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Enhanced in vitro Regeneration in Sugarcane (*Saccharum officinarum* L.) by Use of Alternate High-Low Picloram Doses and Thidiazuron Supplementation

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Abstract—Sugarcane is an important domestic and industrial crop and considerable efforts have been made to improve its yield through conventional as well as biotechnological approaches. Genetic manipulation of sugarcane is dependent on an efficient reproducible in vitro regeneration regime. In the current study, the role of explant position, induced desiccation, picloram (PIC) levels for callogenesis as well as thidiazuron (TDZ) addition during regeneration phase has been appraised. Using an optimum combination of mentioned factors, an enhanced in vitro regeneration system has been established for two elite sugarcane cultivars. Embryogenesis was stimulated in cv. HSF-240 by MS medium augmented with 12.42 μM PIC while cv. CPF-237 exhibited sometic embryo formation when PIC supplementation was combined with induced desiccation (using 12 g L^{-1} agar). A decrease in embryogenesis frequency was recorded from base towards tip. The explants cultured alternately on high (12.42 μM) and low (4.14 μM) PIC medium produced the highest number of nodular calli which later exhibited maximum regeneration potential. Optimal shoot initiation was observed with 9.08 μM TDZ followed by medium having 2.27 μM 2,4-dichlorophenoxyacetic acid plus 4.43 μM benzylaminopurine. However, the shoots produced with former medium composition showed frailty as compared to the one regenerated on later medium. Healthy roots were initiated with 16.11 μM naphthalene acetic acid in the presence of 0.5% activated charcoal. Malondialdehyde content, catalase and peroxidase activity of in vitro and field grown sugarcane plants were analogous, indicating that the in vitro regenerated plants were equally fit for subsequent growth in natural conditions. The reported protocol can be helpful in devising strategies for a robust sugarcane genetic engineering regime.

Keywords: auxins, callogenesis, explant, growth regulators, somatic embryogenesis

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1. INTRODUCTION

Sugarcane is a domesticated perennial grass widely cultivated throughout the world, particularly in tropical and subtropical areas. The main products of sugarcane are sucrose and two industrially important products i.e. bagasse and molasses. About 20 million hectares of sugarcane is cultivated worldwide with Brazil at top in the list of countries contributing a share of about 33% of world's average sugarcane cultivation. Pakistan is ranked at five (with a share of 4%) and according to Economic Survey of Pakistan (2017–2018), sugarcane yield in the country was about 55 thousand tons per hectare with approx. 73.6 million tons of annual cane production. The crop share in value addition of Pakistan's agriculture is 3.4% while in GDP is 0.7%.

Sugarcane is generally propagated using clonal buds and, at farm level, its seeds have non-significant role in its reproduction. The classical breeding program for sugarcane is relatively slow and requires a tedious job for release of new cultivars owing to its very large and complex genome. The vegetative propagation is also conducive to spread of various systemic diseases (Croft et al., 2008). Tissue culture technology made it possible to develop new genotypes with better traits (Lakshmanan et al., 2006) and aslo facilitated in screening of genotypes with greater abiotic/biotic stress resistance at early growth stages (Snyman et al., 2006; Munir and Aftab, 2009). Furthermore, sugarcane yield can be enhanced by employing transformation technology (Altpeter et al., 2005; Hotta et al., 2010) but it requires availability of receptive recipient tissues and a vigorous in vitro regeneration regime (Lakshmanan et al., 2005).

Induction of somatic embryos is desirable in plant cell cultures as somatic embryos usually develop from single cells, so, their ontogeny is comparable with

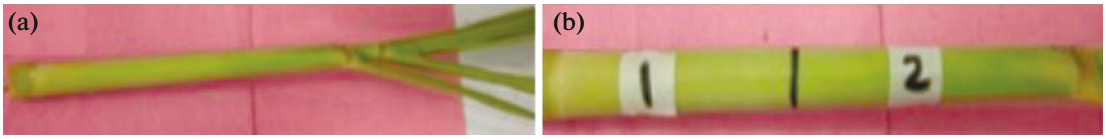

Fig. 1. Source of sugarcane explants used for in vitro culture studies. (a) Top of the field grown sugarcane plant. (b) Division of the top internodal region into two parts (proximal, closer to the base, as P1 and distal, closer to top, as P2).

zygotic embryogenesis. Embryogenesis also represents a regulated development leading to programmed cell division, expansion and differentiation (Taiz and Zeiger, 2010). In vitro regeneration in sugarcane is thought to occur via somatic embryogenesis (Kaur and Kapoor, 2016) and establishment of protocols for enhanced SE is a focused research area (Lakshmanan et al., 2005; Snyman et al., 2006) as somatic embryos are preferred target material for transformation (Lakshmanan et al., 2006). The addition of 2,4-D, in the growth medium is considered essential for embryogenic callus formation (Ali et al., 2008). Yet importance of picloram addition also proved equally effective in some studies (Chengalrayan et al., 2005). Besides growth regulators, physical factors like light and temperature (Garcia et al., 2007) as well as developmental stage of donor plant (Lakshmanan et al., 2006) are very important. Kaur and Gosal (2009) highlighted the importance of induced desiccation for increased SE potential of a particular explant type.

For practical perspective, callogenesis is followed by regeneration. Addition of appropriate cytokinins, alone, or in combinations with an auxin has been tested (Munir and Aftab, 2009) and BAP was found to be the most appropriate for regeneration of diverse explant types. Scientists also appraised the addition of thidiazuron (TDZ) for in vitro regeneration of sugarcane (Gallo-Meagher et al., 2000; Chengalrayan and Gallo-Meagher, 2001). A higher plantlet multiplication was observed in in vitro cultures using apical meristem sections of sugarcane with TDZ addition (Dhawan et al., 2004). Chengalrayan et al. (2005) observed that TDZ addition resulted in direct regeneration of multiple plantlets but no callus formation. Recently, Kumari et al. (2017) has reported that explants primed with TDZ resulted in better micropropagation with highest number of tillers.

Successful in vitro *shoot* regeneration should be followed by in vitro rooting. The boosting effects of hormones (particulalry NAA) on root initiation and development has been well reported (Chengalrayan and Gallo-Meagher, 2001). Furthermore, addition of activated charcoal could also enhance root vigour (Thomas, 2008).

It is evident that a considerable progress has been achived by the scientists for establishement of sugarcane in vitro regeneration systems. Yet there is potential gaps particularly when the exerted affects are found to be genotype dependent (Rikiishi et al., 2008). In view of the recent updates regarding use of PIC or TDZ and influencing effects of induced dessication as well as explant characteristics, we, in the current study, attempted to achieve production of nodular callus with high ability of regeneration for extended periods of time.

2. MATERIALS AND METHODS

2.1. Plant Material and Explant Excision

The explants were obtained from 6–8 month old field grown sugarcane plants of two genotypes i.e. cv. HSF-240 and cv. CPF-237. The healthy and disease free plant tops were selected and the youngest part (ca. 10–12 cm in length) was divided into two fragments. The proximal part (nearer to the base) was designated as P-1 while the distal one (closer to top) as P-2 (Figs. 1a and 1b). The outer leaves were removed to obtain an innermost naturally sterilized stalk. Each part (P-1 and P-2) was then cut into 2–3 mm thick leaf roll disks, the explants.

2.2. Somatic Embryogenesis Sstudies

2.2.1. Effect of medium composition and explant position. Murashige and Skoog (1962) basal salts and organic supplements with addition of 2,4-D at a concentration of 13.62 μM (SEM-1) was designated as a standard medium composition (Aftab and Iqbal, 1999; Ali et al., 2008). Four other medium compositions i.e. SEM-2, SEM-3, SEM-4 and SEM-5 were prepared using picloram (PIC) at aconcentration of 4.14, 8.28, 12.42 or 16.56 μM, respectively. Growth medium pH was set at 5.7 using 0.1 N KOH or HCl. Agar (0.8% w/v, sigma) was used to solidify the medium and cultures were mantained in test tubes (25 × 150 mm) having 20 mL soil medium. Sterilization of the medium was done in an autoclave for 15 min at 121°C and 15 psi. The cultures were maintained under dark conditions at 27 ± 2°C for 42 days with subculturing in 2 weeks intervals to respective medium compositions. The percentage callogenesis was calculated by scoring number of explants exhibiting at least 50% nodular callus formation.

2.2.2. Desiccation effect. Based on the obtained results, three medium compositions (SEM-1, SEM-3 and SEM-4) were selected to investigate the effect of induced desiccation on SE (Heath and Packer, 1968; Garcia et al., 2007). Explants (exclusively from posi-

Table 1. Medium compositions used for in vitro shoot initiation/proliferation of sugarcane cultivars HSF-240 and CPF-237 using callus developed on high-low concentration regime of auxin, 2,4-D or picloram

Sr. No.	Treatment code	2,4-D, μM	BAP, μM	TDZ, μM
1	SIM-1	0.0	4.43	0.0
2	SIM-2	0.0	8.86	0.0
3	SIM-3	2.27	4.43	0.0
4	SIM-4	2.27	8.86	0.0
5	SIM-5	0.0	0.0	4.54
6	SIM-6	0.0	0.0	9.08
7	SIM-7	2.27	0.0	4.54
8	SIM-8	2.27	0.0	9.08

tion 1) were cultured on medium solidified using three different levels (0.8, 1.2 or 1.6%, w/v) of agar. After 4 weeks, frequency of SE was determined by scoring number of explants exhibiting at least 50% nodular callus formation.

2.2.3. Auxin concentration effect. Explants (exclusively from P-1) were incubated on MS basal medium ammended with 13.62 μM 2,4-D or 12.42 μM PIC (high auxin medium) for 14 days. Subsequently, cultures were moved to MS medium having 4.54 μM 2,4-D or 4.14 μM PIC (low auxin medium) for two weeks. The alternate high-low auxin regime was repeated once before scoring cllogenesis frequency. The cultures maintained on continuous high auxin medium (13.62 μM 2,4-D or 12.42 μM PIC) served as control.

2.3. In vitro Regeneration: Effect of BAP and TDZ

Spindle leaf roll explants (exclusively from P-1) were incubated on alternate high-low auxin regime where the medium was solidified with 1.2% agar. After 4 weeks, the calli exhibiting 50% SE were shifted to various kinds of shoot initiation medium compositions (SIM-1 to SIM-8 as given in Table 1), each solidified using 0.8% (w/v) agar. The data were recorded for shoot initiation after 14 days. The calli, exhibiting shoot initiation, were subcultured to respective fresh liquid medium (having cotton plugs at the base of vessel to support growing plantlets). The vigour of *in vitro* grown plantlets were assessed visually as well as by recording length of plantlets after 30 days of incubation.

2.4. In vitro rooting and Soil Accalimatization

The in vitro grown shoots were separated, into a bunch of 2–3 shoots and transferred to fresh liquid MS growth medium having 21.48 μM NAA, sucrose (40 g L^{-1}) and without or with 0.5% activated charcoal. The regenerated plants were successfully accalimitized for growth in soil.

2.5. Environmental Fitness of in vitro Regenerated Plants

The level of lipid peroxidation/malondialdehyde content (Heath and Packer, 1968), catalase activity (Aebi, 1984) and peroxidase activity (Chance and Maehly, 1955) of in vitro regenerated plants of both sugarcane genotypes (i.e. HSF-240 and CPF-237) were determined and compared with the naturally field grown plants of the respective genotype.

2.6. Experimental Design and Data Analysis

Fourty explants were used in each experiment (for each treatment) using a completely randomized factorial design. Data were analyzed for analysis of variance and computated for least significant differences ($p < 0.05$) among treatment means using statistical program "CoStat".

3. RESULTS AND DISCUSSION

3.1. Somatic Embryogenesis

3.1.1. Effect of medium composition and explant position. For somatic embryo induction in cv. HSF-240, the auxin 2,4-D and picloram (PIC) were almost equally effective when used at a concentration of 13.62 and 12.42 μM, respectively. A maximum number of nodular calli were observed from explants of cv. HSF-240 on medium SEM-1 (MS medium composition + 13.62 μM 2,4-D) (Fig. 2). In this medium composition, nodular structures, similar to proembryos, appeared directly at the edges of leaf whorls in initial two weeks of in vitro growth (Fig. 3a). The medium having 8.28 μM picloram (SEM-3) also proved better for SE in cv. HSF-240. The cv. CPF-237 exhibited less SE when picloram was used instead of 2,4-D (Fig. 2a). The results were in accordance with earlier studies (Aftab et al., 1996) where highest callus initiation frequency was observed in MS growth medium having 13.62 μM 2,4-D. In the present study, it was observed that 13.62 μM 2,4-D addition in MS medium had stimulatory effects for callus initiation as well as inducing somatic embryos. The observations were similar as described earlier (Ali et al., 2008). This revealed the genotype independency of

2,4-D for induction of SE. Ramanand et al. (2006) also reported that auxin 2,4-D exerted genotype independent effects while establishing callus initiation and SE in various sugarcane cultivars. Genotype dependent in vitro responses in sugarcane are also reported (Aftab et al., 1996). It is also described that different explant types of a single plant species showed varied in vitro responses. In addition, developmental age of the same tissue/explant type could have modulating effects on in vitro culture responses. We here noted that the leaf roll explants obtained from different positions exhibited different rates of emergence of somatic embryos. Explants obtained from proximal segments (designated as P-1 in Fig. 1b) produced higher number of embryogenic calli. For cv. HSF-240, using medium SEM-1, frequency of SE was 68 and 48% for explants from P-1 and P-2, respectively (Fig. 2a). For cultivar CPF-237, maximum SE frequency (56%) was attained when explants from position 1 were incubated on SEM-1 growth medium, while 41% SE frequency was observed, with explants from position 2, on same medium composition. With PIC supplementation (8.82 or 12.42 μM), the same pattern for the two explant positions was observed, although it was more pronounced in cv. HSF-240. It seems likely that top internodal cells were too immature to differentiate into characteristic embryonic cell type and were unable to withstand in vitro environmental conditions, thus produced highly compact masses of cells instead of nodular cells. It has been suggested earlier that position on parent plant and growth stage of a particular explant type play an important role in rate of callus induction and callus type. Positional effect of leaf roll explants has been documented describing that the upper portion of sugarcane apical region was less responsive for callogenesis than the basal part (Lakshmanan et al., 2006). Similarly, Tiel et al. (2006) reported that highest number of plantlts were produced when explants were obtained from basal segments of sugarcane stem.

3.1.2. Effect of induced desiccation. It was observed that induced desiccation in culture medium had a stimulatory effect on the emergence of somatic embryos. Cultivar CPF-237 which was not responsive for SE using PIC and 0.8% (w/v) agar, produced a good number of somatic embryos when PIC supplementation was combined with 1.2% agar (Figs. 2b, 3d). Stimulatory effects of desiccation have been observed in Brazilian sugarcane genotypes in a study conducted by Garcia et al. (2007) where it has been established that a high regeneration efficiency was recorded in calli which were desiccated in a laminar airflow cabinet. Kaur and Gosal (2009) reported that addition of higher agar concentration (1.6%) induced embryogenic callus formation in three sugarcane genotypes i.e. cv. CoJ 64, cv. CoJ 83 and cv. CoJ 86. Furthermore, they also recorded a higher regeneration potential in calli which were grown on a medium having 1.6% agar in comparison to that solidified with 0.8% agar. Results of the current experiment suggested that addition of agar at a concentration of 1.2% was more effective than agar concentration of 0.8%. We here envisioned that desiccation exerted effects are comparable to the ones that occur naturally during the maturation stage of zygotic embryos development (Ali et al., 2008).

3.1.3. Effect of alternate high-low auxin (2,4-D or picloram) levels. It is reported that auxin 2,4-D may lead to several metabolic pathways within a plant body which are largely dependent on its initial concentration (Grossmann, 2000). To substantiate the potential effects induced by auxin concentrations, two strategies were employed i.e. continuous high auxin (13.62 μM 2,4-D or 12.42 μM PIC) throughout culture or high-low auxin regime repeated after 2 week interval. It was noted that using high-low auxin regime enhanced SE in explants of position 1. This revealed that CPF-237 (a recalcitrant genotype for SE) responded well with the new strategy (induced desiccation together with high-low auxin regime) as was evident from 70% SE in comparison to 56% SE (Figs. 2a–2c).

3.2. In Vitro Regeneration

Of all the medium combinations, medium SIM-6 (Table 1) produced maximum shoot initiation frequency, followed by SIM-4 medium (Fig. 4). For cultivar, HSF-240, the highest shoot induction (82%), was recorded on SIM-6 medium using the calli which were initially developed on high-low 2,4-D treatment. A relatively low regeneration (78%) was noted in calli grown by using high-low PIC treatment. Similar results were obtained for CPF-237 with maximum shoot initiation frequency of 73% on SIM-6 medium from the calli that were initially maintained on alternate high-low 2,4-D regime. While explants which were grown on high-low PIC treatment, before shifting to shoot induction medium, showed 68% shoot initiation frequency on SIM-6 medium. These findings are in agreement with earlier reports where it has been concluded that calli grown on low 2,4-D levels proved better and maintained regeneration potential for an extended period of time (Ali et al., 2008). Use of reduced 2,4-D concentrations for attaining maximum regeneration frequency was also recommended by Garcia et al. (2007). Auxin, like 2,4-D, modulate key regulators of ethylene synthesis; leading to increased production of ethylene and cyanide, a co-product of the pathway. It was assumed that ethylene together with cyanide caused phyto-toxicity to plants, which led to reduced photosynthetic efficiency and thus resulted in less shoot initiation frequency. In addition, auxin also contribute in establishing apical dominance, by making shoot apex a sink for cytokinins and 2,4-D, being a synthetic auxin, is not easily metabolized by a plant (Taiz and Zeiger, 2010). Thus it seems likely that continuous exposure to high auxin (2,4-D or PIC) levels exerted negative effects on the population of self perpetuating pluripotent cells and hence

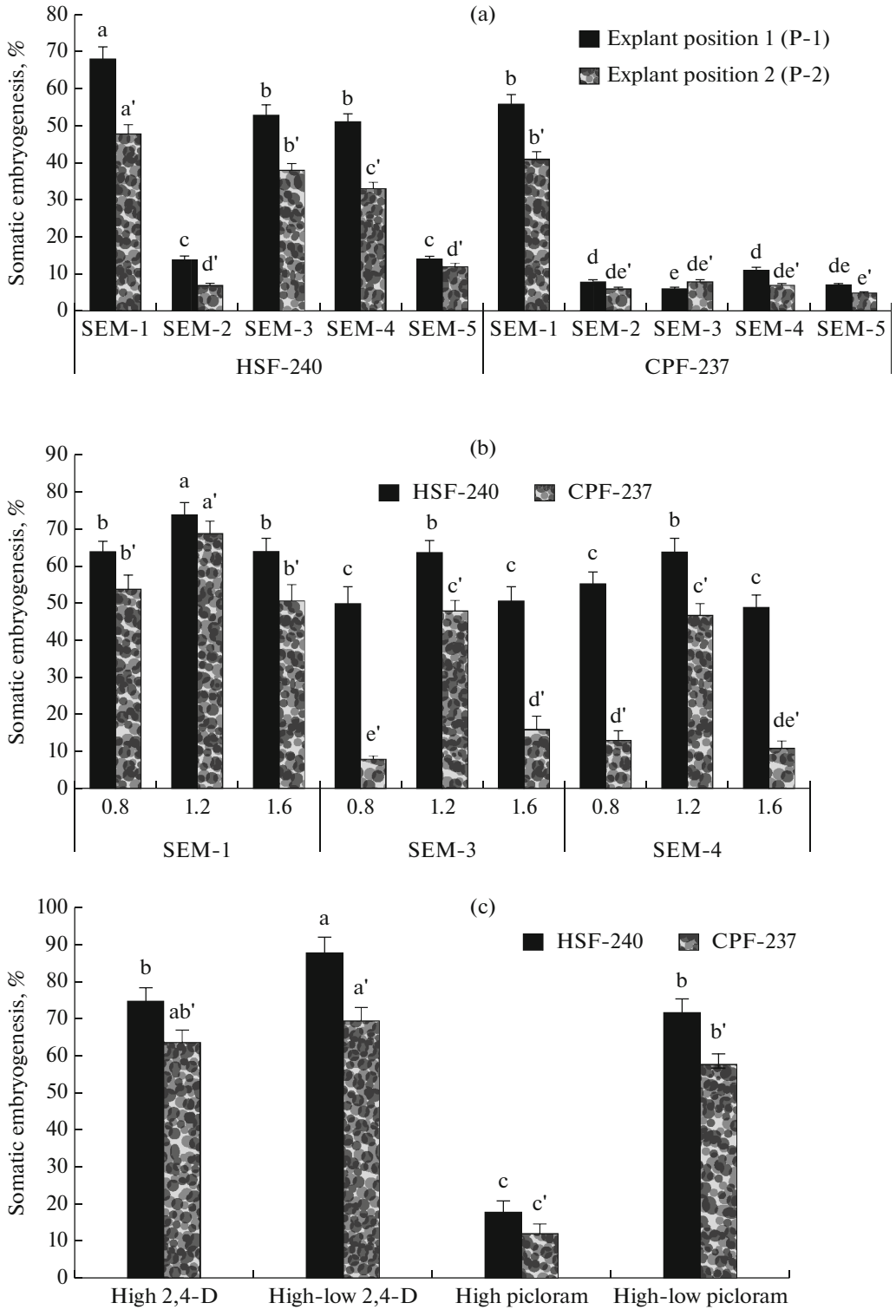

Fig. 2. Somatic embryogenesis in sugarcane cv. HSF-240 and cv. CPF-237 using leaf roll explants. (a) Effect of medium composition and explant position, (b) Effect of induced desiccation by using various levels (0.8, 1.2 or 1.6%) of agar (c) effect of alternate high-low auxin concentrations. Medium was prespared using MS composition with addition of 13.62 μM 2,4 -D (SEM-1), 4.14 μM picloram (SEM-2), 8.28 μM picloram (SEM-3), 12.42 μM picloram (SEM-4), or 16.56 μM picloram (SEM-5). The position 1 (P-1) and 2 (P-2) designated the two positions of the youngest internodal region as shown in Fig. 1. High 2,4-D or picloram indicate continuous exposure of explants to 13.62 μM 2,4-D or 12.42 μM picloram. High-low 2,4-D indicate explants were grown with 13.62 μM 2,4-D followed by growth on 4.54 μM 2,4-D. High-low picloram indicate explants were grown with 12.42 μM picloram followed by growth on a medium having 4.14 μM picloram. The frequency of somatic embryogenesis was calculated after 4 weeks. Different lowercase letters indicate significant difference ($p < 0.05$) among treatment means.

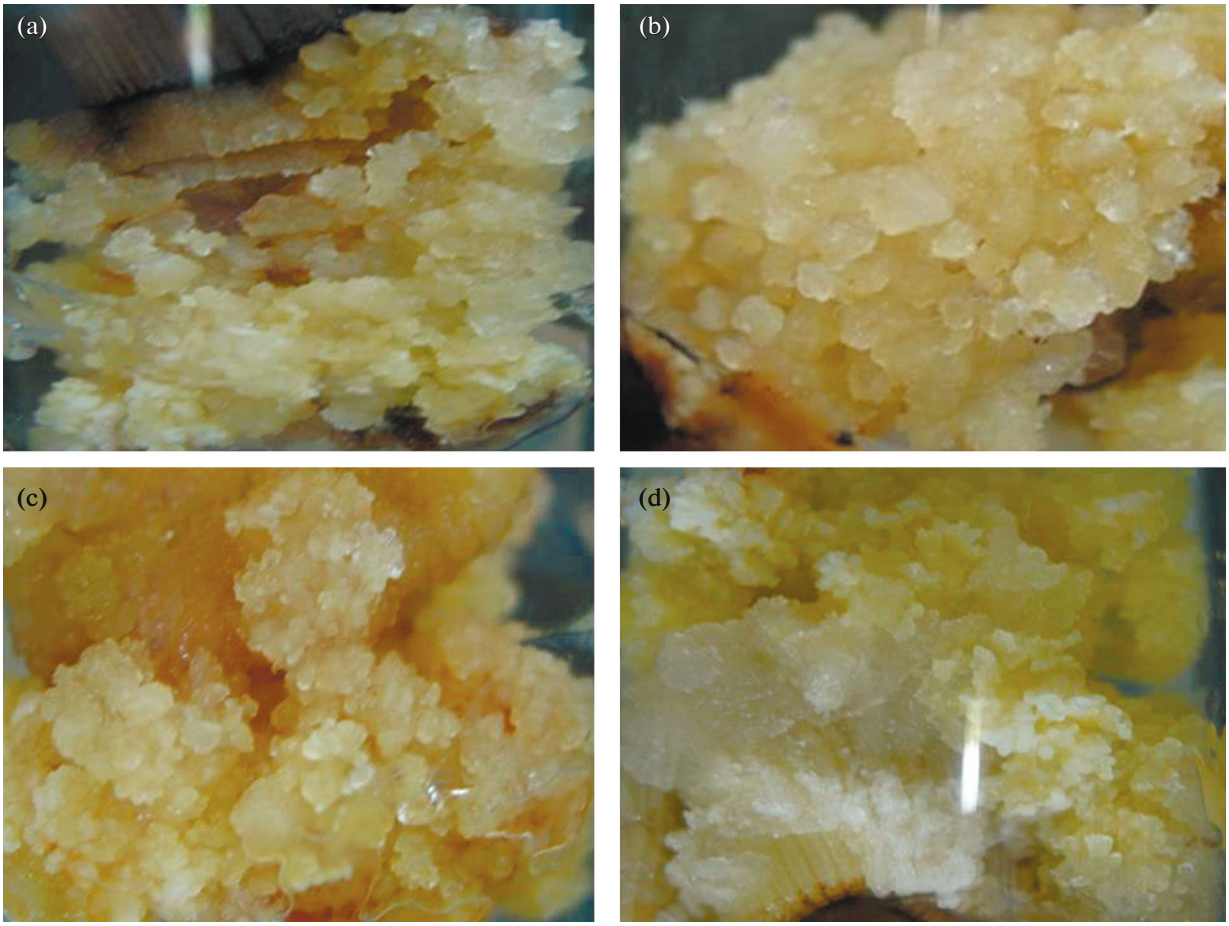

Fig. 3. Somatic embryogenesis in sugarcane cv. HSF-240 (a, b) and cv. CPF-237 (c, d). Spindle leaf roll explants were cultured on alternate high-low 2,4-D (a and c), or picloram (b and d) regime. The medium was soldified using 1.2% agar. The images were taken after 30 days of explant culture.

reduced in vitro regeneration potential. Generally, a high:low ratio of cytokinins and auxin is necessary for regeneration of in vitro grown calli. In earlier studies, it was reported that 1–2 mg L^{-1} BAP supplementation of MS medium having low levels of 2,4-D (or without auxin) proved better for initiation of shoot primordia on sugarcane calli. Contrary to this, in this experiment it was observed that TDZ addition was better than BAP as the addition of former resulted in more number of shoot primordia in comparison to later (Fig. 4a). The studies pertaining to use of TDZ for in vitro shoot multiplication are limited (Jain et al., 2007) but the current results were comparable as reported by Chengalrayan et al. (2005) where maximum shoots were regenerated on MS basal salts with TDZ supplementation. Chengalrayan and Gallo-Meagher (2001) reported that calli grown with TDZ addition exhibited higher shoot initiation frequency and resulted in higher number of plantlets per callus. Similarly, Dhawan et al. (2004) reported that TDZ addition was found to be stimulatory for proliferation of plantlets uisng explants obtained from sugarcane apical domes. In the current experiments, after 45 days of culturing on shoot initiation medium, with 3 subcultures in between, significantly short and weak shoots were observed on SIM-6 than the ones regenerated on SIM-3 medium composition. This indicated that the former medium composition (having TDZ) was not good for long term use and its addition at later culture phase became noxious for plant development. When the explants were initially grown on alternate high-low 2,4-D regime (SE-MS1), the shoot length in cv. HSF-240 was 5.2 and 3.7 cm on SIM-3 and SIM-6 medium, respectively. Initial culture of explants on alternate high-low PIC regime (SE-MS2) resulted in slightly lower shoot length values i.e. 4.5 cm (SIM-3 medium) and 2.9 cm (SIM-6 medium) (Fig. 4). A similar trend was noticed for cv. CPF-237 on SIM-3 and SIM-6 medium compositions indicating that callus growth on high-low auxin regime was better than continuous high 2,4-D/PIC. It was concluded that, for better shoot vigour, SIM-3 (MS medium composition with

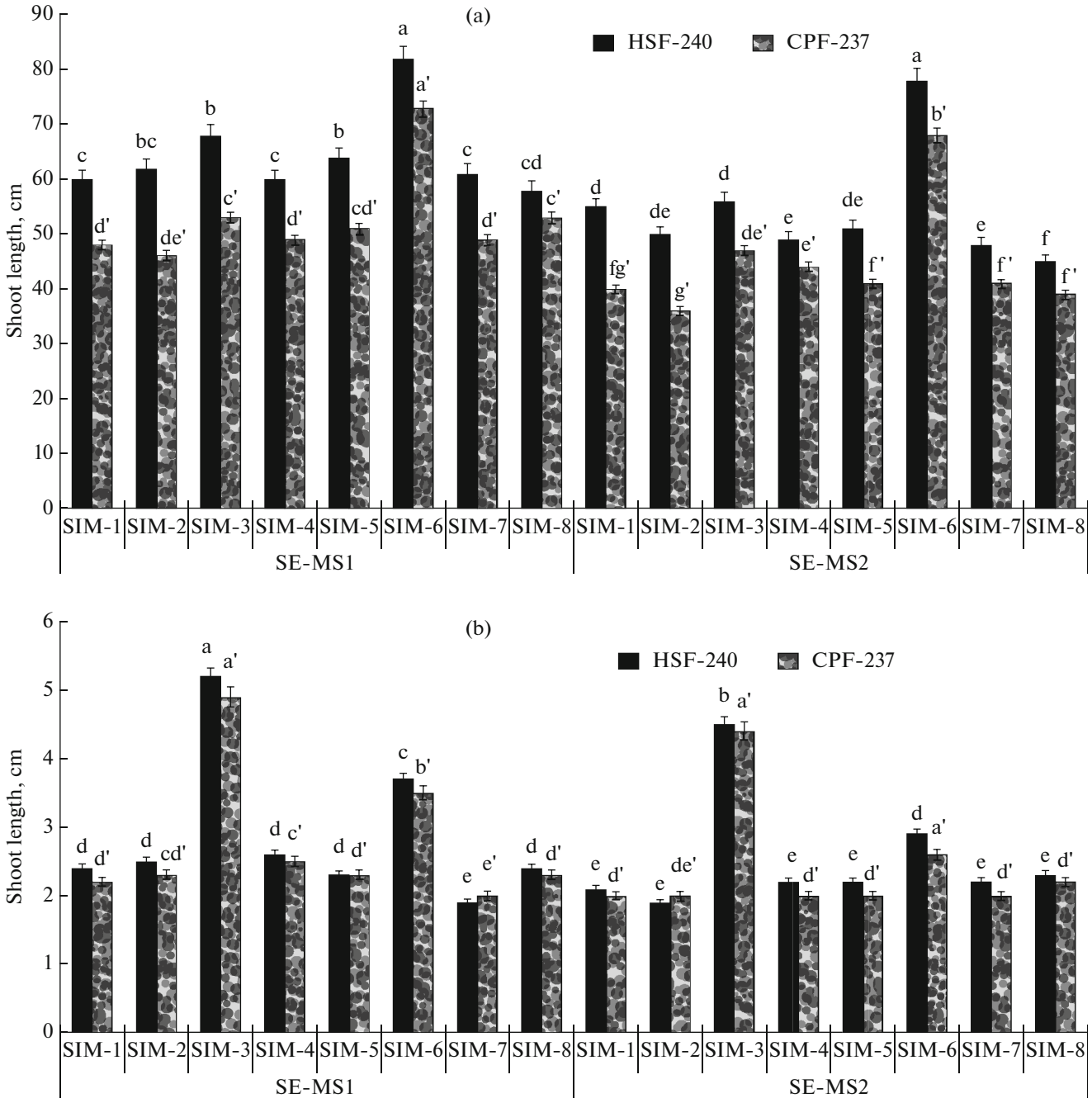

Fig. 4. In vitro regeneration in sugarcane cv. HSF-240 and cv. CPF-237. The explants (from position 1) were grown on alternate high-low regime of 2,4-D (SE-MS1) or picloram (SE-MS2) where medium was soldified using 1.2% agar. Subsequelntly, the calli exhibiting 50% somatic embryogenesis were shifited to shoot initiation medium (SIM-1 to SIM-8 as given in Table 1). Frequency of shoot initiation was calculated after 2 weeks (a) while shoot length of plants were recorded after 4 weeks (b). Different lowercase letters indicate significant difference ($p < 0.05$) among treatment means.

2.27 μM 2,4-D + 4.43 μM BAP) proved better than SIM-6 (MS medium having 9.08 μM TDZ). This highlighted that TDZ addition was only required for initial stages of shoot initiation but, once the shoots were established, its continuous presence in the medium exerted drastic effects on plant's growth. It has been reported that in vitro grown native spearment produced low length shoots when regenerated on TDZ supplemented medium (Charleson et al., 2006). It seems likely that TDZ affected the photosynthetic pig-

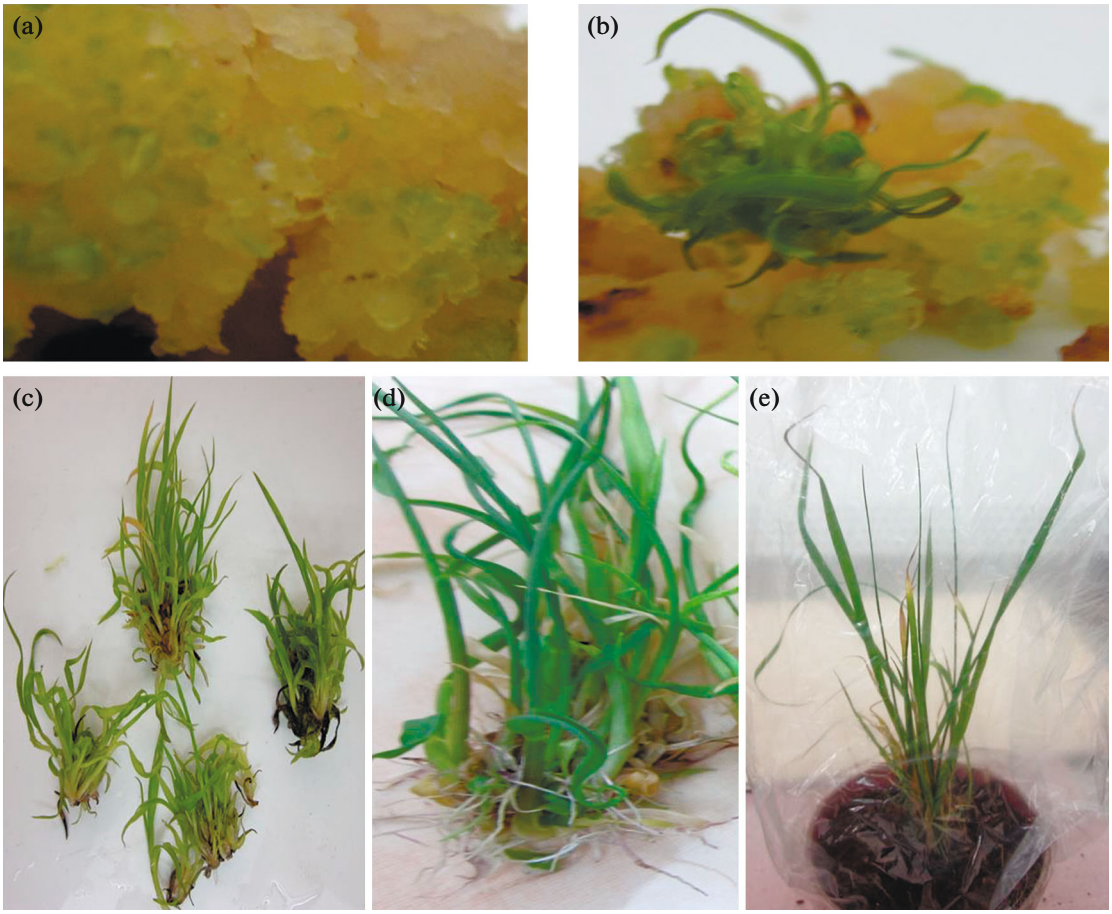

Fig. 5. Representative images showing somatic embryogenesis (a), shoot initiation (b), in vitro grown plantlets (c), root development (d) and soil acclamitization (e) as observed in sugarcane cv. CPF-237 using the modified protocol with initiation of somatic embryogenesis by using high-low picloram regime, shoot initiation on MS medium with 9.08 µM thidiazuron, shoot proiferation on growth medium having 4.43 µM BAP plus 2.27 µM 2,4-D. The roots were developed on MS basal salts having 21.48 µM NAA and activated charcoal (0.5%).

ments directly and, also, it may have initiated cascades leading to induced inhibitory effects on other growth regulators involved in shoot development.

3.3. Root Development and Soil Acclimatization

For in vitro rooting in sugarcane, addition of NAA was found necessary. A vigorus root formation was recorded in sugarcane cv. CoJ 83 when plants were grown with NAA (5.0 mg L^{-1}) and kinetin (0.5 mg L^{-1}) (Gill et al., 2006). Use of 2–3 mg L^{-1} NAA was reported for maximum rooting in sugarcane plantlets regenerated on MS medium (Ali et al., 2008). In the current study, MS medium having 21.48 µM NAA and 60 g L^{-1} sucrose was used for root initiation/proliferation. Furthermore, addition of activated charcoal (AC) at a concentration of 0.5% boosted the root vigour (visual observation). As the AC has high adsorption ability, it is likely that AC adsorbed the growth inhibitory chemicals (Mamun et al., 2004) and thus improved the plant growth. Moreover, AC also provided a darkened environment to the growing roots; hence mimicked the soil environmental conditions (Thomas, 2008). Plants were acclimatized in natural conditions by initially transferring to pots filled with sand, peat moss, and garden soil (2:1:1) where each pot was enclosed in transparent polythene sheet for maintaining humidity (Fig 5). The in vitro regenerated plants showed optimum level of studied physio-biochemical attributes (MDA content, catalase and peroxidase activity) which were compareable to one recorded for field grown plants (data not shown). Finally, the plants were transplanted in soil and grown, till maturity, under natural field conditions.

CONCLUSIONS

It was concluded that for enhanced SE, explants should be obtained from basal region of first internode. Induced desiccation (by 1.2% agar addition) boosted SE potential of young spindle leaf whorl explants. For rapid shoot initiation, calli/somatic

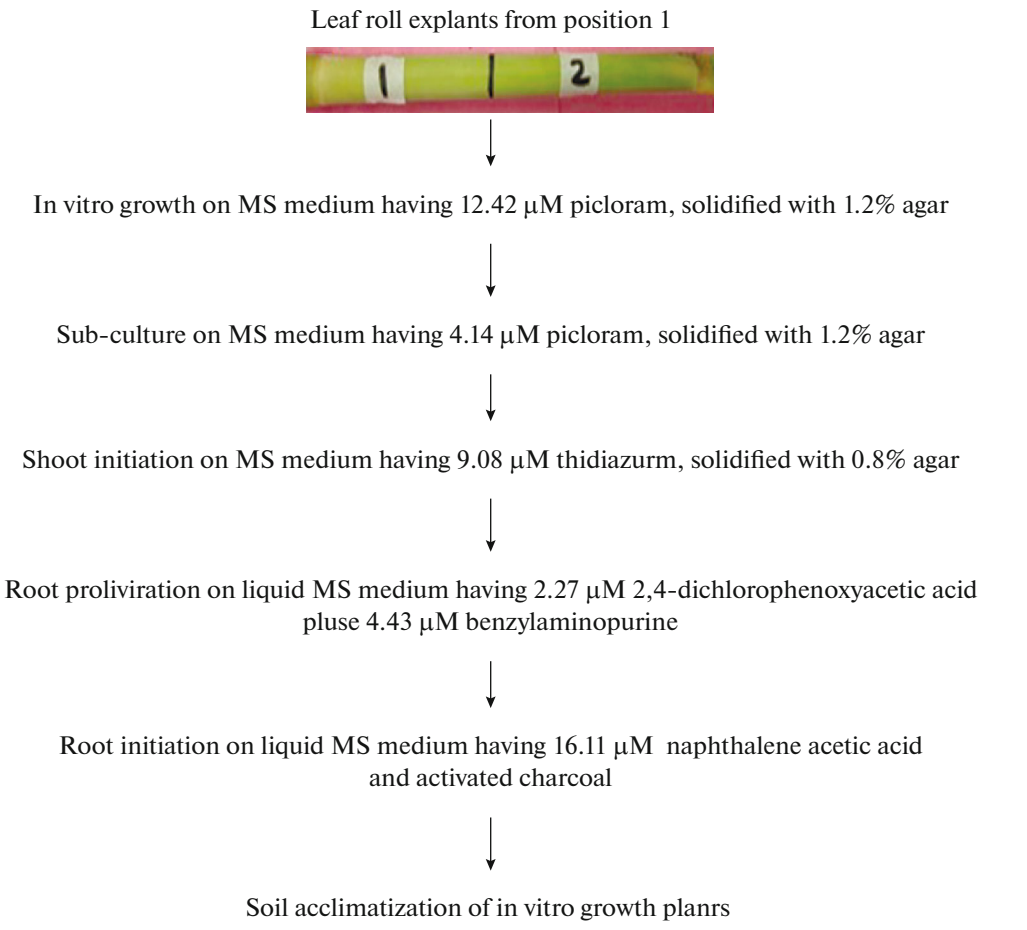

Fig. 6. Graphical representation of modified regime for robust in vitro sugarcane regeneration.

embryos should be initiated on alternate high and low auxin (2,4-D or picloram) concentrations. Subsequently, shifting explants with SE on medium having 9.08 μM TDZ (for 1–2 week) and then on a medium supplemented with BAP (4.43 μM) plus 2,4-D (2.27 μM) could regenerate maximum shoots with better length/vigour. The cv. CPF-237, which was less responsive with our routine procedure, showed a significantly enhanced in vitro regeneration portentail by employing the currently modified approach (Figs. 5, 6).

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interests. This article does not contain any studies involving animals or human participants performed by any of the authors.

AUTHOR CONTRIBUTIONS

JI perceived the idea/s and MSA executed the experiments. MS and AKA helped in statistical analysis of the data and preparation of the manuscript. All the authors read/approved the manuscript for submission.

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