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Ex vitro hardening of sugarcane (*Saccharum* Species Hybrid) clones for rapid multiplication

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ABSTRACT

The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment. In the present study an attempt was made to find out the best *ex vitro* condition (single shade net and double shade net) for hardening of five different clones of sugarcane. The effect of temperature, light intensity and humidity on hardening of elite sugarcane genotypes were evaluated under two different hardening conditions. The results obtained in the present investigation concluded that both the *ex vitro* acclimatization conditions showed almost similar effect on number of leaves per plant. However, for chlorophyll content (mg/g), root characters, *viz.* number of roots, root length (cm), root volume (cm^3) and survival ability, double shade net was found to be optimum for all the genotypes, *viz.* CoVC 09-61-02, CoVC 07-06-05, CoVC 09-61-07, CoC 671 and CoVC 10-38-07. Whereas, CoVC 07-06-05 and CoC 671 produced optimum shoot length under single shade net. Hence, it is clear that the double shade net served as better hardening condition as revealed from better survival and development of appropriate morphological characters of all the sugarcane clones.

Key words: Hardening, Humidity, Light Intensity, Micropropagation, Sugarcane, Temperature

Sugarcane (*Saccharum* Species Hybrid) is a perennial monocot plant is the major source of sugar worldwide. Micropropagation has been extensively used for the rapid multiplication of many plant species. However, its more widespread use is restricted by often high per cent of plants lost or damaged when transferred to *ex vitro* conditions (Rani and Kumar 2017). Tissue cultured plants are prone to transplantation shocks during laboratory to land transfer leading to high mortality rates during the final stage of micropropagation, a major limitation in large scale application of this technology (Dhawan and Bhojwani 1996). This is because the growth conditions inside the culture vials induce abnormal morphology and physiology in the plants (Sutter 1984). In tissue culture condition, light is important to regulate certain morphogenetic processes such as formation of shoots and initiation of roots and in asexual embryogenesis. Similarly, in the acclimatization stage to obtain high survival rates, it is necessary to ensure optimal intensity of light. Microshoots on being transferred to *ex vitro* conditions may wilt rapidly or desiccate and can die as a result of the change in environment (altered temperature, high light intensity and water stress conditions) warranting acclimatization for successful establishment and survival of plantlets (Deb and Imchen 2010). In commercial micropropagation, this step is often the limiting factor (Poole and Conover 1983) and at best, is challenging besides labour intensive and costly (Debergh 1988). Understanding the abnormalities encountered during the transplantation is a prerequisite to develop efficient transplantation protocols. The *in vitro* environments are characterized by a humid atmosphere, relatively low light intensity (average 12-70 $\mu mol\ m^{-2}\ s^{-1}$), and constant temperature of 20-28 ^{0}C, low rates of gas exchange between the culture vials and the external atmosphere and high concentrations of carbohydrate and exogenously applied growth regulators in the medium. In the greenhouse or field, where the relative humidity tends to be less, the ambient light levels are much higher than in culture with fluctuating temperatures and the substrate has a much higher water potential. Hence, it is necessary to convert rapidly from a mixotrophic to a fully autotrophic mode of nutrition to survive. The *ex vitro* survival and physiological

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superiority is important especially to guard against water stress and encourage autotrophy. Considering these facts, the present investigation was undertaken to study the effect of temperature, humidity and light intensity on hardening in sugarcane (*Saccharum* Species Hybrid) under two different conditions; single shade net and double shade net.

MATERIALS AND METHODS

Preparation of basal medium and culture bottles for root regeneration

Sterilized glassware's and double distilled water were used for the preparation of basal medium. All the stock solutions and chemicals listed in Table 1 were added in required amounts at the time of medium preparation. After adding all the components, volume was made up little short of the target volume by using double distilled water. The pH of the medium was adjusted to 7.0 using 0.1 N HCl or 0.1 N NaOH. The liquid medium of 40-50 ml was poured into pre-sterilized culture bottles, capped and sealed. These bottles were autoclaved at 121°C at 15 lb psi for 20 minutes. These bottles were stored in growth room for future use. Fluorescent tubes were used as a light source, and culture bottles were placed 25-30 cm below the fluorescent light. Sub-culturing was done after every 2-3 weeks, according to the need of experiment.

The selected sugarcane genotypes, *viz*. CoVC 09-61-02, CoVC 07-06-05, CoVC 09-61-02, CoC-671 and CoVC 10-38-07 in the culture bottles were obtained through *in vitro* culture of apical meristem taken from top shoot of sugarcane stock plants selected from AICRP on sugarcane, ZARS, Mandya. The *in vitro* raised rooted micro-shoots were transferred to pro trays containing 1:1:1 soil, sand and cocopeat under two different *ex vitro* conditions (Double shade net and Single shade net) for their acclimatization. The experiment was laid out in a Completely Randomized Factorial design (CRFD). After two weeks of planting in hardening cage structure, the response of genotypes for mortality percentage, shoot characters, *viz*. shoot length, number of leaves, chlorophyll content and root characters like number of roots, root length and root volume was measured in 15 randomly selected plants from each replication in different hardening condition.

Table 1 Preparation of basal medium for root induction

Stock solution	Quantity (ml/l)
Macronutrient stock A	20
Macronutrient stock B	20
Micronutrient stock C	5
Micronutrient stock D	5
Micronutrient stock E	5
Micronutrient stock F	5
Glycine	2
Pyridoxine HCL	0.50
Nicotinic acid	0.50
Thiamine HCL	0.10
NAA	5.0
Meso inositol	0.10g
Sucrose	30.00g
Coconut water	100

Hardening cages

The process of acclimatization of the *in vitro* grown plants to the normal environment is called hardening where heterotrophic *in vitro* cultured delicate plantlets become autotrophic and gets acclimatised. This process is carried out in the structures called hardening cage. In the present investigation, two types of hardening cages were used as explained below.

Single shade net: The shade net house constructed with 50 per cent light interception shade net and the average temperature, relative humidity and light intensity were 27.33°C, 67.66 per cent and 4983.3 lux, respectively.

Double shade net: Hardening cage structure made with two layers of green shade net and the average temperature, relative humidity and light intensity were 25.86°C, 74.66 % and 2771.6 lux, respectively (Table 2).

Thermometer, hygrometer and lux-meter were used to monitor temperature, relative humidity and light intensity respectively in hardening cage structures. Observations were recorded on following characters.

Shoot length (cm)

Length of shoots on 15 randomly selected plants was recorded as average shoot length of plantlets under two different hardening conditions and shoot length was expressed in centimetres.

Number of leaves per shoot

Mean number of leaves per plant was recorded from 15 randomly selected acclimatized plants under two different conditions.

Number of roots

Mean number of roots per plant was recorded as average number of roots produced from acclimatized plants at two different conditions.

Root volume

Root volume was recorded by immersing the roots of micro-shoots in 100 ml measuring cylinder filled with double distilled water. The volume of roots was calculated as follows:

Root volume = volume of the water after submerging

Table 2 Different types of hardening conditions

Condition	Temperature (°C)	Humidity (%)	Light Intensity (Lux)
Single shade net	27.33	67.66	4983.3
Double shade net	25.86	74.66	2771.6

the roots into the cylinder – volume of the water before submerging the roots.

Mean length of roots

Mean length of roots was observed as average length of all roots produced from 15 randomly selected hardened seedlings and expressed in centimetres.

Chlorophyll content (mg/g)

Leaf chlorophyll content of plantlets were recorded from 15 randomly selected plants in each replication under both the conditions by using soil plant analysis development (SPAD) and expressed in mg/g.

RESULTS AND DISCUSSION

The results of present study demonstrate the optimum *ex vitro* condition for different genotypes of sugarcane. The data subjected to analysis of variance revealed that interaction effects of genotypes and conditions were significant for parameters such as mortality percentage, shoot characters, *viz*. shoot length (cm), chlorophyll content (mg/g) and root characters like number of roots, root length (cm) and root volume (cm^3) except for number of leaves. Single shade net was found to be optimum in producing highest shoot length for two genotypes, *viz.* CoVC 07-06-05 (20.15) and CoC 671 (27.76) (Table 3). Scaranari *et al.* (2009) reported that shade net (50% shade) facility was optimum for hardening of banana genotypes. They obtained superior performance of plantlets maintained under black coloured 50% shade cloth for nine weeks, both in the summer and winter seasons. In contrast, three genotypes, *viz*. CoVC 09-61-02 (24.71), CoVC 09-61-07 (30.45) and CoVC 10-38-07 (26.50) showed optimum response for highest shoot length under double shade net. This result is also in line with findings of Scaranari *et al.* (2009) where in along with 50% shade net, they also obtained superior outcomes of banana plantlets cultivated under red coloured 70% shade cloth, for six weeks in the summer. Our results can also be supported by findings of Meziani *et al.* (2015) who reported that the light intensity ranging from 2000-3000 lux induces shoot elongation in date palm. No significant difference was observed between two *ex vitro* conditions and genotypes for number of leaves it is probably due to the response of sugarcane genotypes which is solely due to genotypic effect further the genotypic effect is independent and specific (Table 3). Double shade net as compared to single shade, was found to be optimum for chlorophyll biosynthesis in all the genotypes used in this study, *viz*. CoVC 09-61-02 (32.21), CoVC 07-06-05 (20.15), CoVC 09-61-07 (33.54), CoC 671 (24.58) and CoVC 10-38-07 (26.33) (Table 4). The results obtained were in close agreement with the findings of Arun and Bhaishnab (1998) who reported that higher temperature was not optimum for chlorophyll biosynthesis as it was partly due to impairment of 5-aminolevulinic acid biosynthesis and protochlorophyllide. Jo *et al.* (2007) reported that plantlets of *Alocasia amazonica* showed better growth and development with higher amount of chlorophyll a and chlorophyll b in lower photon flux density than those grown under higher photon flux density (PFDs). In conclusion, light intensity affects growth and development of stomata. Optimum survival ability was recorded under double shade net hardening condition (RH 74.0%) for all the genotypes studied, *viz*. CoVC 09-61-02, CoVC 07-06-05, CoVC 09-61-07, CoC-671 and CoVC 10-38-07 with mortality percentage of 4.4, 3.89, 8.33, 3.33 and 4.11, respectively whereas, under single shade net it was 21.00, 5.00, 17.00, 9.74 and 12.54, respectively. Our findings are in harmony with the report of Short *et al.* (1987) confirming that high humidity supports growth and hardening of plants. Higher mortality (%) observed under single shade net might be due to high light intensity and temperature during hardening, which causes charring and wilting of plantlets (Chandra *et al.* 2010) (Table 4). Double shade net was found to be optimum for root volume for all the genotypes studied, *viz*. CoVC 09-61-02 (1.75), CoVC 07-06-05 (1.12), CoVC 09-61-07 (1.33), CoC-671 (1.42) and CoVC 10-38-07 (1.58) (Table 5). In contrast, the genotypes had lower root volume under single shade net condition, *i.e.* 1.05, 1.00, 1.16, 1.33

Table 3 Effect of different hardening conditions on shoot length (cm) and number of leaves on *in vitro* raised sugarcane clones

Genotype	Shoot length (cm)		Mean	Number of leaves		Mean
	Double shade net	Single shade net		Double shade net	Single shade net	
CoVC 09-61-02	24.71	22.82	23.76	3.23	3.05	3.14
CoVC 07-06-05	20.06	20.15	20.11	3.04	3.05	3.05
CoVC 09-61-07	30.45	25.88	28.16	3.25	3.05	3.15
CoC 671	26.34	27.76	27.05	3.33	2.95	3.14
CoVC 10-38-07	26.50	23.59	25.04	3.16	3.37	3.27
Mean	25.61	24.04		3.20	3.09	
	S.Em±	*CD@1%*	*CV%*	*S.Em±*	*CD@1%*	*CV%*
Condition (C)	0.21	0.84	3.28	0.05	NS	6.42
Genotypes (G)	0.33	1.33		0.08	NS	
G×C	0.47	1.88		0.11	NS	

Table 4 Effects of different hardening conditions on chlorophyll content (mg/g) and mortality % on *in vitro* raised sugarcane clones

Genotype	Chlorophyll content (mg/g)		Mean	Mortality %		Mean
	Double shade net	Single shade net		Double shade net	Single shade net	
CoVC 09-61-02	32.21	21.72	26.96	4.44	21.00	12.72
CoVC 07-06-05	20.15	19.92	20.03	3.89	5.00	4.44
CoVC 09-61-07	33.54	22.69	28.11	8.33	17.67	13.00
CoC 671	24.58	21.08	22.83	3.33	9.74	6.53
CoVC 10-38-07	26.33	23.08	24.71	4.11	12.51	8.31
Mean	27.36	21.70		4.82	13.18	
	SE.m±	*CD@1%*	*CV%*	*SE.m±*	*CD@1%*	*CV%*
Condition (C)	0.22	0.90	3.55	0.21	0.84	9.07
Genotypes (G)	0.35	1.42		0.33	1.33	
G×C	0.50	2.02		0.47	1.89	

Table 5 Effects of different hardening conditions on root volume (cm^3) and number of roots on *in vitro* raised sugarcane clones

Genotype	Root volume (cm^3)		Mean	Number of roots per shoot		Mean
	Double shade net	Single shade net		Double shade net	Single shade net	
CoVC 09-61-02	1.75	1.05	1.40	12.08	7.58	9.83
CoVC 07-06-05	1.12	1.00	1.06	7.83	7.53	7.68
CoVC 09-61-07	1.33	1.16	1.25	8.70	8.68	8.69
CoC 671	1.42	1.33	1.38	8.92	8.78	8.83
CoVC 10-38-07	1.58	1.50	1.54	10.78	10.47	10.63
Mean	1.44	1.20		9.66	8.60	
	S.Em±	*CD@1%*	*CV%*	*S.Em±*	*CD@1%*	*CV%*
Condition (C)	0.03	0.11	8.03	0.10	0.40	4.33
Genotypes (G)	0.04	0.17		0.16	0.64	
G×C	0.06	0.24		0.23	0.91	

and 1.50, respectively. For root length also double shade net was found to be optimum for all the genotypes used, *viz.* CoVC 09-61-02 (16.91), CoVC 07-06-05 (13.82), CoVC 09-61-07 (13.72), CoC-671 (14.88) and CoVC 10-38-07 (15.58). The genotypes showed comparatively lower root length under single shade net condition (Table 6). Similarly for optimum number of roots double shade net was better for all the genotypes, *viz.*, CoVC 09-61-02 (12.08), CoVC 07-06-05 (7.83), CoVC 09-61-07 (8.70), CoC 671 (8.92) and CoVC 10-38-07 (10.78). The genotypes produced comparatively less number of roots, *i.e.* 7.58, 7.53, 8.68, 8.78 and 10.47, respectively under single shade net (Table 5). The low cholorophyll content in genotypes grown under single shade net resulted in less photosynthesis which restricted the root growth and hence low root volume. These results imply that the rate of root growth under certain conditions is determined by the supply of carbohydrates from the leaves. Results obtained by Wassink and Richardson (1951) and Richardson (1953) with first-year seedlings of *Acer pseudoplatanus* and *Acer saccharinum* indicate that root growth sometimes is dependent on carhohydrates currently produced in the leaves.

Under double shade net, the genotype CoVC 09-61-07 showed highest shoot length (30.45), chlorophyll content (mg/g) (33.54) and root volume (1.75), whereas lowest shoot length (20.06), chlorophyll content (20.15) and root volume (1.12) was observed in genotype CoVC 07-06-05.

Table 6 Effects of different hardening conditions on root length (cm) on *in vitro* raised sugarcane clones

Genotype	Root length (cm)		Mean
	Double shade net	Single shade net	
CoVC 09-61-02	16.91	10.63	13.77
CoVC 07-06-05	13.82	13.58	13.70
CoVC 09-61-07	13.72	11.55	12.63
CoC 671	14.88	13.63	14.25
CoVC 10-38-07	15.58	10.10	12.84
Mean	14.98	13.87	
	S.Em±	*CD@1%*	*CV%*
Condition (C)	0.19	0.77	5.68
Genotypes (G)	0.30	1.22	
G×C	0.43	1.73	

Genotype CoVC 09-61-02 showed optimum root length (16.91) and number of roots (12.08) while least root length was observed in genotype CoVC 09-61-07 (13.72) and number of roots (7.83) in genotype CoVC 07-06-05. The optimum survival response was shown by genotype CoC-671 with least mortality (3.33%) followed by CoVC 07-06-05 (3.89%). In contrast, highest mortality was observed in genotype CoVC 09-61-07 (8.33 %) followed by CoVC 09-61-02 (4.44%) (Table 7). Under single shade net, the genotype CoC 671 showed highest shoot length (27.76) and root length (13.63) followed by genotype CoVC 09-61-07 with 25.88 cm shoot length and 13.58 cm root length. Highest chlorophyll content (mg/g) (23.08), number of roots (10.47) and root volume (1.50) was observed in genotype CoVC 10-38-07 whereas, lowest chlorophyll content (mg/g) (19.92), number of roots (7.53) and root volume (1.00) was observed in genotype CoVC 07-06-05. The optimum survival response was shown by genotype CoVC 07-06-05 with least mortality (5.00%) followed by CoC 671 (9.74%). In contrast, highest mortality was observed in genotype CoVC 09-61-02 (21.00%) followed by genotype CoVC 09-61-07 (17.67%) (Table 7).

Double shade net was found to be optimum for all the genotypes, *viz*. CoVC 09-61-02, CoVC 07-06-05, CoVC 09-61-07, CoC 671 and CoVC 10-38-07 for the characters such as, chlorophyll content (mg/g), root characters, *viz*. number of roots, root length, root volume and survival ability. Whereas, for shoot length, single shade net was found to be optimum for genotypes CoVC 07-06-05 and CoC-671. For number of leaves there was no significant difference observed between two *ex vitro* conditions and genotypes. The optimum response of genotypes under double shade net might be due to lower average temperature (25.86°C), higher relative humidity (74.66 %) and lower light intensity (2771.6 lux) than single shade net with the average temperature, relative humidity and light intensity of 29.33°C, 67.66 per cent and 4983.3 lux, respectively. Short *et al.* (1987) also reported that optimum growth of cultured cauliflower and chrysanthemum occurred when plantlets were cultured with 80% relative humidity. Also the leaves of chrysanthemum and sugarbeet developed at relative humidity below 100 % had increased epicuticular wax, reduced leaf dehydration and improved stomatal functioning (Ritchie *et al.* 1991). Higher mortality (%) observed under single shade net might be due to high light intensity (4983.3 lux) and temperature (29. 33°C) during hardening period. Chandra *et al.* (2010) reported that high temperature and light intensity during hardening cause charring and wilting of plantlets. Further, higher temperature under single shade net might be one of the reasons for lower chlorophyll content (mg/g) in genotypes studied. Arun and Bhaisnab (1998) reported that higher temperature was not optimum for chlorophyll biosynthesis as it was partly due to impairment of 5-aminolevulinic acid biosynthesis and protochlorophyllide. Bhojwani and Razdan (1983) also reported that high humidity conditions are required during the initial days for successful transplantation and temperature during hardening plays an important role in the survival rate and growth of transplanted plants. High humidity during the monsoon months showed better survival rate and growth of the plantlets of *Chlorophytum borivilianum* under agro-shadenet conditions compared to plantlets hardened *in vitro* and subsequently transferred to the greenhouse for acclimatization (Dave *et al.* 2003). In *Alocasia amazonica* plantlets regenerated under *in vitro* condition under photon flux density (PFD) of 15 or 30 μmol m^{-2} s^{-1} showed better growth and development than those grown under higher PFDs. The amount of chlorophyll a and chlorophyll b decreased while the number of stomata increased with increasing PFD. In conclusion, light intensity affects growth and development of stomata (Jo *et al*., 2007). Our result also matches with findings of Scaranari *et al.* (2009). They obtained superior outcomes of banana plantlets cultivated under red 70% shade cloth, for six weeks in the summer.

Based on the results obtained in the present investigation it can be concluded that both the *ex vitro* acclimatization conditions (double shade net and single shade net) showed almost similar effect on number of leaves per plant (Table 7). However, for chlorophyll content (mg/g), root characters, *viz*. number of roots, root length (cm), root volume (cm^3) and survival ability, double shade net was found to be optimum for all the genotypes, *viz.* CoVC 09-61-02, CoVC 07-06-05, CoVC 09-61-07, CoC 671 and CoVC 10-38-07. Whereas, CoVC 07-06-05 and CoC 671 produced optimum shoot length under single shade net (Plates 1 to 5). From these observations, it is clear that the double shade net was optimum for development of appropriate morphological characters during hardening and is better for survival of all

Table 7 Effects of different hardening conditions on *in vitro* raised sugarcane clones for all the parameters

Genotype	Double shade net							Single shade net						
	SL	RL	CH	NL	RV	NR	M%	SL	RL	CH	NL	RV	NR	M%
CoVC 09-61-02	24.71	16.91	32.21	3.23	1.75	12.08	4.44	22.82	10.63	21.72	3.05	1.05	7.58	21.00
CoVC 07-06-05	20.06	13.82	20.15	3.04	1.12	7.83	3.89	20.15	13.58	19.92	3.05	1.00	7.53	5.00
CoVC 09-61-07	30.45	13.72	33.54	3.25	1.33	8.70	8.33	25.88	11.55	22.69	3.05	1.16	8.68	17.67
CoC 671	26.34	14.88	24.58	3.33	1.42	8.92	3.33	27.76	13.63	21.08	2.95	1.33	8.78	9.74
CoVC 10-38-07	26.50	15.58	26.33	3.16	1.58	10.78	4.11	23.59	10.10	23.08	3.37	1.50	10.47	12.51

SL- Shoot length, RL- Root length, CH- Chlorophyll content, NL- Number of leaves, RV- Root volume, NR- Number of roots per shoot and M%- Mortality %

the genotypes used in the present study which is in contrast with the findings of Yadav *et al.* (2019). They reported single shade net to be ideal for most of the sugarcane genotypes. The variation in response of genotypes to two different hardening conditions is almost entirely due to genotypic variation in sugarcane. Hence, it is of paramount importance to optimize genotype specific protocols for popular leading sugarcane clones. Thus, the developed protocol can be used for not only rapid mass multiplication but also production of low-cost and healthy sugarcane plantlets on a commercial scale in short period of time which will supplement the conventional propagation.

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