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Production of Somaclone *In vitro* for Drought Stress Tolerant Plantlet Selection in Sugarcane (*Saccharum officinarum* L.)

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Abstract

An experiment was carried out at the Biotechnology Laboratory of Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna for the development of drought tolerant somaclones. Five sugarcane varieties viz. Isd 20, Isd 35, Isd 36, Isd 37 and Isd 38 were used as plant material. Unexpanded spindle leaf sheaths were used as explants in tissue culture. In the first culture, the MS medium (BM) was supplemented with 2,4-D (3 mgL^{-1}) and coconut water (10 %) for callus induction. The callus was then sub-cultured on fresh BM with BAP (2.0 mgL^{-1}) and Kinetin (1.0 mgL^{-1}) for plantlet initiation (2nd culture). In the third culture, initiated plantlets were sub-cultured again on fresh BM contained NAA (5.0 mgL^{-1}) for root development. In all cultures BM was supplemented with 0.0, 5.0, 7.5 and 10.0% poly ethylene glycol (PEG) and was semi solidified with 0.6% agar to select somaclone variant plantlets of sugarcane *in vitro*. In the first culture 100% explants initiated callus on medium supplemented with no PEG. Callus induction, proliferation and plantlet regeneration decreased with increased level of PEG. At 7.5% PEG, the callus induction was highest (80%) in varieties Isd 35 and Isd 38. Callus was induced but became reddish black and senescence within 40 days on BM supplemented with 10.0 % PEG. Both shoot and root production decreased with increased PEG level in the medium. At 7.5 % PEG in BM, the highest shoot number was in Isd 38 (5.5 per culture), root number (7.6 per shoot) and root length (1.2 cm) were in the variety Isd 38. The highest shoot length was in Isd 37 and Isd 38 (1.8 cm). Survival percentage of *in vitro* regenerated plantlets was 100 % during hardening in low cost polythene house and in establishment in the field.

Keywords*: Somaclone, sugarcane, drought stress, plantlet, *in vitro

1. Introduction

Sugarcane (*Saccharum officinarum* L.) requires ten to twelve months from planting to harvesting and faces various adverse environmental conditions such as drought, waterlogging, flood, salinity, high temperature, toxicity etc. (Wang *et al.*, 2003). These abiotic stresses significantly decrease quantity and quality of production and limits plant growth (Bayer, 1982), which eventually result in lower cane yield as well as sugar production in Bangladesh. Drought is one of the principal environmental stress which constraints sugarcane production in Bangladesh. To overcome this barrier, development of drought tolerant cultivar is an important task to improve sugarcane production.

Several techniques may be adopted to develop drought tolerant cultivars. Selection of drought tolerant plantlets from *in vitro* regenerated plantlets is one of the prospective methods. Tissue culture with stress inducing chemicals generates a wide range of genetic variation in plant species, which can be incorporated in plant breeding programs. By *in vitro* selection,

mutants with useful agronomic traits, e.g. salt or drought tolerance or disease resistance can be isolated in a shorter duration (El-Aref, 2002). Sugarcane is often considered as a model crop in which cultivar improvement has been possible through tissue culture technique. This method refers to heritable changes that occur in callus during somatic embryogenesis and are expressed in progeny of *in vitro* regenerates in course of culture. In plant cell, genetic variability develops spontaneously during tissue culture and creates somaclonal variation. Somaclonal variation has provided important genetic material both for genetic studies and for *in vitro* selection of desired traits in plants (Scowcroft *et al*., 1983; Maddock and Semple, 1986 and Taghian and El-Aref, 1997). In sugarcane, tissue culture induces considerable phenotypic variability (Burner and Grishan, 1995).

Polyethylene glycol (PEG) has long been used in research programs to induce water deficit stress in plants (Dolgikh *et al*., 1994; Adkins *et al*., 1995 and Chazen *et al*., 1995). PEG neither enters nor substantially degrades and is not absorbed by cells in culture. The cells are left under the stress of water deficits in a manner similar to that they would be under actual desiccation condition (El-Aref, 2002).

In vitro selected plants with a significant improvement for drought tolerance were reported for maize (Dolgikh *et al.,* 1994), sorgham (Duncan *et al.,* 1995), wheat (Almansouri *et al.,* 2001), rice (Adkins *et al.,* 1995), *Vigna radiata* (Gulati and Jaiwal, 1994) and Tagetes (Mohamed *et al.,* 2000). However, report on sugarcane grown *in vitro* for plantlets production to select ambient cultivar in Bangladesh is little. The present experiment was undertaken for the first time in Bangladesh on *in vitro* regeneration of sugarcane for selection of drought tolerant plantlet. The experiment was undertaken to provide information on tissue culture, plantlet regeneration and somaclone selection under drought stress *in vitro* to establish drought tolerant plantlet selection in sugarcane.

2. Materials and Methods

2.1. Plant material

Five sugarcane varieties viz. Isd 20, Isd 35, Isd 36, Isd 37 and Isd 38 were used as plant material. Unexpanded spindle leaf sheaths collected from 3-4 months old sugarcane plants grown in Bangladesh Sugarcane Research Institute (BSRI) experimental field were used as explants.

2.2. Treatment and culture method

In the first culture the MS medium (BM) was supplemented with 2,4-D (3 mgL^{-1}) and coconut water (10 %) for callus induction. In the second culture, callus was sub-cultured on fresh BM with BAP (2 mgL^{-1}) and Kinetin (1 mgL^{-1}) for plantlet initiation. In the third culture, initiated plantlets were separated as healthy and unhealthy groups and sub-cultured again on fresh BM contained NAA (5 mgL^{-1}) for root development. Plantlets having distinct shape and shoot with leaves were selected as healthy plantlets. In all cultures, BM was supplemented with 0.0, 5.0, 7.5 and 10.0% poly ethylene glycol (PEG) as treatment. The culture medium was semi solidified with 0.6% agar. The pH of the medium was adjusted to 5.8 prior to autoclave under 1.2 $kgcm^{-2}$ at 121 ºC for 20 minute. The culture vessels were 14 cm x 1.2 cm (length x dia.) test tubes, which contained 20 ml BM and maintained in 25±1 ºC at 30 $\mu molm^{-2}s^{-1}$ lighting for 16 hours provided by fluorescent tubes.

2.3. Acclimatization of plantlets

In vitro healthy and rooted plantlets were washed to remove medium adhered with roots and planted in earthen pots containing sterilized soils-press mud mixture and were kept under polythene shed with higher moisture (>90% RH) for 3 weeks to harden (Fig. 4a & 4b). The hardened plantlets were ready for transplantation in the field.

2.4. Data collection and analysis

The data were collected on callus induction percent, plantlet regeneration per culture and acclimatization of plantlets and were analyzed

following factorial Completely Randomized Block Design (CRBD) using computer software MSTAT-C. The analysis of variance was performed and means were compared by Duncan's New Multiple Range Test (DNMRT) at 5 % level of probability.

3. Results and Discussion

3.1. Callus initiation and its features

Calli initiation revealed after 3 weeks culture at cut ends of explants. Explants produced callus varied depending on PEG concentration of medium. In control (0.0% PEG), almost all explants initiated callus. With the increased level of PEG the percentage of callus initiation decreased differently (Table 1a-1c, Fig. 1). Explants of all varieties induced callus vigorously in medium with no PEG. Among the varieties callus initiation percentage were varied distinctly. The highest number of explants induced callus in the variety Isd 35 (75.8%) followed by Isd 38 (75.0 %) and the lowest was for Isd 36 (Table 1a). Higher PEG supplement into medium significantly decreased percent callus induction. In control 99.0 % explants induced callus which was reduced to 86.0% at 5% PEG into medium. With increasing level of PEG supplement into medium callus induction decreased gradually. At 10.0 % PEG only 10.0 % explants induced callus.

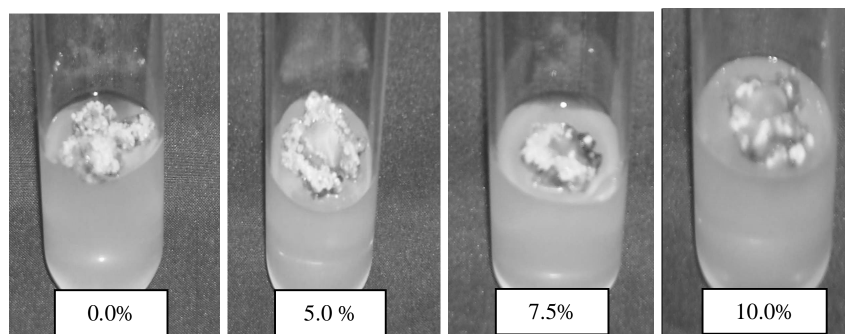

Fig. 1. Effects of % PEG supplemented into BM on callus induction in variety Isd 35.

Table 1a. Main effect of sugarcane varieties on callus induction in medium supplemented with different concentrations of PEG.

Name of varieties	% explants induced callus
Isd 20	67.1b
Isd 35	75.8
Isd 36	43.6
Isd 37	64.6
Isd 38	75.0
$LSD_{0.05}$	2.561

Table 1b. Main effect of different concentrations of PEG on callus formation of some sugarcane varieties.

% PEG into medium	% explants induced callus
0.0 % PEG	99.0a
5.0% PEG	86.0b
7.5 % PEG	65.9c
10.0 % PEG	10.0d
$LSD_{0.05}$	2.291

The interaction effects of PEG to varieties on callus induction were significant. Almost in all varieties 100% explants initiated callus in control (Table 1c). At 5.0 % PEG into medium, 100 % explants of Isd 35 initiated callus and was followed by Isd 20 and Isd 38 produced 95 % callus. At 7.5 % PEG, explants of Isd 35 and Isd 38 initiated the highest of 80 % callus which decreased to 50 % at 10 % PEG. The varieties Isd 20, Isd 36 and Isd 37 did not initiate any callus at 10 % PEG. Cells grown under stress may have to spend more metabolic energy than those grown in the absence of stress. The extra energy is probably used up in regulating osmotic adjustment resulting in declined callus growth. (Babu *et al.*, 2007). The quality of callus varied with PEG supplement into medium. Initiated callus was comparatively bigger, brighter and more whitish yellow in medium with no or low level PEG and was turned into reddish, gritty, poor and black with increased level of PEG into medium (Fig. 1, Table 2).

The morphology of callus is taken as a criterion for selection of callus for regeneration under stress condition (Nabors and Dykes, 1985; Narayanan & Rangasamy, 1991). The callus initiation was very poor on medium with 10.0 % PEG, became black and died after 40-45 days culture. The present results agree with those of Taghian (2002) in sugarcane. In triticale, Birsin and Özgen (2004) indicated that the effect of genotypes on callusing from mature embryo varied significantly. Gandonou *et al.* (2005) observed significant differences between nine sugarcane genotypes for callus induction capacity, embryogenesis and plant regeneration ability and indicated that these criteria are genotype dependent. Similarly, Badawy *et al.* (2008) and Burner (1992) also reported that the callus induction capacity in sugarcane is genotype dependent. In the present investigation, different sugarcane varieties responded differently in callusing to various levels of PEG concentration into medium. Resistant varieties showed minimum reduction in callus initiation even at high level PEG. This result confirms that the genetic constitution plays a major role in callus induction in sugarcane.

Table 1c. Interaction effect of varieties x PEG concentrations into the medium on callus formation.

Variety x % PEG	% explants * induced callus
Isd 20 x 0.0	98.30ab
Isd 20 x 5.0	95.00b
Isd 20 x 7.5	75.00e
Isd 20 x 10.0	0.00i
Isd 35 x 0.0	98.30ab
Isd 35 x 5.0	100.00a
Isd 35 x 7.5	80.00d
Isd 35 x 10.0	25.00h
Isd 36 x 0.0	100.00a
Isd 36 x 5.0	50.00g
Isd 36 x 7.5	24.30h
Isd 36 x 10.0	0.00i
Isd 37 x 0.0	98.30ab
Isd 37 x 5.0	90.00c
Isd 37x 7.5	70.00f
Isd 37x 10.0	0.00g
Isd 38 x 0.0	100.00a
Isd 38 x 5.0	95.00ab
Isd 38 x 7.5	80.00c
Isd 38 x 10.0	25.00f
$LSD_{0.05}$	4.436

* For each treatment 20 explants were cultured.

3.2. *Shoots regeneration from callus under PEG induced drought stress*

The calli sub-cultured on new fresh BM supplemented with different levels of PEG and PGR regenerated plantlets 4-5 weeks culture. The total number of shoots, healthy shoot per culture, and shoot length significantly varied among different varieties (Table 3a and Fig. 2). Isd 38 produced the highest number of shoots per culture. Number of healthy shoots per culture also varied significantly among varieties. The highest number of healthy shoots were produced in Isd 20 and Isd 38. Shoot length was the highest in Isd 37 followed by Isd 38 (Table 3a). Number of shoots, healthy shoots and shoot length were decreased significantly with increasing level of PEG supplemented into medium (Table 3b). The interaction effects of varieties to PEG concentrations on number of shoot, healthy shoot and shoot length varied significantly (Table 3c). All varieties regenerated the highest number of shoots, healthy shoots and shoot length in control compared to 5.0 % and 7.5 % PEG supplemented into medium. At 5.0 % PEG into medium, the highest number of shoots (10.1 per culture) and healthy shoot were produced in Isd 20 (Table 3c, Fig. 2). Similarly, healthy shoot number was also the highest in Isd 20, followed by Isd 38. The highest shoot length at 5% PEG was in Isd 37 (4.1 cm) followed by Isd 38 (3.1 cm). Isd 20 and Isd 36 produced less shoot at 7.5 % PEG. The reasons could be attributed to the presence of non-viable cells in the callus, which later on affected the formation of shoots. This finding is in agreement with the findings of Khan *et al.* (2004).

Table 2. Effects of PEG into BM on morphological features of callus.

% PEG supplemented into BM	Morphological features visually estimated in different varieties				
	Isd 20	Isd 35	Isd 36	Isd 37	Isd 38
0.0% (control)	Vigorous, whitish, healthy and no dead tissue	Vigorous, whitish, healthy and no dead tissue	Vigorous, whitish, healthy and no dead tissue	Vigorous, whitish, healthy and small dead tissue	Vigorous, whitish, healthy and small dead tissue
5.0 % PEG	Vigorous light reddish and with dead tissue	Vigorous light reddish and with dead tissue	Vigorous light reddish and with big dead tissue	Vigorous reddish and with dead tissue	Vigorous reddish and with dead tissue
7.5 % PEG	Poor, reddish and with big black patch	Poor, reddish and with big black patch	Poor, reddish and with black patch	Very poor, reddish and with big black patch	Less vigorous reddish and with small dead patch
10.0 % PEG	Callus did not induced	Very poor, reddish, gritty, with big dead patch	Callus did not induced	Callus did not induced	Very poor, reddish, gritty, with big dead patch

At 7.5 % PEG, the highest number of shoot was produced in Isd 38 (5.5 per culture) followed by the variety Isd 37 (5.3 per culture) and Isd 35 (5.1 per culture, Table 3c). Under same PEG level, the highest number of healthy shoots was produced in Isd 36 (1.7 per culture) followed by Isd 20 (1.6 per culture) and the shoot length was highest in Isd 37 (1.8 cm) and Isd 38 (1.8 cm). The variable number of shoot formation also indicates that the callus initiated in first culture was embryogenic with differential regeneration potential. The present results showed the presence of significant variation among different genotypes for the production of embryogenic calli that might be resulted due to the genetic variability. These findings agree with those reported by Ather *et al.* (2009) and Raza *et al.* (2010) for sugarcane. Those authors showed significant difference in embryogenic callus production and shoot regeneration. Similar results were also reported by Gandonou *et al.* (2005) in sugarcane. On the contrary, Khan *et al.* (2009) observed non-significant difference in shoot induction from callus of three different sugarcane cultivars. Different growth performances of varieties under increasing PEG level in the present experiment might be due to genetic potentiality of the varieties or due to somaclonal variation. Bower and Birch (1992) also used different genotypes and found that potentiality of different genotypes to produce the embryogenic calli is the most critical parameters. It reveals the capacity of calli to regenerate plantlets from one or few cells.

3.3. Root initiation in shoots regenerated under PEG induced drought stress

Root initiation in mini shoots sub-cultured on fresh BM supplemented with different level of PEG started after one week. After three weeks the number and length of root varied significantly depending upon PEG supplement into medium (Table 4a-4c, Fig. 3). The number of roots per shoot was the highest in Isd 38 followed by Isd 37 and the lowest was in Isd 36. Similarly, the root length was the highest in Isd 38 followed by Isd 37. The concentration of PEG supplemented into medium affected the growth of roots significantly (Table 4b). Both the number of roots and length of roots per plantlet gradually decreased with increase level of PEG. The interaction effects of varieties to different concentrations of PEG on the number of roots and root length varied significantly (Table 4c). In control treatment, the highest number and length of roots were recorded in Isd 38 (13.1 per shoot and 2.3 cm). At 5 % PEG the highest number of roots were produced in the variety Isd 38 (9.6 per shoot) followed by Isd 37 (9.5 per shoot) and the highest root length was produced in the variety Isd 37 (1.2 cm) and Isd 38 (1.2 cm).

Table 3a. Main effect of varieties on shoot regeneration from callus after 60 days culture.

Name of varieties	Total number of shoot per culture	Number of healthy shoot per culture	Avg. shoot length (cm)
Isd 20	8.4b	3.9a	2.0e
Isd 35	8.1b	3.0d	3.0c
Isd 36	7.6c	3.5c	2.4d
Isd 37	8.2b	2.3e	4.3a
Isd 38	9.0a	3.7b	3.5b
LSD $_{0.05}$	0.3372	0.1777	0.1532
CV (%)	4.29	5.66	5.30

Table 3b. The effect of PEG level into medium on shoot regeneration from callus after 60 days culture.

PEG % in medium	Total number of shoot per culture	Number of healthy shoot per culture	Shoot length (cm)
0.0 %	12.2a	5.3a	5.0a
5.0 %	8.3b	3.2b	2.7b
7.5 %	4.2c	1.4c	1.3c
$LSD_{0.05}$	0.2612	0.1377	0.1187

Table 3c. Interaction effect of PEG levels into medium to varieties on callus derived shoot regeneration after 60 days culture.

Variety x % PEG	Number per culture		Shoot length (cm)
	shoot	healthy shoot	
Isd 20 x 0.0 %	13.0b	6.1a	3.9d
Isd 20 x 5.0 %	10.1e	4.2d	1.5i
Isd 20 x 7.5 %	2.1j	1.6hi	0.7k
Isd 35 x 0.0 %	11.0d	4.9c	4.9c
Isd 35 x 5.0 %	8.2f	2.7f	2.8f
Isd 35 x 7.5 %	5.1h	1.5hi	1.4i
Isd 36 x 0.0 %	12.0c	5.5b	4.1d
Isd 36 x 5.0 %	7.5g	3.3e	2.1g
Isd 36 x 7.5 %	3.2i	1.7gh	1.0j
Isd 37 x 0.0 %	11.2d	4.0d	6.9a
Isd 37 x 5.0 %	8.2f	2.0g	4.1d
Isd 37x 7.5 %	5.3h	1.0j	1.9h
Isd 38 x 0.0 %	13.9a	6.1a	5.5b
Isd 38 x 5.0 %	7.7fg	3.9d	3.1e
Isd 38x 7.5 %	5.5h	1.3ij	1.8h
$LSD_{0.05}$	0.5841	0.3079	0.2653
CV (%)	4.29	5.66	5.30

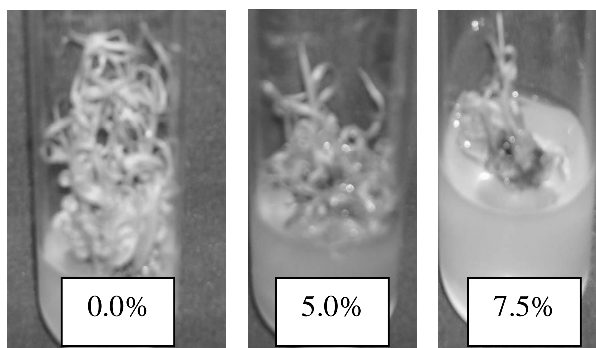

Fig. 2. Effects of PEG concentrations on shoot regeneration in the variety Isd 20.

Table 4a. Main effect of varieties on root initiation in plantlets.

Name of variety	Avg. number of root per shoot	Avg. root length (cm)
Isd 20	6.1d	0.7d
Isd 35	6.4c	1.0c
Isd 36	4.6e	0.7d
Isd 37	6.9b	1.1b
Isd 38	7.6a	1.2a
$LSD_{0.05}$	0.167	0.0795

Table 4b. Main effect of PEG level supplemented in medium on root initiation in plantlets.

% PEG in medium	Number of root per shoot	Avg. root length (cm)
No PEG	11.3a	1.9a
5 % PEG	7.7b	0.9b
7.5 % PEG	0.0c	0.0c
$LSD_{0.05}$	0.1296	0.06157

Table 4c. Interaction effect of PEG concentrations to sugarcane varieties on root growth.

Variety x % PEG	Number of root per shoot	Root length (cm)
Isd 20 x 0.0%	10.0c	1.4c
Isd 20 x 5.0%	8.4f	0.7f
Isd 20 x 7.5%	0.0i	0.0g
Isd 35 x 0.0%	13.0a	2.1b
Isd 35 x 5.0%	6.1g	1.0e
Isd 35 x 7.5 %	0.0i	0.0g
Isd 36 x 0.0%	9.0e	1.4c
Isd 36 x 5.0%	4.9h	0.7f
Isd 36 x 7.5%	0.0i	0.0g
Isd 37 x 0.0%	11.2b	2.2ab
Isd 37 x 5.0%	9.5d	1.2d
Isd 37 x 7.5 %	0.0i	0.0g
Isd 38 x 0.0%	13.1a	2.3a
Isd 38 x 5.0%	9.6d	1.2d
Isd 38 x 7.5%	0.0i	0.0g
$LSD_{0.05}$	0.2897	0.1377

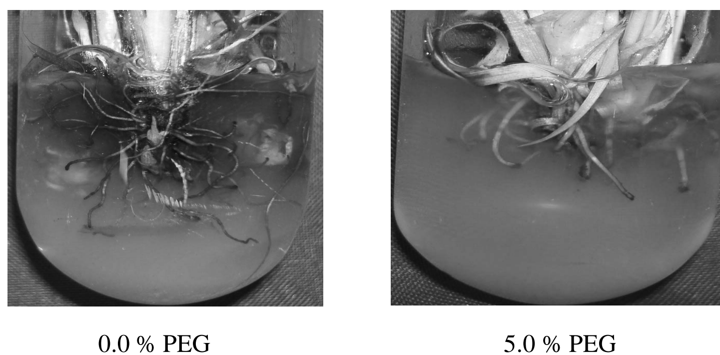

Fig. 3. Effects of PEG level into medium on rooting in the plantlets *in vitro* (var. Isd 20).

Table. 5. Survival percentage of the plantlets under hardening in polythene shed.

Sl. No.	Name of the varieties	Survival Percentage
1.	Isd 20	95
2.	Isd 28	98
3.	Id 35	98
4.	Isd 36	97
5.	Isd 37	99
6.	Isd 38	98

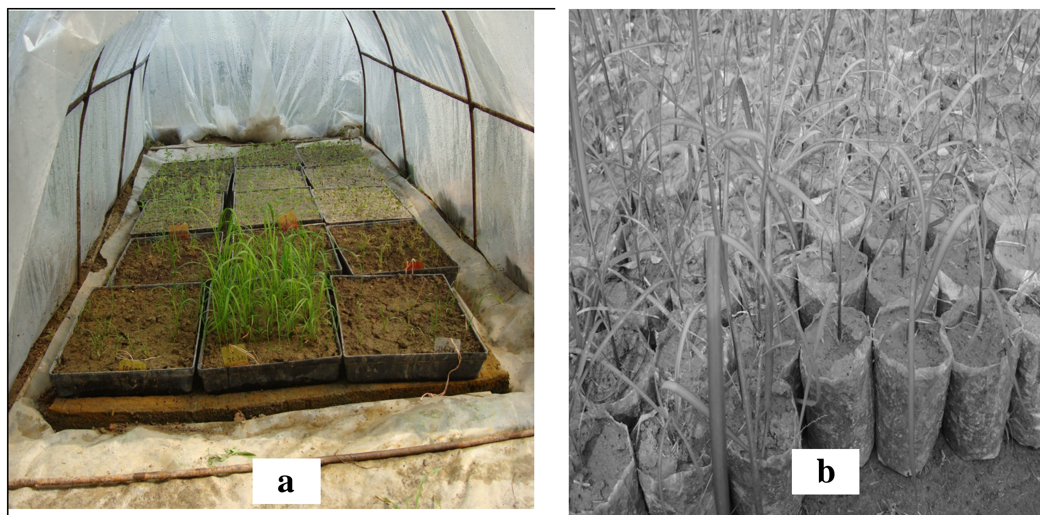

Fig. 4. Hardening of plantlets in locally made low cost polythene shed (a) in plastic trays (b) plantlets are ready for transplantation in the field.

Survival of plantlets was 95-99 % during hardening in low cost polythene house (Table 5). Temperature and humidity of the polythene house were 36 ºC, and above 90 %, respectively. Duration of hardening was 2 weeks under polythene-shed covering (Fig. 4a) and one week in open field in polybag soil (Fig. 4b). Selected plantlets were transplanted in the field for further drought tolerance studies.

4. Conclusions

The results of present investigation indicate a procedure and feasibility of *in vitro* regenerated plantlet production and selection *in vitro* by adding PEG in the medium for screening drought tolerant plantlets. Callus selected from culture medium supplemented with 10 % PEG might be highly tolerant. Shoot and root formation obtained at 5 % PEG level might also be drought tolerant. However, for confirmation of the results, the growth performance of selected plantlets in field is to be evaluated.

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