage of plants lost or damaged when transferred to *ex vitro* conditions (greenhouse or field). During *in vitro* culture, plantlets grow under very special conditions in relatively air-tight culture vessels, e.g., air humidity is higher and irradiance lower than in conventional culture. Furthermore, the plantlets are usually supplied with large doses of carbon, energy sources and growth regulators. These conditions result in the formation of plantlets of abnormal morphology, anatomy and physiology (Pospíšilová *et al.,* 1992; Kozai and Smith, 1995). The final stage of micro-propagation involves the transfer of *in vitro* rooted plantlets from the aseptic environment to soil media, to function as independently growing plantlets. At this stage of the *ex vitro* transfer the plantlets have to correct the above-mentioned abnormalities, after *ex vitro* transplantation plantlets usually need some weeks of *in vitro* acclimatization with gradual lowering in air humidity (Preece and Sutter, 1991; Kadleček, 1997; Bolar *et al.,* 1998). Therefore, Rooting plantlets directly into potting medium is preferable because: 1) it eliminates the time, material and labor required for an *in vitro* rooting step, 2) roots developed *in vitro* can be difficult to manipulate and are easily broken (Norton and Skirvin, 17), 3) *ex vitro* formed roots are anatomically and morphologically superior to those formed *in vitro* (McClelland *et al.,* 1990*;* Barlass, M. and Skene, 1978). Poor acclimatization and establishment of plantlets in the greenhouse have been a noticeable complication to commercial production of grapevine (Swartz and Lindstrom, 22 and Bigger, 5). The transition from test tube to the soil has also considered as a handicap in his report because the *in vitro* produced plants are not well adapted to the *in vivo* conditions. However, there is a need to standardize the requirements of *in vitro* hardening of tissue cultured grape plantlets. Gribaudo *et al.,* (1995), suggested that *in vitro* formed roots contribute substantially to the growth of some grape rootstock species during acclimatization in plastic pots with cover. Singh *et al.,* (2004), successfully hardened 3-week-old *in vitro* raised grape plantlets on rooting medium in glass-jars filled with peat: soilrite® (1:1) mixture. Shatnawi *et al.,* (2011), found that survival rates of 95% were achieved when rooted grape plantlets were acclimatized *ex vitro* in a mixture of 1 soil: 1 perlette: 1 peat. Acclimatized plants grew in the greenhouse and were maintained as virus-free plants. Jamwal *et al.,* (2013), found most suitable potting media for *in vitro* raised plantlet hardening of grape cv. Perlette constituted sand: soil: FYM: vermiculite (1:1:1:1), which resulted in 73.33 per cent plantlet survival.

Materials and Methods

The experiments were conducted at the Central Tissue Culture Laboratory, LBS Building, Indian Agricultural Research Institute (IARI), New Delhi. Newly emerged vine segments from field-grown 20-year-old mother plants of the four grapes (*Vitis vinifera* L.) genotypes, *viz.,* Pusa Navrang, Hybrid 76-1 (Hur x Cardinal), Pearl of Csaba and Julesky Muscat were taken from the Main Orchard, Division of Fruits and Horticultural Technology, IARI, New Delhi. Murashige and Skoog (17), medium supplemented with growth regulator combinations, 2.0, 4.0 mg l^{-1} BAP and Kinetin individually and in combination with 0.2 mg l^{-1} NAA were tried for culture initiation. Thereafter, the proliferated cultures in different genotypes were sub-cultured using repetitive two node micro-cutting techniques (Singh *et al.,* 2004) on MS medium supplemented with auxin 2.0 and 4.0 mg l^{-1} IBA singly and in combination with 200 mg l^{-1} activated charcoal (AC) supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar-agar was also used for multiplication, shoot elongation and rooting.

Thereafter, rooted plantlets were *in-vitro* hardened using hardening medium consisting coco peat + vermiculite + perlite (2:1:1) ratio and two different types of hardening pots *viz.* Glass jars with polypropylene (PP) cap and plastic pots with polyethylene cover. The potting mixture moistened with 1/4th MS salts. The observation related to per cent plantlets survival and days took to *in vitro* hardening was noted. The rooted plantlets (30-day-old) were then shifted to glass-house conditions. Parameters related to plantlet survival (15 and 30 days), plantlet growth, chlorophyll content (leaf) and total phenols (vine) were recorded at 30 and 45 days of the transfer. In general, about 25 cultures were taken for each treatment. The percentage data were subjected to angular transformation before analysis. ANOVA was calculated to partition the variance as reported by Gomez and Gomez (1984).

Results and Discussion

In-vitro hardening

The rooted plantlets (30-day-old) were transferred to *in vitro* primary hardening following two different strategies. Parameters were recorded at 30 and 45 days of transfer to the hardening pot. The plantlets in the sterilized coco peat+ vermiculite + perlite (2:1:1) (T_1) in the glass jar were found to be the effective means of *in-vitro* plantlets hardening which gave the highest survival (85.97%) and minimum duration for acclimatization (23.5 days) (Table 1). Plastic pot with the cocopeat + vermiculite + perlite (2:1:1) (T_2) was found non-significant since it required a longer duration for acclimatization (27.93 days) and also giving minimum (63.46%) survival (Table 1). The transparent polypropylene (PP) cap was found most effective as light reached the plantlets under the jar (Plate 1). Furthermore, the plantlets under the jar had higher CO_2 concentration along with higher and constant humidity level, thus, improving the vegetative growth and recovery of plantlet (Gribaudo *et al.,* 1995).

Ex vitro hardening under glass-house conditions

The plantlets were grown in coco peat + vermiculite + perlite (2:1:1) in the glass jar (T_1) gave the highest survival (79.63 and 72.64%) after 30 and 45 days of transfer under glasshouse condition, respectively. Plastic pot with the cocopeat + vermiculite + perlite (2:1:1) (T_2) gave the minimum (58.76 and 50.31%) survival after 30 and 45 days of a transfer, respectively. Mean survival after 30 days (71.13%) and 45 days (65.25%) was found to be significantly higher in Pusa Navrang followed by H-76-1 (69.95% and 61.98%) than other genotypes (Table 2 and Plate 2). The interaction effect between genotype and hardening strategy indicate that maximum survival was in Julesky Muscat after 30 days (85.60%) and 45 days (74.70%) followed by H-76-1 (80.43 and 73.57%, respectively) with T_1.

In-vitro structures vary in their abilities to be transferred to soil, according to the method used during this stage. The acclimatization of the *in vitro* developed plants are a crucial stage to make them survive under the *ex vitro* conditions. The transparent polypropylene cap was found most effective as light reached the plantlets under the jar. Furthermore, the plantlets under the jar had higher CO_2 concentration along with higher humidity, improving the growth and recovery of the plantlet. A similar strategy has earlier been reported by Singh *et al.,* (2004), and Alizadeh *et al.,* (2010). These results are in conformity with the earlier results reported by Barlass and Skene (1978) and Lakso *et al.,* (1986). When plantlets were transferred to the potting mixture, removal of sticking agar-agar

improved the hardening success and also reduced the infection of pathogens. The potting mixture moistened with 1/4th MS salts improved the acclimatization of plantlets. Earlier, similar results were reported by Singh *et al.,* (2004) and Khawale *et al.,* (2006); Alizadeh *et al.,* (2010) and Abido *et al.,* (2013).

Plantlets were grown in a plastic pot (T_1) filled with a mixture of coco peat + vermiculite + perlite (2:1:1) and polythene cover exhibited the highest mean vine and root length compared to plantlets grown in a glass jar with polypropylene (PP) cap (T_2). Mean vine length of H-76-1 (26.48 cm) plantlet was found to be significantly higher than other genotypes (Table 3). The interaction effect between genotype and hardening strategy showed maximum vine length in H-76-1 (28.87 cm) followed by Pusa Navrang (28.07 cm), whereas, the minimum was in Julesky Muscat (27.0 cm) with T_1, though they differed significantly with each other. Plantlets hardened with T_2 produced a higher number of leaves (21.09) which was significantly high compare to T_1 (12.79) in all the genotypes (Table 3). With this treatment (T_2), numbers of leaves were recorded from 19.33 (H-76-1) to 23.37 (Pearl of Csaba). Plant growth was reduced by the lowest levels of RH, but reducing the RH in the vessels proved to ameliorate plant water relations and therefore facilitate acclimatization (Gribaudo *et al.,* 2001).

Two-way interaction indicated, maximum root length was in Julesky Muscat (23.47 cm) with T_2 (Plastic pot with polythene cover), this was significantly higher than other genotypes, whereas shortest roots (8.93 cm) were found in Pearl of Csaba with T_1 (Glass jar with PP cap). The mean number of roots per plant was maximum in Pusa Navrang (24.92 cm), which was highest among the genotypes (Table 4). While significant, the lowest number of roots per plant (18.08 cm) was recorded in Julesky Muscat. A higher number of roots (26.64) were observed in all four genotypes was recorded T_2 strategy compared to T_1 (16.84). The number of roots produced in T_2 was significantly different with T_1. Furthermore, after shifting the plantlets in the potting mixture, root growth was found to be better. Gradual removing of the PP cap helped the plant to withstand the outer environmental conditions better, *i.e.* under increased light intensity and low humidity. As for several other species, acclimatization of grapevine plants is often difficult because of malfunctioning stomata and poor epicuticular wax deposition (Iacono and Martinelli, 1998; Gribaudo *et al.,* 2001). These abnormalities are mostly due to the water saturated atmosphere in the culture vessels.

Higher root, fresh: dry weight ratio was recorded in H-76-1 (9.25) followed by Pusa Navrang (9.20), Julesky Muscat (8.85) and Pearl of Csaba (8.75). The root, fresh: dry weight ratio was higher (9.18) in plants grown in the T_2 compared to plantlets grown in T_1 (8.85) (Table 5). The interaction effect showed the highest root, fresh: dry weight ratio (9.50) in H-76-1 with T_2 followed by Pusa Navrang (9.30). While the minimum ration was registered in Pearl of Csaba (8.60) with T_1.

Irrespective of genotypes, the content of total chlorophylls synthesized in the leaves was positively affected by the better vegetative growth obtained in T_2 (Fig. 1). Higher mean leaf total chlorophyll contents were observed in plantlets with T_2 (2.88) significantly different from T_1 (2.69). Whereas, interaction effects between treatment and genotype was non-significant. They further reported that in sealed vessels, inadequate gas exchange contributes to the generally low rate of photosynthesis.

Table.1 Effect of hardening strategies on plantlet survival (%) and day taken for hardening in grape genotypes under controlled conditions

Strategy	Plant survival (%)					Days taken for hardening				
	PN	H 76-1	POC	JM	Mean	PN	H 76-1	POC	JM	Mean
Glass jar with polypropylene (PP) cap (T_1)	82.03 (64.90)*	83.60 (66.09)	89.33 (70.91)	88.90 (70.51)	85.97 (68.10)	22.87 ±0.12#	24.10 ±0.35	22.67 ±0.30	24.60 ±0.29	23.56
Plastic pot with Polythe Cover (T_2)	69.40 (56.39)	62.9 (52.48)	61.37 (51.55)	60.13 (50.83)	63.46 (52.81)	28.07 ±0.22	28.87 ±0.12	27.77 ±0.20	27.00 ±0.25	27.93
Mean	75.72 (60.65)	73.27 (59.28)	75.35 (61.23)	74.52 (60.67)		25.47	26.48	25.22	25.80	
CD at 5%										
Treatment (T)					0.32					0.37
Genotype (G)					0.45					0.52
T X G					0.64					0.73

*Arc Sin √% transformed values

Table.2 Effect of hardening strategies on plantlet survival (%) under glasshouse conditions

Strategy	Plantlet survival (30 DAP)					plantlet survival (45 DAP)				
	PN	H -76-1	POC	JM	Mean	PN	H- 76-1	POC	JM	Mean
Glass jar with polypropylene (PP) cap (T_1)	76.77 (61.17)*	80.43 (63.73)	75.70 (60.44)	85.60 (67.68)	79.63 (63.25)	72.93 (58.65)	73.57 (59.04)	69.37 (56.37)	74.70 (59.78)	72.64 (58.46)
Plastic pot with Polythene Cover (T_2)	65.50 (54.01)	59.47 (50.44)	56.47 (48.70)	53.60 (47.05)	58.76 (50.05)	57.57 (49.33)	50.40 (45.21)	48.93 (44.37)	44.33 (41.73)	50.31 (45.16)
Mean	71.13 (57.59)	69.95 (57.08)	66.08 (54.57)	69.60 (57.36)		65.25 (53.99)	61.98 (52.13)	59.15 (50.37)	59.52 (50.76)	
CD at 5%										
Treatment (T)					0.61					0.81
Genotype (G)					0.86					1.15
T x G					1.21					1.62

*Arc Sin √% transformed values

Table.3 Effect of hardening strategies on no. of leaves per plantlet and vine length (cm) in grape genotypes

Strategy	No. of leaves/ plantlet					Vine length (cm)				
	PN	H 76-1	POC	JM	Mean	PN	H 76-1	POC	JM	Mean
Glass jar with polypropylene (PP) cap (T_1)	11.17 ±0.44#	10.33 ±0.60	13.33 ±0.55	16.33 ±0.71	12.79	22.87 ±0.54	24.10 ±0.55	22.67 ±0.61	24.60 ±0.46	23.56
Plastic pot with Polythene Cover (T_2)	21.33 ±0.55	19.33 ±0.55	23.37 ±0.47	20.33±0. 66	21.09	28.07 ±0.33	28.87 ±0.58	27.77 ±0.64	27.00 ±0.29	27.93
Mean	16.25	14.83	18.35±	18.33		25.47	26.48	25.22	25.80	
CD at 5%										
Treatment (T)					0.86					0.37
Genotype (G)					1.21					0.52
T x G					1.71					0.73

#Data represent the mean ± standard error of three independent determinates

Table.4 Effect of hardening strategies on no. of roots/shoot and root length (cm) in grape genotypes

Strategy	No. of roots/shoot					Root length (cm)				
	PN	H 76-1	POC	JM	Mean	PN	H 76-1	POC	JM	Mean
Glass jar with polypropylene (PP) cap (T_1)	17.30 ±0.46#	18.53 ±0.38	18.17 ±0.43	13.37 ±0.46	16.84	14.43 ±0.46	16.53 ±0.38	8.93 ±0.26	17.93 ±0.81	14.46
Plastic pot with Polythene Cover (T_2)	32.53 ±0.03	29.47 ±0.41	21.77 ±0.37	22.80 ±0.36	26.64	16.90 ±0.56	17.60 ±0.49	16.00 ±0.29	23.47 ±1.08	18.49
Mean	24.92	24.00	19.97	18.08		15.67	17.07	12.47	20.70	
CD at 5%										
Treatment (T)					1.59					0.90
Genotype (G)					2.25					1.27
T x G					3.18					1.80

#Data represent the mean ± standard error of three independent determinates.

Table.5 Effect of hardening strategies on shoot fresh: dry wt. ratio and root fresh and dry wt. ratio in grape genotypes

Strategy	Shoot fresh: dry wt. ratio					Root fresh: dry wt. ratio				
	PN	H 76-1	POC	JM	Mean	PN	H 76-1	POC	JM	Mean
Glass jar with polypropylene (PP) cap (T_1)	5.32 ±0.01#	5.24 ±0.02	5.35 ±0.03	5.30 ±0.06	5.30	9.10 ±0.09	9.00 ±0.06	8.60 ±0.06	8.70 ±0.06	8.85
Plastic pot with Polythene Cover (T_2)	5.43 ±0.01	5.47 ±0.04	5.62 ±0.01	5.40 ±0.09	5.48	9.30 ±0.12	9.50 ±0.10	8.90 ±0.10	9.00 ±0.17	9.18
Mean	5.38	5.36	5.49	5.35		9.20	9.25	8.75	8.85	
CD at 5%										
Treatment (T)					0.01					0.06
Genotype (G)					0.01					0.09
T x G					0.01					0.12

#Data represent the mean ± standard error of three independent determinates

Fig.1 Effect of hardening strategies on total chlorophyll (mg/g FW) in grape genotypes. Data represent the mean ± standard error of three independent determinates

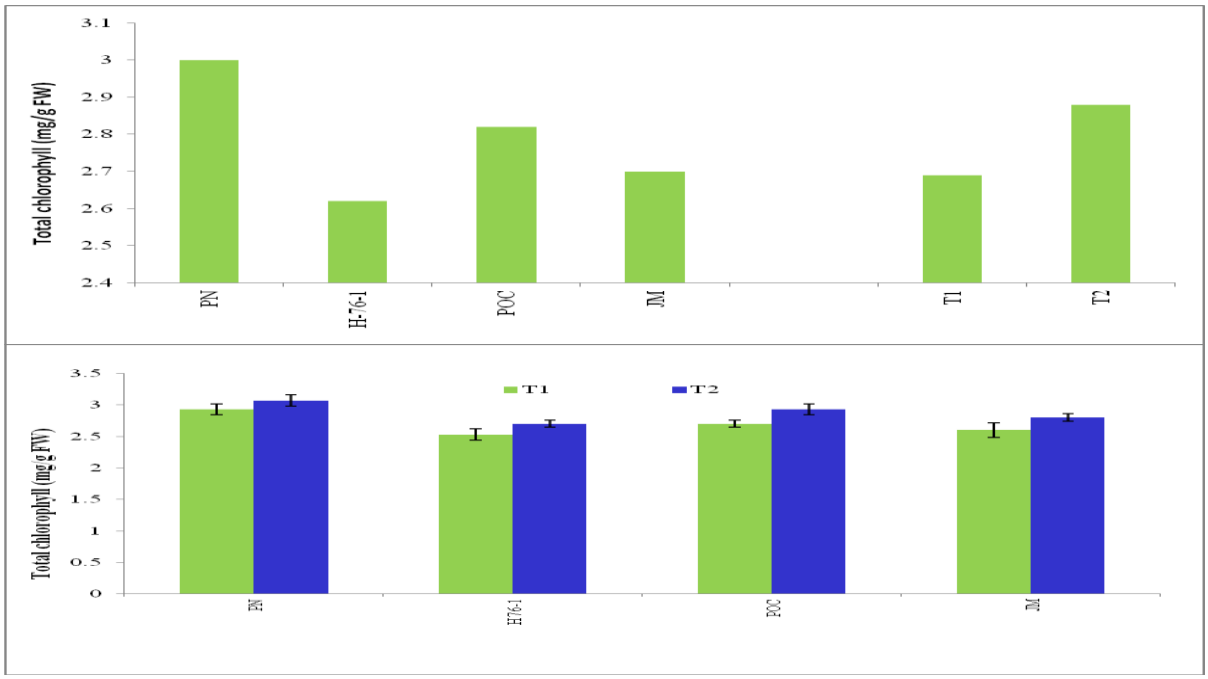

Fig.2 Effect of hardening strategies on total phenols (mg/g FW) in grape genotypes. Data represent the mean ± standard error of three independent determinates

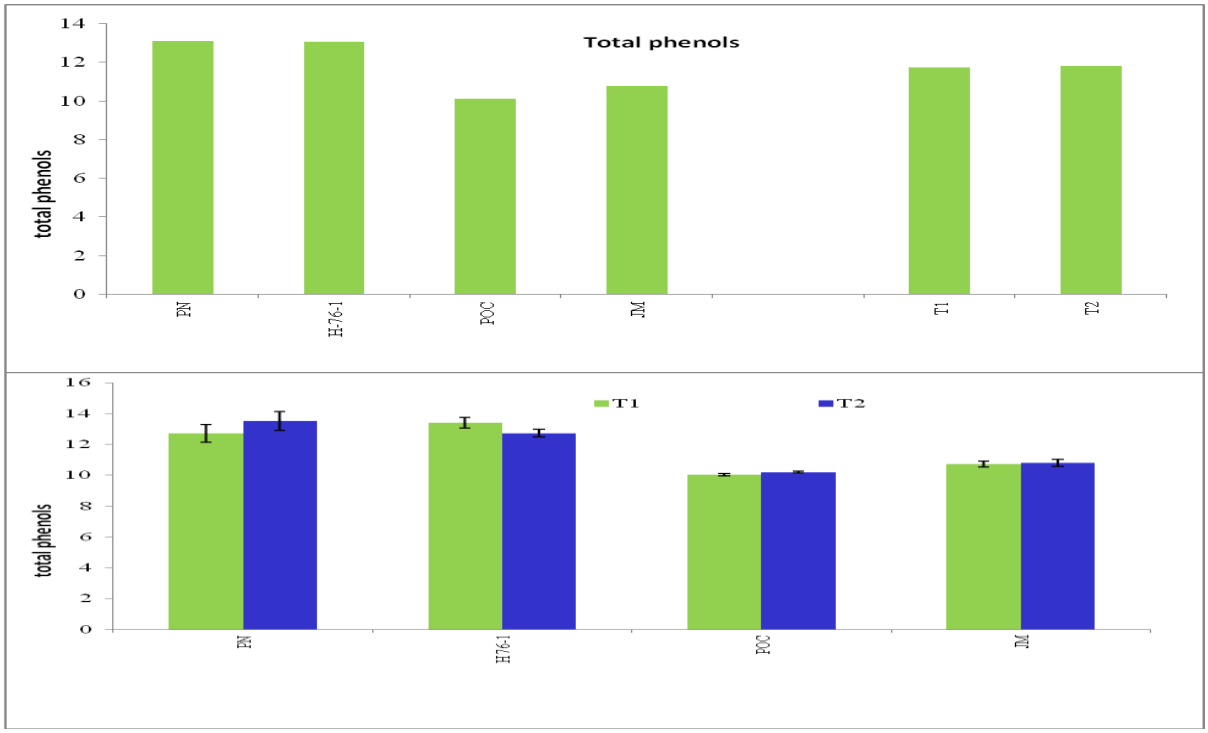

Plate.1 Strategies for primary hardening of *in vitro* raised grape plantlets. (a) Plastic pot with polythene cover, (b) glass jar with PP cap

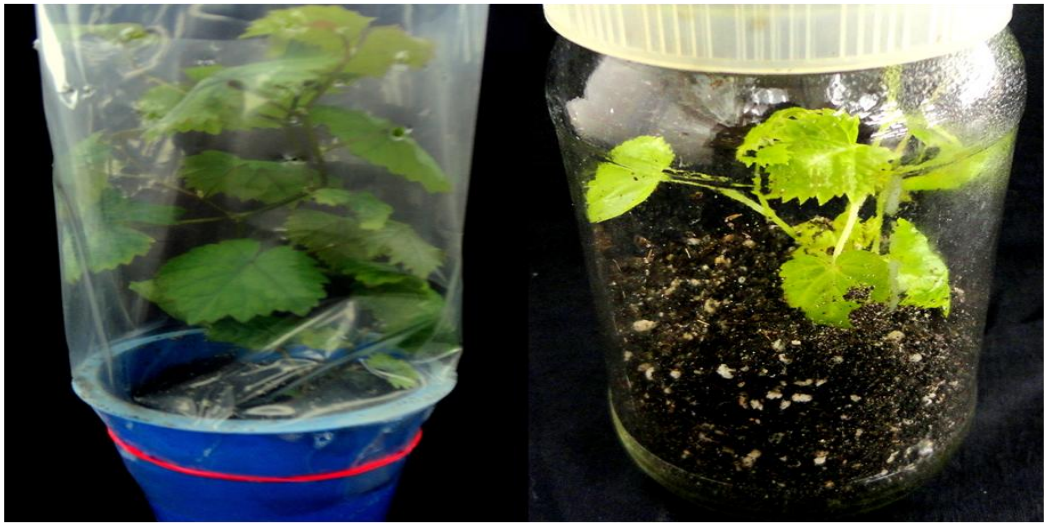

Plate.2 Pusa Navrang plantlets during hardening in glass jars with PP cap

The data in Figure 2 suggested that *in vivo* total phenols content in plantlets hardened in T_2 was higher (11.81) but non-significantly different to T_1 (11.72). Phenol content of plantlets grown in plastic pots with polyethylene cover (T_2) was slightly higher than plantlets raised in a glass jar with PP cap (T_1). The two-way interaction between hardening treatment and genotype was non-significant, but Pusa Navrang plantlets

exhibited maximum phenol content (13.10) but non-significantly followed by H-76-1 (13.07), whereas the lowest phenol content was estimated in Pearl of Csaba (10.03).

These results indicate that the plantlets in the sterilized coco peat+ vermiculite + perlite (2:1:1) on the glass jar was the effective means of *in vitro* plantlets hardening which gave the highest surviving. The transparent polypropylene (PP) cap was found most effective as light reached the plantlets under the jar. Furthermore, the plantlets under the jar had higher CO_2 concentration along with higher and constant humidity level, thus, improving the vegetative growth and recovery of plantlet.

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