Plant Cell, Tissue and Organ Culture (PCTOC)
https://doi.org/10.1007/s11240-019-01603-9

ORIGINAL ARTICLE

Reduction in hyperhydricity and improvement in in vitro propagation of commercial hard fibre and medicinal glycoside yielding *Agave sisalana* Perr. ex Engelm by NaCl and polyethylene glycol

Tukaram D. Nikam[1] · Ketki V. Mulye[1] · Mahadev R. Chambhare[1] · Harichandra A. Nikule[1] · Mahendra L. Ahire[2]

Received: 24 November 2018 / Accepted: 21 March 2019

Abstract

Agave sisalana is hapaxanthic monocotyledonous plant yielding a hard fibre of commercial value. It is also a source of medicinally important glycosides such as hecogenin and tigogenin. Sisal plantations can play a significant role in reforestation of hilly barren land. Unfortunately, natural propagation is not sufficient to fulfill the demands; besides, hyperhydricity is a severe problem in the in vitro propagation method of *A. sisalana*. The present study for the first time demonstrates that, the hyperhydricity problem can be solved by inclusion of osmotic stress inducing agents like sodium chloride (NaCl) and polyethylene glycol (PEG 6000) (0.0, 0.1, 0.2, 0.3, 0.4, or 0.5% w/v) in MS medium. The response of hyperhydric shoots, normal shoots and hyperhydric reverted shoots with the treatments was analyzed for water content, chlorophyll content, osmolyte accumulation and oxidative damage. The principal component analysis showed the significant positive and negative variations in the net photosynthesis, stomata conductance, internal CO_2, transpiration, and water use efficiency between normal, hyperhydric and hyperhydric reverted shoots among all the time points of day and night period. Besides, detailed anatomical and ultra-structural observations were made to discern the changes that occurred in epidermis, mesophyll cells, vascular bundles, and stomata. About 85 and 58% of hyperhydric shoots were reverted on medium fortified with 0.2% NaCl and 0.1% PEG, respectively. All reverted shoots were normal and survived when transferred to the field conditions. The improved protocol with the treatment of NaCl for recovery of hyperhydric shoots was not cost intensive and without any harmful effects on shoots and plantlets.

Key message

First report on change in physiology and recovery of hyperhydricity with NaCl and PEG inshoots and somatic embryos of Agave sisalana which allows additional 38% plantletsavailable for plantation.

Keywords Asparagaceae · CAM plant · Hardening · Reforestation · Osmotic stress · Sisal · Shoot regeneration · Somatic embryo

Communicated by Sergio Rosales-Mendoza.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11240-019-01603-9) contains supplementary material, which is available to authorized users.

✉ Tukaram D. Nikam
tdnikam@unipune.ac.in

Ketki V. Mulye
kvmulye@gmail.com

Mahadev R. Chambhare
madhavc04@gmail.com

Harichandra A. Nikule
hanikule@gmail.com

Mahendra L. Ahire
mlahire@gmail.com

[1] Department of Botany, Savitribai Phule Pune University, Pune, MS 411 007, India

[2] Department of Botany, Yashavantrao Chavan Institute, Satara, MS 415 001, India

Published online: 01 April 2019

Introduction

Sisal (*Agave sisalana* Perrine; Family—Asparagaceae APG 2009), is a perennial, xerophytic, hapaxanthic monocotyledonous plant, which yields hard fibre of commercial value and is the sixth most important fibre crop at global level (FAO 2012). It is native to Mexico and widely cultivated in Central America, Asia, Brazil, Africa and many other tropical countries. The properties of Sisal fibre such as strength, durability, ability to stretch, interaction with dyestuff and resistance to deteriorate, give the economic importance (McLaughlin and Schuck 1991; Gonzalez et al. 2015). The species may be used for live fence and ornamental purposes (FAO 2012). The plant is the source of medicinally the important glycosides hecogenin and tigogenin (Debnath et al. 2010). Besides, due to its crassulacean acid metabolism (CAM), the plant has an most important role in restoration of barren land as it grows well on hilly, dry and sandy soil, coastal dunes and in deserts (Arizaga and Ezcurra 2002; Weber et al. 2016). Due to their underground propagating structures (rhizomes, tubers, corms, or bulbs), massive spreading root system, shape of the leaves direct the rain water or water condensed to the root system, bunch of leaves, and colonial nature, the plants receive the protection during the forest fire and drought. Such strategies are rarely observed in other plants. Therefore, Sisal was found to be the best plant species for reforestation program, conservation of water, prevention of soil erosion, prevention of spread of forest fire and improvement of economy in arid barren hilly regions as observed in Brazil, Tanzania, Kenya (Mwaniki et al. 2017), Madagascar, Mexico, Haiti, South Africa, Mozambique China (Sarkar and Jha 2017), India (Sarkar et al. 2018) and some countries in Asia (Debnath et al. 2010; Santos et al. 2015; Kundu et al. 2018).

Sisal is a monocarpic in nature, it flowers after 10–15 years of vegetative growth, and life of the plant ends with flowering. However, Sisal does not produce seeds; if rarely produced these are non-viable (Tewari et al. 2014). Naturally, Sisal is propagated by suckers and formation of bulbils from on inflorescence axis. However, this is slow process and it is not useful for large scale propagation and plantation of Sisal (Debnath et al. 2010). These factors are also not permitting the genetic improvement of Sisal using conventional methods. Using plant tissue culture technique, during the last few decades, the in vitro propagation protocols has been developed (Binh et al. 1990; Nikam 1997; Hazra et al. 2002; Reyes-Zambrano 2016, Rodriguez-Garay 2016). However, the protocols for *A. sisalana* (Nikam et al. 2003), *A. tequilana* (Santacruz-Ruvalcaba and Portillo 2009), *Dianthus chinensis* (Gao et al. 2017) and different blueberry cultivars (*Vaccinium* spp.) (Gao et al. 2018) were associated with the problem of hyperhydricity which did not allow plants to transfer to the field condition easily and their survival was reducing by 50–60%. This is possible because hyperhydricity causes morphological, anatomical, and physiological abnormalities in the plantlets (Olmos and Hellıin 1998; Piqueras et al. 2002).

Therefore, in the present investigation, to overcome a severe problem of hyperhydricity in in vitro propagation of *A. sisalana*, the effect of different concentrations of the ionic, penetrating, osmotic agent, sodium chloride (NaCl), and the non-ionic, non-penetrating agent polyethylene glycol (PEG) on hyperhydric plant material was studied. Inclusions of these agents in the medium were investigated in terms of reversion of hyperhydric shoots and their growth, morphological, physiological and biochemical responses. Besides, the anatomical changes were studied in detail to understand the mechanism of NaCl and PEG on the reversion of hyperhydric shoots and the elimination during in vitro propagation of *A. sisalana*.

Materials and methods

Plant material and growth condition

In vitro cultures of *A. sisalana* were raised from the central meristematic part of the bulbils. The bulbils were collected from naturally grown plants in the village area of Bornar (altitude 213 meters above the sea level; and coordinates 20.8447° N, 75.4367° E) of the Jalgaon district of Maharashtra, India. The explants were surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 min and rinsed seven times with sterilized distilled water. The explants were cultured onto the callus induction medium containing MS salts (Murashige and Skoog 1962) in combination with 1.5 mg/l BA + 0.5 mg/l NAA, MS + 0.25 mg/l NAA + 1.5 mg/l Kin and MS + 0.25 mg/l 2,4-D + 1.0 mg/l BA (Nikam 1997). The pH of the medium was adjusted to 5.8 and solidified with 0.8% agar–agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) prior to autoclaving at 121 °C for 15 min. The cultures were incubated at 25 ± 2 °C under a 8 h photoperiod (30 µmol m^{-2} s^{-1} PFD) using cool white fluorescent tube lights (Philips Ltd., Mumbai, India), and 70% relative humidity. After 4 weeks of culture incubation, in order to maintain and multiply the cultures, the callus was regularly sub-cultured on freshly prepared callus induction medium. The part of the callus produced on MS + 1.5 mg/l BA + 0.5 mg/l NAA was transferred onto shoot regeneration medium MS + Kin (0.2–2.0 mg/l). The somatic embryogenic special masses developed from callus on medium MS + 0.25 mg/l NAA + 1.5 mg/l Kin and MS + 0.25 mg/l 2,4-D + 1.0 mg/l BA (Fig. 1b) were transferred to MS + Kin

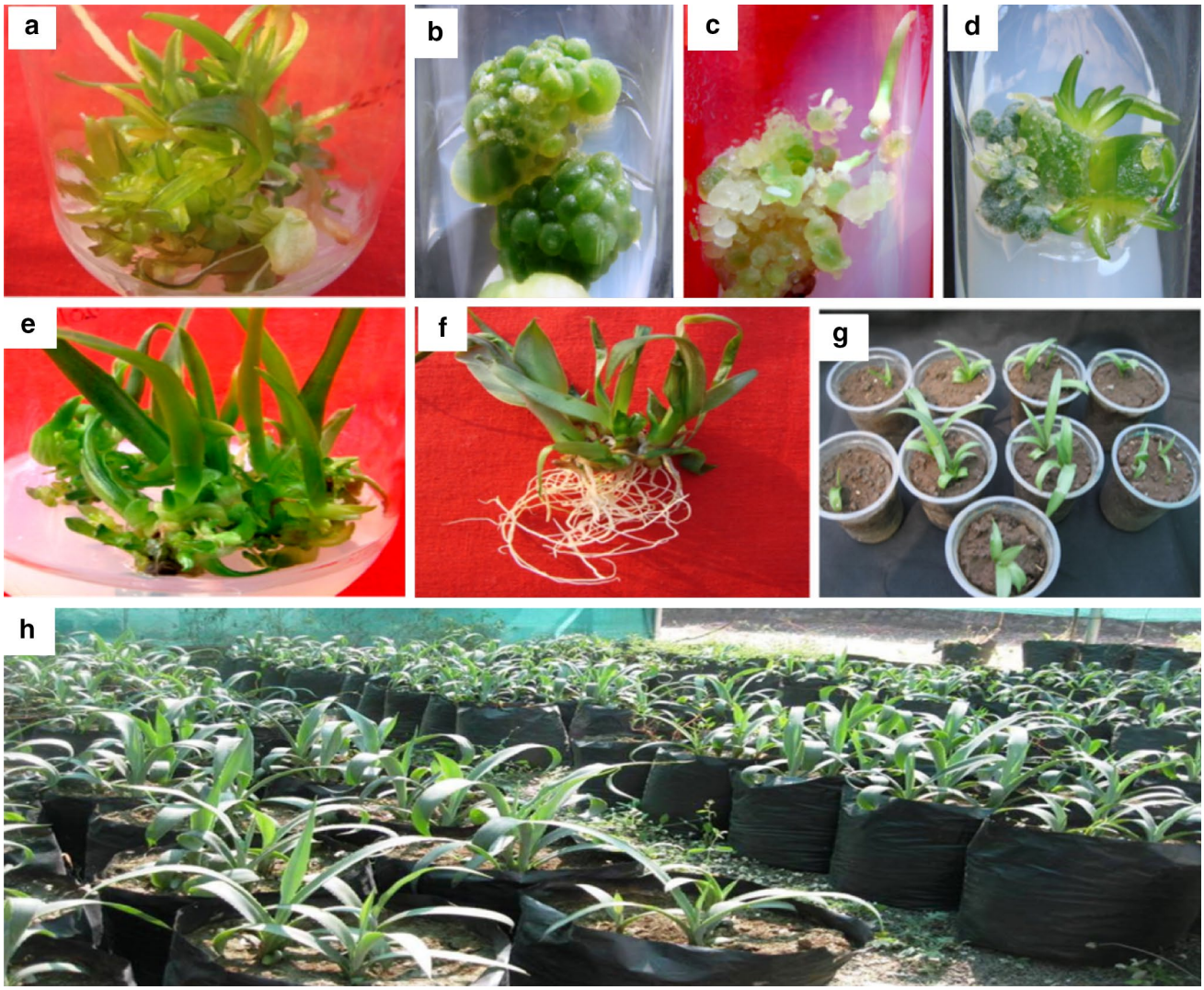

Fig. 1 Effect of NaCl on hyperhydric shoots and in vitro rooting and acclimatization of hyperhydric recovered plantlets. Hyperhydric shoots developed on MS + 2.0 mg/l BA (**a**) and embryogenic callus on MS + 0.25 mg/l 2,4-D + 1.0 mg/l BA (**b**), germination of somatic embryos on MS + 0.1 mg/l Kin (**c, d**), showing turgid and translucent or glassy leaves (**a, c, d**), effect of 0.2% NaCl treatment on hyperhydric shoots of *A. sisalana* (**e**), rooting of recovered hyperhydric shoots on MS + 0.1 mg/l NAA medium (**f**), hardening of *in vitro* grown hyperhydric recovered plantlets in the small plastic glass containers (**g**), field transferred acclimatized plantlets (**h**)

(00. 0.1, 0.2 mg/l) (Fig. 1c, d). Among subculture, about 38% of regenerated shoots and somatic embryos showing stunted growth with turgid, translucent, and glassy leaves (Fig. 1c) and shoots, were considered as hyperhydric shoots (Fig. 1a, c, d). These hyperhydric shoots were used to study the effect of different concentrations of NaCl and PEG on their recovery.

NaCl and PEG treatment to hyperhydric shoots

The hyperhydric shoots and somatic embryos were cultured on shoot multiplication medium consisting of MS salts together with 3% sucrose, 2.0 mg/l BA, 0.8% agar–agar and various concentrations (0, 0.1, 0.2, 0.3, 0.4, or 0.5%) of NaCl and PEG 6000. The cultures were incubated for 4 weeks under controlled conditions as described earlier. After 4 weeks, hyperhydricity recovery percentage was recorded (Fig. 1e) and also evaluated for physiological, biochemical and anatomical parameters.

In vitro rooting and acclimatization of hyperhydricity recovered shoots

The shoots reverted from hyperhydricity after treatment with NaCl and PEG were sub-cultured on MS medium containing 0.1 mg/l NAA and 3% sucrose. The cultures were incubated for 4 weeks under controlled condition as described earlier. Well rooted plantlets were carefully removed from the culture

bottles and washed under tap water to remove the traces of agar and were initially transferred to small plastic glass containers (200 ml) containing sterilized sand: soil (1:1) mixture. The potted plantlets were subsequently transferred to the shade net house with 50% cut off of natural light intensity. Four week hardened plantlets were then transferred to the field conditions (Average humidity 40–50%; light intensity 3442 µmol m^{-2} s^{-1} PFD; and temperature minimum 19–23 °C and maximum 36–38 °C).

Determination of tissue water status

Tissue water status was determined by recording the fresh weight (FW) and dry weight (DW) of the leaf of normal, hyperhydric and reverted shoots. The leaves were dried at 80 °C in a hot air oven until constant weight. The tissue water status was expressed as percent tissue water content (TWC %) calculated using the equation: $TWC\ \% = [(FW - DW)/FW] \times 100$.

Determination of pigments content, relative electrolytic leakage (REL), lipid peroxidation, total soluble sugars (TSS) and free titratable acidity

Chlorophylls and carotenoids content were estimated by the method of Lichtenthaler and Buschmann (2001). Damage to the membrane was determined in terms of relative electrolytic leakage according to the method of Sullivan (1972). Level of lipid peroxidation was estimated by measuring the levels of malondialdehyde (MDA) content using the thiobarbituric acid (TBA) method explained by Heath and Packer (1968). Total soluble sugar in samples was estimated by using the anthrone method (Watanabe et al. 2000). Total free titratable acidity was determined as malate and citrate content in the fresh mature leaf tissue samples (Castillo 1996).

Gas exchange parameters measurement

The photosynthetic rate (PN), internal CO_2 (Ci), stomatal conductance (gS), transpiration rate (E) and water use efficiency (WUE) were measured in the second pair of fully expanded leaves using a portable photosynthesis system Infrared Gas Analyzer (LI-6400XT, LI-COR Inc., USA). The parameters were measured under a constant flow of 300 µmol mol^{-1} CO_2 and 800 µmol m^{-2} s^{-1} PAR, 3.9 kPa vapor pressure deficits, and 25 °C block temperature. The WUE was calculated as the ratio of net photosynthesis rate and transpiration rate ($WUE = PN/E$) (Shelke et al. 2017).

Measurement of stomatal density, stomatal index, and stomata size

Leaf peel was taken from the abaxial surface and stomatal densities were determined at 100X magnification by using Axio scope (Zeiss scope A1) microscope. Stomatal index (SI) for each sample were determined by using the formula; $SI = [S/(E + S)] \times 100$, where, number of stomata (S), and number of epidermal cells (E) per unit leaf area (Pompelli et al. 2010). The stomata size was measured at ×40 magnification using stage (0.01 mm) and ocular scale with microscope (Olympus OIC—612086, Japan).

Light and scanning electron microscopy (SEM)

The transverse sections of leaf samples were stained with 1% (v/v) aqueous safranin and observed under a light microscope at ×100 magnification, the structural variation among the samples were digitized using a digital camera. For scanning electron microscopy, leaf samples of in vitro grown normal, hyperhydric and reverted shoots were cut into 0.5 cm^2 pieces and fixed in 2.5% glutaraldehyde (Fluka chemicals, Buchs, Switzerland) in phosphate buffered saline. The leaves were dehydrated by increasing grades of ethanol from 10 to 100% for 60 min in each grade (Steinmacher et al. 2011). The plant material was further dried and mounted on stub and coated with platinum in an auto fine coater (JEOL JFC-1600, Tokyo, Japan) and subjected to SEM (JEOL JSM-6360A, Japan) for leaf surface observations.

Experimental design and statistical analyses

The experiments were arranged in completely randomized design (CRD). For NaCl and PEG treatment, 20 replicates per treatment were used. Anatomical, physiological and biochemical analyses were performed with three replicates and repeated at least thrice. The data were analyzed by one-way ANOVA using the statistical software (SPSS 16.0) and Duncan's multiple range test (DMRT) was applied to reveal significant differences at ($p < 0.05$). The data observations variability was expressed as mean ± SE.

Results

Effect of NaCl and PEG on recovery of hyperhydric shoots

About 38% of callus and somatic embryo derived shoots with shorter, brittle, thick, tuft, curled, glossy, light green and translucent leaves (Tables 1, 2) were collectively referred to be of a hyperhydric nature (Fig. 1a, c, d, and Supplementary Fig. S1a). The effect of NaCl and PEG on hyperhydric shoots was measured at the end of the 3rd week after the beginning of the experiment. The hyperhydric shoots were characterized by average higher FW, lower DW and high moisture content compared to normal shoots and hyperhydric shoots treated with NaCl and PEG

Table 1 Normal and hyperhydric shoot regeneration in callus of *A. sisalana*

Callus produced on	Callus transferred on MS + Kin (mg/l)	Regeneration of normal shoots	Regeneration of hyperhydric shoots
MS + 1.5 mg/l BA + 0.5 mg/l NAA	0.2	11.1 ± 1.20^{b}	4.01 ± 0.91^{b}
	0.5	19.0 ± 0.93^{a}	8.74 ± 0.70^{a}
	1.0	17.2 ± 1.41^{a}	7.05 ± 1.0^{a}
	2.0	7.80 ± 0.62^{c}	3.30 ± 1.12^{b}

Data are mean ± SE scored and followed by same letter are not significantly different at $P \leq 0.05$ level by Duncan's multiple range test

Table 2 Normal and hyperhydric somatic embryo formation in callus of *A. sisalana*

Embryogenic Callus produced on	Embryogenic Callus transferred on MS + Kin (mg/l)	Formation of normal somatic embryo	Formation of hyperhydric somatic embryo
MS + 0.25 mg/l NAA + 1.5 mg/l Kin	00	3.41 ± 1.43^{b}	1.10 ± 0.40^{a}
	0.1	5.23 ± 1.0^{a}	2.31 ± 0.86^{a}
	0.2	4.18 ± 0.92^{a}	2.01 ± 0.51^{a}
MS + 0.25 mg/l 2,4-D + 1.0 mg/l BA	00	3.90 ± 1.0^{b}	1.60 ± 0.30^{a}
	0.1	6.24 ± 1.63^{a}	2.23 ± 0.93^{a}
	0.2	5.13 ± 1.21^{a}	2.41 ± 0.72^{a}

Data are mean ± SE scored and followed by same letter are not significantly different at $P \leq 0.05$ level by Duncan's multiple range test

(Supplementary Table S1). NaCl and PEG treatment significantly influenced the tissue water content in hyperhydric shoots. No differences were observed in tissue water content between shoots subjected to NaCl and PEG treatments (Supplementary Table S1). The hyperhydric shoots did not produce proper roots and were unable to survive on transfer to field conditions.

Visible appearance in *A. sialana* plantlets was quantified by the ability of the plant to grow on transfer to the field conditions with the treatment of NaCl and PEG. The hyperhydric plants subjected to the moderate treatment of NaCl and PEG showed the minimum hyperhydricity symptoms (Table 3, Fig. 1e, and Supplementary Fig. S1 b). Compared to PEG, the recovery in shoots from hyperhydricity symptoms was earlier and significantly higher in the presence of NaCl. Results also evidenced the influence of high concentration of NaCl and PEG on shoots growth, showing declining in recovery from hyperhydricity and gradual yellowing. At the end of the 4th week, all shoots were died under NaCl stress situation (0.5%) (Table 3).

Table 3 Effect of NaCl and PEG 6000 on hyperhydric shoots of *A. sisalana* grown on MS medium supplemented with 2.0 mg/l BA

Treatments	Concentration (%)	Number of plants recovered	Percent recovery
Control	0.0	2.1^{d}	10.3^{d}
NaCl	0.1	9^{b}	45^{b}
	0.2	16.7^{a}	85^{a}
	0.3	7.8^{c}	38.3^{c}
	0.4	4.3^{d}	21.65^{d}
	0.5	0^{e}	0^{e}
PEG 6000	0.1	11.9^{a}	58.3^{a}
	0.2	7.6^{b}	38^{b}
	0.3	5.3^{c}	26.65^{c}
	0.4	2.6^{d}	11.7^{d}
	0.5	1^{e}	5^{e}

Data are mean ± SE scored and followed by same letter are not significantly different at $P \leq 0.05$ level by Duncan's multiple range test

In vitro rooting and acclimatization of reverted hyperhydric plantlets

After the treatment of NaCl and PEG, the reverted hyperhydric shoots were subjected to rooting media showed the best result on MS medium containing 0.1 mg/l NAA; an average 3–4 roots were produced from the base of each shoot by the end of the 3rd week of culture (Fig. 1f). The plantlets transferred to sand–soil mix and maintained under the shade-net house, attained a height of 7–8 cm at the end of a month (Fig. 1g). All acclimatized plantlets survived transferred to field conditions and were morphologically similar to the parental plants (Fig. 1h).

Influence on chlorophyll and carotenoid content

The hyperhydric shoots raised on BA containing medium exhibited a reduction in chlorophyll a and b of 30 and 50% respectively, and loss of approximately 30% of the carotenoids (Supplementary Table S2). The treatments of NaCl and PEG to hyperhydric shoots significantly influenced the content of photosynthetic pigments; the average content of chlorophylls and carotenoids reached the level of non-hyperhydric leaves at the end of the fourth week after initiation of the treatments (Supplementary Table S2). In the preliminary experiment, we observed that, the hyperhydric shoots treated with higher level of NaCl (0.5%) and PEG (0.5%) did not show the reversion in content of photosynthetic pigments.

Effect on oxidative damage

Lipid peroxidation was measured in terms of malondialdehyde (MDA) accumulation. MDA content and relative electrolyte leakages (REL) was notably high (69.38%) in hyperhydric shoots compared to non-hyperhydric shoots (Supplementary Table S3). The reduction in MDA content and REL was observed in response to moderate level of NaCl and PEG.

Effect on accumulation of TSS

The content of total soluble sugars in hyperhydric shoot was low, compared to non-hyperhydric shoot (Supplementary Table S3). The reverted shoots obtained from transfer of hyperhydric shoots on medium fortified with moderate levels of NaCl and PEG, the restoration of the level of TSS were accomplished at the end of the 4th week and the accumulation was the same as normal shoots.

Influence on titratable acid number (TAN)

Titratable acid number (TAN) was measured at the morning 6 am and 9 am, noon 12, afternoon 3 pm, evening 6 pm, night 9 pm, in the middle of night 12 am and 3 am (Supplementary Fig. S3, 4) in leaf of natural, in vitro normal and hyperhydric shoots and reverted hyperhydric shoots after 4th weeks treatment of NaCl and PEG. In leaf of natural, in vitro normal and reverted hyperhydric shoots, TAN values were gradually increasing from evening to the end of the dark period, and reverse is the case from morning to the end of the light period indicating CAM diurnal acidification. This difference was prominent in naturally grown plants to that of in vitro grown normal and hyperhydricity reverted shoots. There was no marked difference observed between in vitro grown normal and hyperhydricity reverted shoots. While in hyperhydric shoots no significant changes were observed in TAN values during dark and light period.

Principal component analysis revealed the variation among normal, hyperhydric and reverted hyperhydric shoots

Results of photosynthesis rate (PN), stomatal conductance (gS), internal CO_2 (Ci), transpiration rate (E) and water use efficiency (WUE) of leaf tissues of normal shoots, hyperhydric shoots and reverted hyperhydric shoots at field and in vitro conditions during day and night period are shown in Supplementary Fig. S5. Auto-scaling data depicts the relative distribution of the cultures condition for their physiological response. The first axis i.e. (PC1) was identified as distribution of plants as per their normal and treated conditions (NaCl and PEG), while the second axis PC2 was considered as the variation with physiological attributes of CAM metabolism in terms of PN, gS, Ci, E and WUE during the light and dark periods (Supplementary Fig. S5 a,b). The PCA bi-plots results revealed that the first component accounted for 82.23% of variance and the second component for 17.76% the variance during day times (Supplementary Fig. S5 a), while PC1 99.99% variance and PC2 0.001% variance during night times (Supplementary Fig. S5b). Therefore, the significant positive and negative variations were observed in the PN, gS, Ci, E and WUE among normal, hyperhydric and hyperhydricity reverted plants.

Influence on stomatal characteristics

The stomata of *A. sisalana* are of anomocytic type. The guard cells are dumbbell shaped. The stomata in hyperhydric

Table 4 Stomatal characteristics (size, density, and stomatal index) of control, hyperhydric, NaCl and PEG 6000 treated plants of *A. sisalana*

In vitro developed shoots	Size of stomata		Stomatal (density/mm^2)	Stomatal index (%)
	Width (µm)	Length (µm)		
Normal shoots	0.38 ± 0.02^{a}	0.41 ± 0.01^{b}	30 ± 1.78^{a}	7.2^{a}
Hyperhydric shoots	0.35 ± 0.02^{b}	0.56 ± 0.03^{a}	11.58 ± 0.64^{c}	4.97^{c}
NaCl (0.1%) treated shoots	0.38 ± 0.02^{a}	0.40 ± 0.01^{b}	16.84 ± 1.96^{b}	5.77^{b}
PEG 6000 (0.2%) treated shoot	0.38 ± 0.02^{a}	0.39 ± 0.01^{b}	15.27 ± 0.56^{b}	5.20^{b}

Data are mean ± SE scored and followed by same letter are not significantly different at $P \leq 0.05$ level by Duncan's multiple range test

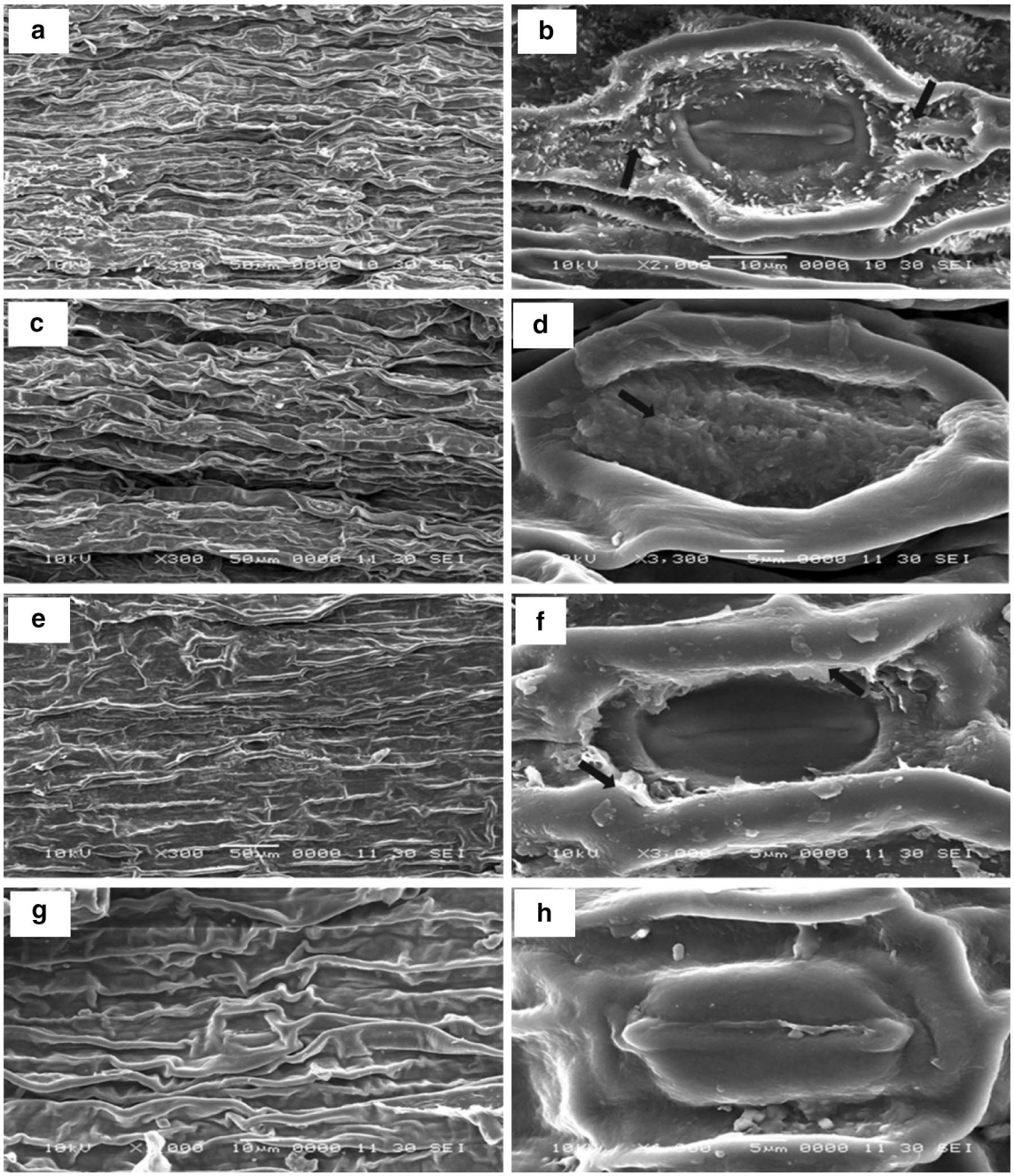

Fig. 2 Scanning electron microscopy (SEM) micrographs of stomata in normal, hyperhydric and NaCl and PEG treated hyperhydric leaves. Leaf surface overview of normal plants (**a**). Stomata of normal leaf showing parallel oriented platelets of epicuticular wax (**b**). Leaf surface overview of hyperhydric plants (**c**). Stomata of hyperhydric leaf occluded by epicuticular wax plug which is in dendrite form (**d**). Leaf surface overview of NaCl treated hyperhydric plant (**e**). Stomata of NaCl treated hyperhydric leaf showing parallel oriented platelets of epicuticular wax (**f**). Leaf surface overview of PEG treated hyperhydric plant (**g**). Stomata of PEG treated hyperhydric leaf without epicuticular wax plug (**h**)

shoots were larger in size and oval in shape with low width and longer length compared to normal shoots (Table 4, Fig. 2a–h). In normal and hyperhydric shoots reverted after the treatment of NaCl and PEG, the stomata were spherical in shape and almost similar in length and width. The stomatal density and stomatal index of hyperhydric shoots was significantly lower compared to the normal shoots (Table 4) and reverted hyperhydric shoot with NaCl and PEG. The stomatal size, shape, density, and index were

similar in 1 year old reverted hyperhydric and normal plantlets grown in the field condition (Table 4).

Influence on anatomical and ultra-structural characteristics

The epidermis of non-hyperhydric shoot leaf of *A. sisalana* is continuous, consisting of uniform size spherical epidermal cells (Supplementary Fig. S2d). The mesophyll is homogenous, and has compactly arranged cells with abundant chloroplasts. The vascular bundles are conjoint, collateral, and open type. They are of various sizes and embedded in mesophyll tissue (Supplementary Fig. S2d). On the other hand, hyperhydric shoot leaf epidermis is discontinuous, consisting uneven and large epidermal cells. The mesophylls are composed of globular large cells, less number of chloroplasts and possess numerous intercellular spaces. The vascular bundles conjoint collateral and open, but xylem organization and lignifications were very poor (Supplementary Fig. S2b, c, e, f).

The observations of leaf surface carried out using SEM showed that, in hyperhydric shoot leaf, the stomata were exist at the surface along with the epidermal cells (Fig. 2a, c). On the other hand, the stomata were sunken in position in normal shoots. Similarly, the stomata were become sunken in position in leaf of hyperhydric shoot grown on medium containing moderate level of NaCl after 3 weeks of culture (Fig. 2b, d). However, these features of leaf of hyperhydric shoots grown in PEG fortified medium still did not reach the sunken position as those of in leaf of non-hyperhydric and NaCl treated shoots, and the differences between the two groups of shoots were still statistically significant even after 3 weeks of culture. Parallel oriented platelets of epicuticular wax which form distinct pattern around the stomata were observed in non-hyperhydric leaf (Fig. 2a). In contrast, in hyperhydric leaf, the stomata occluded by epicuticlar wax plug of spherical form or otherwise in dendrite form (Fig. 2d). There was no difference in orientation and pattern of epicuticular wax deposition in leaf of NaCl treated shoots (Fig. 2f) and leaf of non hyperhydric shoots. While in leaf of PEG treated shoots, around stomata the platelets and plugs of epicuticular wax were absent (Fig. 2 h).

Discussion

In vitro propagation of *Agave* using liquid medium has been limited success (Santacruz-Ruvalcaba and Portillo 2009). Direct and indirect method of shoot regeneration and somatic embryo formation has been reported for several commercially important *Agave* species; however, these protocols are different due to species specific responses (Reyes-Zambrano 2016; Rodriguez-Garay 2016). The somatic embryogenesis system in *A. sisalana* (Nikam et al. 2003) and *A. tequilana* (Portillo and Santacruz-Ruvalcaba 2006) were associated with hyperhydric embryo formation. In the present investigation, the increased proliferation of shoot and somatic embryos were accompanied by hyperhydricity (Table 1 and Fig. 1) as has been reported in several in vitro propagation systems (Rossetto et al. 1992; Gao et al. 2018). To minimize the hyperhydricity in vitro, different approaches have been applied such as changes in type and concentration of gelling agents, cytokinins and ventilation of culture vessels and certain improvements have been achieved in in vitro propagation systems (Ivanova and Van Staden 2011; Gao et al. 2018).

Hyperhydricity is one of the major barriers in large scale in vitro propagation of *A. sisalana*. Once the regenerated shoots or plantlets become hyperhydric, it would be very rarely revert to its normal state (Gao et al. 2018). In this context, the role of NaCl and PEG described in the present investigation is a new approach for the reversion of in vitro regenerated hyperhydric shoots to normal ones. Hyperhydric shoots of *A. sisalana* could be brought to normal type on subculture to MS medium containing 0.2% NaCl and 0.1% PEG. At the end of four weeks of culture, no significant differences in morphological characteristics were observed between reverted and normal shoots (Fig. 1e and Supplementary Fig. S1b). At low concentrations of NaCl (0.1–0.3%), hyperhydric shoots might be maintaining osmotic balance through modulated uptake of sodium ion and their sequestration into vacuole.

The mechanism of osmosis may allow withdrawn of excess water from hyperhydric shoots and helps to exhibit normal growth. At the high concentrations of NaCl (0.4–0.5%), hyperhydric shoots may not be able to sequester excess amount of Na^+ ions into vacuole, resulting accumulation in the cytosol and causes adverse physiological and biochemical effects. This might be the reason why hyperhydric shoots turned senescent at the end of the 4th week of culture. PEG is a non-phytotoxic and non-penetrating inert osmoticum, helps to lower down the water potential in shoots (Ghuge et al. 2010; Kacem et al. 2017). The excess water from hyperhydric shoots might be withdrawn by osmotic activity imposed by PEG (0.1–0.3%) and the shoots reverted into normal shoots. At higher levels of PEG (0.4–0.5%), the shoots suffered due to desiccation caused by excess osmosis. Compared to use of higher concentrations of costlier agar–agar, phytagel or gelrite, incorporation of NaCl and PEG in the medium is beneficial as higher concentration of gelling agent affect the availability of components of nutrient medium and PGR to the growing cells (Ghuge et al. 2010; Badr-Elden et al. 2012; Kacem et al. 2017). These results revealed that inclusion of low levels of NaCl and PEG in the medium is beneficial for reverting of hyperhydric shoots and 38% increase in healthy plantlets production in in vitro propagation system of *A. sisalana*. Besides, this is a user

friendly procedure over the application of cooling devices for minimizing the relative humidity. The osmotic stress inducing agents such as NaCl or PEG will act as a choice of chemicals for reducing the problem of hyperhydricity in the tissue culture of *A. sisalana* during large scale production of tissue culture plants.

Physiological and biochemical responses of hyperhydric and reverted shoots

The excess accumulation of water in the apoplast during hyperhydric condition can disturb the gas exchange by cells and may not have the adequate oxygen supply to the cells (Van den Dries et al. 2013). However, the reason for excess accumulation of water in the tissue of hyperhydric plants is still not clear (Gao et al. 2018). Active functioning of stomata regulates the gas exchange and water loss. Stomata in hyperhydric leaves showed abnormal and elongated guard cells which give oval appearance rather than spherical as observed in the control and NaCl and PEG reverted shoot leaves. Poor organization and functioning of stomata structure might be one of the reasons for induction of hyperhydricity (Chakrabarty et al. 2006). In a study carried out on hyperhydric plants, the stomatal closure or reduced stomatal aperture has been observed (Fig. 2c, d, f, h).

Results of the present investigation also support the thought that *A. sisalana* is a susceptible plant to excess water, which was evident by exhibition of high oxidative stress. The oxidative stress at initial level can be detected in terms of membrane damage rate as well as through the peroxidation of membrane lipid in terms of MDA content. In this case, the REL and MDA content were significantly higher in hyperhydric shoots (Supplementary Table S3) and indicated an increase oxidative damage under hyperhydricity (Ghuge et al. 2010). This might be possible due to poor functioning of antioxidant components in *A. sisalana* under hyperhydric situation. Whereas, shoots reverted after the treatments with NaCl and PEG showed lower levels of REL and MDA compared to hyperhydric shoots and almost near to the control. This was likely associated by reducing the hypoxia condition and lipid peroxidation due to osmotic stress. For instance, lower levels of REL and MDA were observed as a result of treatment with NaCl and PEG in *Solanum tuberosum* plant leaves (Ghuge et al. 2010). It has also been demonstrated that in hyperhydric leaves, the activity of antioxidant enzymes (like catalase, superoxide dismutase, ascorbate peroxidase, glutathione reductase) increased to scavenge ROS (Chakrabarty et al. 2006). Moreover, the oxidative damage observed in hyperhydric shoots (Supplementary Table S3) could be related to the influence the photosynthetic pigments and photosynthesis (Ardelean et al. 2014). In the present study, compared to the control, hyperhydric shoots showed reduced Chlorophyll *a*, *b*, total chlorophyll, and carotenoid content. However, shoots reverted after treatment with NaCl and PEG showed an increased total chlorophyll and carotenoid content. It is also corroborated by an earlier report that content of photosynthetic pigments decline in hyperhydric shoots and increased in reverted shoots of apple (*Malus*) species (Chakrabarty et al. 2006). Therefore, in the present investigation, these consequences may be responsible for reduced photosynthetic efficiency of hyperhydric plants and thereby affecting the growth rate of *A. sisalana*.

Existence of hypoxia under hyperhydricity eventually affects the respiration, and plants shift their metabolic pathway from aerobic to anaerobic fermentation pathway (Taiz and Zeiger 2010). Fermentation pathway is a safe route that allows the synthesis of 2 mol of ATP against 36 per mole of glucose produced during aerobic respiration. Hence to compensate the energy deficit, glycolysis is accelerated, leading to the depletion of carbohydrate reserves (Parent et al. 2008). These physiological changes are what make the reduction of total soluble sugar one of the most sensitive effects on shoots of *A. sisalana* caused by hyperhydricity. However, improvement in total soluble sugar content was recorded in NaCl and PEG reverted shoots; this might be possible by maintaining the osmotic balance of the cell during the oxidative stress generated by NaCl and PEG. Fontes et al. (1999) reported a reduction in starch accumulation in chloroplast of hyperhydric plantlets. It is worth noting that physiological events such as accumulation of total soluble sugar and starch may be associated with accumulation of water in the cell.

Principal component analysis

The results employed on PCA in the present study, might be useful in predicting and understanding correlation between the parameters responsible for hyperhydricity and reversion of hyperhydricity problems in in vitro cultures of *A. sisalana* and other plant species (Badr-Elden et al. 2012). The bi-plot (Supplementary Fig. S5a) illustrates that PC1 provide FGP (6 am) as the foremost dominant variable appearing to be a positive PC1 value and Ci as the dominant variable with a positive value for the second component. During day time at 6 am all the parameters (such as PN, gS, Ci and E) and FGP showed dominant positive results in terms of improved WUE, while at other time points the FGP, IvNS and HS showed negative reverse effect in terms of WUE. The internal CO_2 (Ci) and water use efficiency (WUE) were the dominant variables of PC2, while FGP at 6 am, 12 pm were the dominant variables of PC2. Both components revealed significant difference between the normal plants and hyperhydricity reverted shoots and their physiological response under CAM metabolism during light and dark period.

Anatomical responses of hyperhydric and reverted shoots

In general, the leaves of reverted shoots were almost similar to those of the normal shoots. However, the anatomic structure of leaves of hyperhydric shoots was much distinct from the leaves of reverted and normal shoots. The leaves showed the characteristics of discontinuous epidermis with large and uneven epidermal cells. As an overall, mesophyll cells were larger in size with abundant intercellular spaces which gave spongy appearance to hyperhydric leaves. Similarly to our results, an increase in large intercellular spaces has been observed because of schizogenous processes (Picoli et al. 2008). Concurrently with other studies (Fauguel et al. 2008), the vascular elements in the hyperhydric leaves were poorly developed and characterized by hypolignification of xylem than normal and reverted shoot leaves. Decreased lignification due to reduction in peroxidase activity was reported in *Prunus avium* (Franck et al. 2001; Picoli et al. 2008), *Dianthus* (Saher et al. 2004), and *Origanum vulgare* (Picoli et al. 2008).

The stomata of normal and reverted shoots were quite similar with spherical guard cells, whereas in hyperhydric leaves showed abnormal and elongated guard cells due to which stomata appeared oval shaped. Likewise elongation of guard cell was observed and is the result of deformation of the cell plate or alteration in the pattern of deposition of cellulose micro fibrils occurs during division of the primary stomata mother cells (Chakrabarty et al. 2006). The major cause of desiccation and failure of in vitro raised plantlets on transfer to *ex vitro* conditions is the presence of non-functional stomata. The presence of epicuticular wax limits the non-stomatal water loss and gas exchange, repel lipophilic pathogenic spores and dust and provide protection from UV light (Lee et al. 2009). Therefore, it is also a vital component in plants. Its poor development results in poor survival rate when transferred from in vitro to field conditions mainly due to water loss. The scanty formation of epicuticular wax in leaves of *A. sisalana* may be one of major reason for failure of survival of in vitro raised plantlets to the field conditions. The results of the present investigation revealed that some physiological disturbances may have affected the stomatal structures and epicuticular wax deposition which can be normalizing with the treatment of NaCl and PEG to the hyperhydric shoots.

Conclusion

In conclusion, the results of the studies on physiological, biochemical and anatomical parameters of hyperhydric shoots, hyperhydric reverted shoots with the treatment of NaCl and PEG and normal shoots suggest that the hyperhydricity problem in in vitro propagation protocols could be overcome by the simple inclusion of NaCl in the shoot subculture medium. The finding of the present study will be useful to reduce the cost and time of in vitro propagation and to increase about 38% more number of hyperhydricity reverted plantlets availability of the *A. sisalana* for plantation. As the *Agave* is having CAM metabolism and manages growth even with sandy soil and scanty water on plantation in hilly barren land may be helpful for reforestation where water availability is scanty or no water is available and watering for plants is not economically feasible. Thus, the barren land may be utilized for production of commercially important hard fibres and pharmaceutically important glycosides hecogenin and tigogenin. The protocol may be applicable to other important species of genus *Agave* which also affected due to hyperhydricity in in vitro propagation system such as *A. tequilana*, *A. americana*, *A. fourcroydes*, and *A. victoria-reginae* etc.

Acknowledgements The authors are grateful to University Grant Commission (UGC) for financial support for the Project F. No. 37-468/2009 (MS) (SR).

Author Contributions TDN and MLA conceived the experiments. KVM, MRC, and HAN performed the experiments. TDN, KVM, MLA, and MRC analyzed the data and wrote the manuscript. All authors have reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Angiosperm Phylogeny Group (APG III) (2009) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants. Bot J Linn Soc 161:105–121

Ardelean M, Cachita-Cosma D, Ardelean A, Ladasiu C, Mihali VC (2014) The effect of heat stress on hyperhydricity and guaiacol peroxidase activity (Gpox) at the Foliar Lamina of *Sedum telephium* L. Ssp. Maximum (L.) Krock Vitroplantlets. An Stiintifice Univ Al. I. Cuza din Iasi 60(2):21–31

Arizaga S, Ezcurra E (2002) Propagation mechanisms in *Agave macroacantha* (Agavaceae), a tropical arid-land succulent rosette. Am J Bot 89(4):632–641

Badr-Elden AM, Nower AA, Ibrahim IA, Ebrahim MK, Elaziem TMA (2012) Minimizing the hyperhydricity associated with in vitro growth and development of watermelon by modifying the culture conditions. Afr J Biotech 11(35):8705–8717

Binh LT, Muoi LT, Oanh HTK, Thang TD, Phong DT (1990) Rapid propagation of *Agave* by in vitro tissue culture. Plant Cell Tiss Org Cult 23(1):67–70

Castillo FJ (1996) Antioxidative protection in the inducible CAM plant *Sedum album* L. following the imposition of severe water stress and recovery. Oecologia 107(4):469–477

Chakrabarty D, Park SY, Ali MB, Shin KS, Paek KY (2006) Hyperhydricity in apple: ultrastructural and physiological aspects. Tree Physiol 26:377–388

Debnath M, Mukeshwar P, Sharma R, Thakur GS, Lal P (2010) Biotechnological intervention of *Agave sisalana*: a unique fibre yielding plant with medicinal property. J Med Plants Res 4(3):177–187

FAO W (2012) IFAD. The state of food insecurity in the world 65

Fauguel CM, Vega TA, Nestares G, Zorzoli R, Picardi LA (2008) Anatomy of normal, and hyperhydric sunflower shoots regenerated in vitro. Helia 31(48):17–26

Fontes MA, Otoni WC, Carolino SMB, Brommonschenkel SH, Fontes EPB, Fari M, Louro RP (1999) Hyperhydricity in pepper plants regenerated in vitro: involvement of BiP (binding protein) and ultrastructural aspects. Plant Cell Rep 19(1):81–87

Franck T, Gaspar T, Kevers C, Penel C, Dommes J, Hausman JF (2001) Are hyperhydric shoots of *Prunus avium* L. energy deficient? Plant Sci 106(6):1145–1151

Gao H, Xia X, An L, Xin X, Liang Y (2017) Reversion of hyperhydricity in pink (*Dianthus chinensis* L.) plantlets by AgNO3 and its associated mechanism during in vitro culture. Plant Sci 254:1–11

Gao H, Li J, Ji H, An L, Xia X (2018) Hyperhydricity-induced ultrastructural and physiological changes in blueberry (*Vaccinium* spp.). Plant Cell Tiss Org Cult 133:65–76

Ghuge SA, Rai AN, Suprasanna P (2010) Comparative effects of NaCl, PEG and mannitol iso-osmotic stress on solute accumulation and antioxidant enzyme system in potato (*Solanum tuberosum* L.). Plant Stress—Glob Sci Books 4(1):50–55

Gonzalez JTC, Dillon AJP, Perez-Perez AR, Fontana R, Bergmann CP (2015) Enzymatic surface modification of Sisal fibres (*Agave sisalana*) by *Penicillium echinulatum* cellulases. Fibres Polym 16(10):2112–2120

Hazra SK, Das S, Das AK (2002) Sisal plant regeneration via organogenesis. Plant Cell Tiss Org Cult 70:235–240

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125(1):189–198

Ivanova M, Van Staden J (2011) Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. Plant Cell Tiss Org Cult 104:13–21

Kacem NS, Delporte F, Muhovski Y, Djekoun A, Watillon B (2017) *In vitro* screening of durum wheat against water-stress mediated through polyethylene glycol. J Genet Eng Biotechnol 15:239–247

Kundu DK, Sarkar S, Saha AR, Jha AK, Behera MS (2018) Application of micro-irrigation and micronutrients to improve fibre yield and water use efficiency in sisal (*Agave sisalana* Perr. Ex Engelm.). Int J Curr Microbiol App Sci 7(10):2101–2108

Lee SB, Jung SJ, Go YS, Kim HU, Kim JK, Cho HJ, Suh MC (2009) Two Arabidopsis 3-ketoacyl CoA synthase genes, KCS20 and KCS2/DAISY, are functionally redundant in cuticular wax and root suberin biosynthesis, but differentially controlled by osmotic stress. Plant J 60:462–475

Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids: measurement and characterization by UV-Vis spectroscopy. In: Wrolstad RE, Acree TE, An H, Decker EA, Penner MH, Reid DS, Schwartz SJ, Shoemaker CF, Sporns P (eds) Current protocols in food analytical chemistry (CPFA). Wiley, New York, pp F4.3.1–F4.3.8

McLaughlin SP, Schuck SM (1991) Fiber properties of several species of Agavaceae from the southwestern United States and northern Mexico. Econ Bot 45:480–486

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Mwaniki AM, Kisangau DP, Musimba NLR (2017) Socio-economic factors affecting Sisal cultivation and adoption in Kiomo Division, Kitui County. Int J Agron Agri R 11(3):97–103

Nikam TD (1997) High frequency shoot regeneration in *Agave sisalana*. Plant Cell Tiss Org Cult 51:225–228

Nikam TD, Bansude GM, Kumar KA (2003) Somatic embryogenesis in Sisal (*Agave sisalana* Perr. ex. Engelm). Plant Cell Rep 22:188–194

Olmos E, Hellıin E (1998) Ultrastructural differences of hyperhydric and normal leaves from regenerated carnation plants. Sci Hort 75:91–101

Parent C, Capelli N, Berger A, Crevecoeur M, Dat JF (2008) An overview of plant responses to soil waterlogging. Plant Stress 2(1):20–27

Picoli EAT, Paiva EAS, Xavier A, Aguiar RM, Carolino SMB, Fari MG, Otoni WC (2008) Ultrastructural and biochemical aspects of normal and hyperhydric Eucalypt. Int J Hort Sci 14:61–69

Piqueras A, Cortina M, Serna MD, Casas JL (2002) Polyamines and hyperhydricity in micropropagated carnation plants. Plant Sci 162:671–678

Pompelli MF, Martins SCV, Celin EF, Ventrella MC, DaMatta FM (2010) What is the influence of ordinary epidermal cells and stomata on the leaf plasticity of coffee plants grown under full-sun and shady conditions? Braz J Biol 70:1083–1088

Portillo L, Santacruz-Ruvalcaba F (2006) Factibilidad de uso de un nuevos sistema de inmersion temporal (Orbitabion®) para embriogenesis somatica de *Agave tequilana* Weber cultivar azul. Bol Nakari 17:43–48

Reyes-Zambrano SJ, Lecona-Guzman CA, Barredo-Pool FA, Calderon JDA, Abud-Archila M, Rincon-Rosales R, Gutierrez-Miceli FA (2016) Plant growth regulators optimization for maximize shoots number in Agave americana L. by indirect organogenesis. Gayana Bot 73(1):124–131

Rodriguez-Garay B (2016) In: Loyola-Vargas V, Ochoa-Alejo N (eds) Book chapter- somatic embryogenesis: fundamental aspects and applications. Springer International Publishing Switzerland, pp 267–282

Rossetto M, Dixon KW, Bunn E (1992) Aeration: a simple method to control vitrification and improve in vitro culture of rare Australian plants. In Vitro Cell Dev Biol Plant 28:192–196

Saher S, Piqueras A, Hellin E, Olmos E (2004) Hyperhydricity in micropropagated carnation shoots: the role of oxidative stress. Physiol Plant 120(1):152–161

Santacruz-Ruvalcaba F, Portillo L (2009) Thin cell suspension layer as a new methodology for somatic embryogenesis in *Agave tequilana* Weber cultivar azul. Ind Crop Prod 29:609–614

Santos J, Vieira I, Braz-Filho R, Branco A (2015) Chemicals from *Agave sisalana* biomass: isolation and identification. Int J Mol Sci 16(4):8761–8771

Sarkar S, Jha AK (2017) Research for sisal (*Agave* sp.) fibre production in India. Int J Curr Res 9(11):61136–61146

Sarkar S, Jha AK, Majumdar B, Saha AR (2018) Influence of drip irrigation, manure and fertilizers on production of planting materials in sisal (*Agave sisalana* Perr. Ex Engelm.). Int J Curr Microbiol Appl Sci 7(9):1934–1941

Shelke DB, Pandey M, Nikalje GC, Zaware BN, Suprasanna P, Nikam TD (2017) Salt responsive physiological, photosynthetic and biochemical attributes at early seedling stage for screening soybean genotypes. Plant Physiol Biochem 118:519–528

Steinmacher DA, Guerra MP, Saare-Surminski K, Lieberei R (2011) A temporary immersion system improves in vitro regeneration of peach palm through secondary somatic embryogenesis. Ann Bot 108:1463–1475

Sullivan CY (1972) Mechanisms of heat and drought resistance in grain sorghum and methods of measurement. In: Rao NGP, House LR (eds) *Sorghum* in Seventies. Oxford & IBH Pub. Co., New Delhi, pp 247–264

Taiz L, Zeiger E (2010) Plant physiology. Sinauer Associates Inc., Sunderland, MA

Tewari D, Tripathi YC, Anjum N (2014) *Agave sisalana*: a plant with high chemical diversity and medicinal importance. World J Pharm Res 3(8):238–249

Van den Dries N, Gianni S, Czerednik A, Krens FA, de Klerk GJM (2013) Flooding of the apoplast is a key factor in the development of hyperhydricity. J Exp Bot 64:5221–5230

Watanabe S, Kojima K, Ide Y, Sasaki S (2000) Effects of saline and osmotic stress on proline and sugar accumulation in *Populus euphratica* in vitro. Plant Cell Tiss Org Cult 63:199–206

Weber B, Bowker M, Zhang Y, Belnap J (2016) Natural recovery of biological soil crusts after disturbance. In: Weber B, Budel B, Belnap J (eds) Biological soil crusts: an organizing principle in drylands, ecological studies (analysis and synthesis), vol 226. Springer International Publishing Switzerland, Cham, pp 479–498