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RESEARCH ARTICLE

Efficient Regeneration in Sugarcane Using Thin Cell Layer (TCL) Culture System

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Abstract In vitro micropropagation using transverse thin cell layer (tTCL), very small and thin section of explant, has now emerged as a useful tool in plant tissue differentiation and regeneration. Sugarcane, a difficult-to-regenerate crop, lacks an efficient regeneration system. The present study evaluated the TCL technique as a procedure for somatic embryo production and plantlet regeneration of sugarcane plant. Explants from young leaf whorls (tTCLs) were cultivated in MS culture medium supplemented with 3% sucrose and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and incubated under dark condition at 25 + 2 °C for callus formation. The innermost layer of the explants was found more responsive to callogenesis than the outer layers of the tTCLs. The explants cultured on 2.0 mg/l 2,4-D produced good-quality callus as compared to other concentrations. The calli were further evaluated for regeneration. MS medium supplemented with 2.0 mg/l BAP (Benzyl aminopurine) and 0.2 mg/l NAA (naphthalene acetic acid) was found suitable where 100% calli regenerated maximum number of shoots per callus mass (25 ± 1.6) with highest shoot length of 6 ± 0.5 cm. The highest number of root emergence (28.2 ± 0.8) and maximum root length (2.8 ± 0.09 cm) were achieved on MS medium supplemented with 4.0 mg/l NAA.

Keywords Sugarcane · TCL (thin cell layer) · Callus induction · Somatic embryogenesis · Regeneration

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Introduction

Sugarcane (*Saccharum* species hybrids), from the family Poacae, is the most important sugar crop worldwide which is widely cultivated in the tropical and subtropical regions (Raza et al. 2010). It contributes approximately 80% of the sucrose produced globally and thus is regarded as a source of sustainable energy (Hofsetz and Silva 2012; FAO 2018; Iqbal et al. 2021). It is an efficient crop in converting sunlight and water into biomass, and researchers have find it as ideal crop for co-production of specific substances for industrial and medical uses. The whole plant can be made as a bio-factory by directing the genetic mechanisms within the cells to produce valuable compounds. Genetically modified sugarcane has been reported to produce chemicals of high value like therapeutic proteins and natural precursors of biopolymers (Gómez-Merino et al. 2014; Hoang et al. 2015). The sugarcane researchers are optimistic that planting genetically modified sugarcane will have potential benefits far better than the risks. Identification of useful genotypes for callogenesis and successful in vitro plant regeneration is required for such genetic improvement in sugarcane (Hussnain et al. 2011; Srikanth et al. 2011; Sanghera and Malhotra 2019). Studies have shown that callus induction and its transformation efficiency and regeneration are not only affected by genotype, sources of transformation, the type of explant used for callus induction but also by the composition of the culture medium including plant growth hormones and different cultural conditions.

The central dogma of the tissue culture: the ability to regulate the consequences of a developmental event through manipulation of a plant tissue, or to manipulate in vitro micro environment where it is placed, and to generate something novel and unique. From the

consideration of these central theme, thin cell layer (TCL) technique was initiated in tobacco (Van 1973). TCL is one of the tissue culture techniques that help culturists understand the growth and development of the plant using thin layers of cells and tissues cut longitudinally and transversally. One-mm-thick layer of cells with variable area dimensions was defined as a longitudinal TCL or lTCL, while a transverse slice, a few mm thick, was termed a transverse TCL or tTCL. This technique has wide applications in the field of in vitro propagation of ornamental, horticultural, and medicinal plants. TCL is an effective model system to study the control morphogenesis mechanism and transformation and has been proved as an efficient system in some monocotyledonous and dicotyledonous species (Golds et al. 1993; Nhut et al. 2003a, b). TCLs, very small explants derived from a limited cell number of uniform tissue, are useful for reducing the time period and can produce high number of shoots with more competence than primary in vitro culture techniques (Azadi et al. 2017). The thin cell layer system is a highly organized (and considered to be an essential) system to study the growth and development of plants. The actual regeneration capacity of TCL explants is often much higher than thicker conventional explants partly due to having a higher ratio of morphogenic cells and better transport between the medium and these cells (Teixeira da Silva et al. 2007). TCL culture system could be used for the large scale production required for plant conservation. For instance, protocorm-like bodies (PLBs) of *Dendrobium malones* 'Victory' (Anjum et al. 2006) and *Xenikophyton smeeanum* (Reichb.f.) (Mulgund et al. 2012) were successfully induced from thin sections of leaf and shoot tips, respectively, in a short period of time. Vyas et al. (2010) also revealed that the secondary PLBs were induced from tTCL of primary PLBs of Cymbidium Sleeping Nymph.

TCL technique also contributes to mass propagation of plants which are used in genetic transformation, micropropagation or bioreactors. Over 45 years since the birth of the TCL concept (Van 1973), morphogenesis in many different plant species or hybrids including many horticultural, ornamental, orchid species, and a few crop plants were successfully reported using TCLs as the explant (Nhut et al. 2003c, d, e, f, 2006; Teixeira da Silva and Dobránszki 2015). Keeping in view the efficacy of TCL and importance of callus induction, callus-mediated regeneration, the present investigation was carried out for optimizing a complete plant tissue culture protocol in sugarcane.

Materials and Methods

Plant Material

Sprouts of sugarcane cv. Israr Shaheed were obtained from sugar crops research institute Mardan, Pakistan. The present research was carried out in laboratory of plant biotechnology, Abdul Wali Khan University, Mardan, Pakistan.

After removing the outer mature leaves, the leaf whorls/basal part of the stem were thoroughly washed several times with tap water to remove soil particles and other impurities. The sprouts were cut into small pieces (2–3 cm) and surface sterilized (Khan and Khatri 2006). Briefly, the explants were soaked in 70% ethanol in a flask for 1–2 min with continuous shaking. The plant materials were rinsed with sterilized distilled water to remove the ethanol and then incubated in sodium hypochlorite (1% active chlorine) solution supplemented with few drops of Tween 20. The explants were washed 4–5 times (for 1, 3, 5, and 10 min, respectively) with sterilized distilled water with continuous shaking.

Explants Preparation and Culturing

The sterilized plant material (leaf sheaths of young sprouts) was cut transversely into small thin sections (1–2 mm wide), so-called tTCLs (as shown in Fig. 1) with sterile surgical blade. Excess water droplets from the explants were dried on sterile tissue paper. The MS (Murashige and Skoog 1962) media containing 3% sucrose, different concentrations (2, 3, 4 mg/l) of 2,4-D and solidified with 1.0% agar (Technical Agar) were prepared (Table 1).

The explants were then cultured on the MS media in the Petri plates with 9–12 explants in a Petri plate. The plastic Petri plates containing the explants were placed in plant culture room at 25 + 2 °C under 16 h (light)/8 h (dark) with 70–80% relative humidity. The explants were subcultured in flasks containing fresh media after 2–3 weeks.

Plantlet Regeneration, Shoot Elongation and Root Formation

Embryogenic calli, induced from explants, were transferred to flasks containing regeneration media prepared with different combinations of plant growth regulators (PGRs), i.e., BAP and NAA were used for plantlet regeneration (Table 1). Cultures were placed in growth room at 28 °C under 16 h light/8 h dark (1000 lx). Regeneration of plants from each of the calli was recorded consecutively for 4 weeks. Data were recorded on number and length of regenerated shoots for four weeks and media were changed

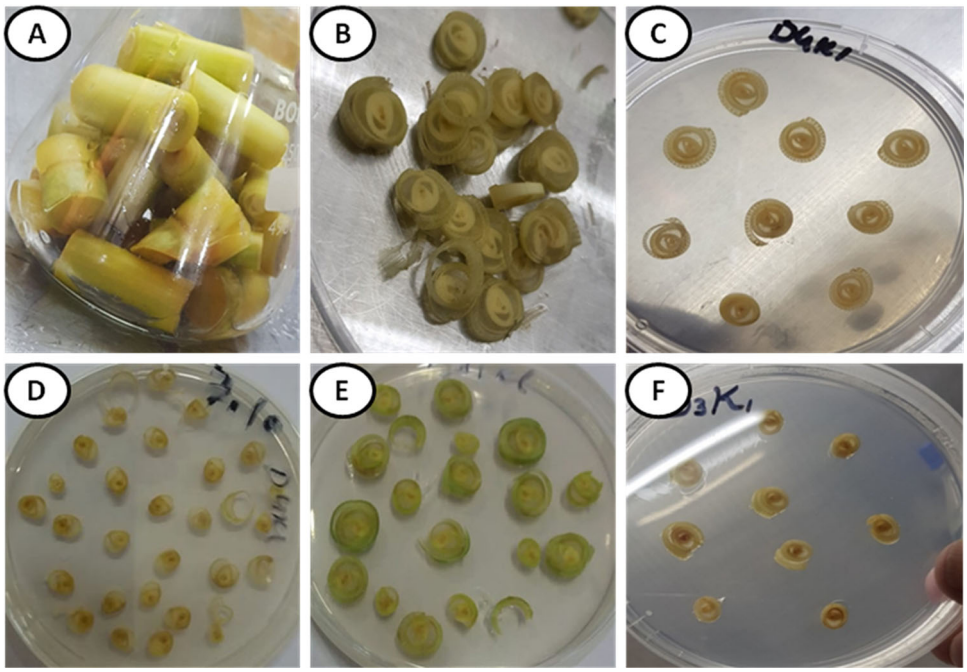

Fig. 1 Small sections of young tops/sprouts of sugarcane surface sterilized (**a**). The sterilized plant materials were cut into small thin transverse sections (**b**). The thin cell layers as explants were cultured on MS medium containing 2,4-D as plant growth regulator (**c–f**). Data represent average of five replicates, and each replicate consists of 30 cultures. Mean ± standard error. Mean followed by the superscript in a column is significantly different from each other according to ANOVA

Table 1 Composition of callus induction and regeneration media with MS salts and vitamins according to manufacturer instructions (4.43 g/l) in all three types of media

S.No	Callus induction media		Regeneration media			Rooting Media	
	Sucrose (g/L)	2, 4-D (mg/L)	Sucrose (g/L)	BAP (g/L) + NAA (mg/L)		Sucrose (g/L)	NAA (mg/L)
1	30.00	1.00	30.00	2.00	1.00	30.00	1.00
2	**30.00**	**2.00**	30.00	2.00	2.00	30.00	2.00
3	30.00	3.00	**30.00**	**2.00**	**0.20**	30.00	3.00
4	30.00	4.00				**30.00**	**3.00**

after every second week. Regenerated plantlets were then shifted onto different combinations of NAA-containing root induction media (Table 1). Data were recorded for number and length of roots induced in four weeks.

Results

An efficient system for in vitro propagation of sugarcane has been optimized, and results obtained in the above experiments are summarized here.

Callogenesis

Transverse TCLs (tTCLs) were prepared from young leaf-whorls of sugarcane which were then used as starting material to generate callus on simple MS (as control) and medium supplemented with different concentrations of 2,4-D (Table 1). Callus induction was observed within three weeks after inoculation of the explants on the medium (Fig. 2). The 2,4-D, used at concentrations of 2–4.0 mg/l, induced callus with varying frequencies. However, the concentration of 2.0 mg/l induced callus within 3 weeks of inoculation (Fig. 3) with 100% frequency (Fig. 4). High concentration proved ineffective towards callus induction. Weight of fresh and dry callus was calculated and found

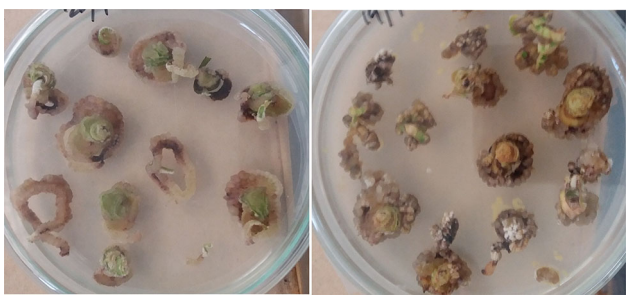

Fig. 2 Callus induced from thin cell layer. Inner section of young leaf rolls produced good quality callus with yellowish and compact stature

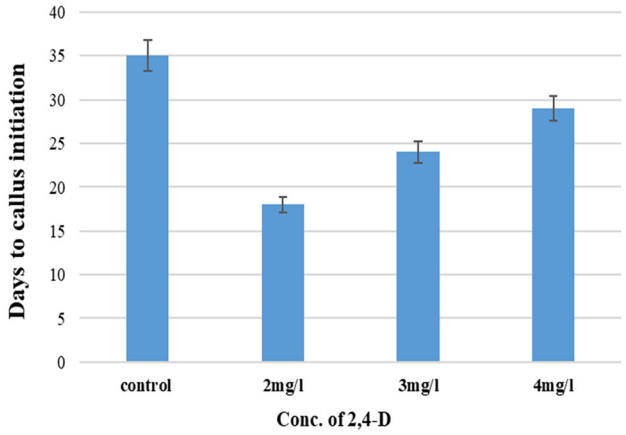

Fig. 3 Effect of different concentration of 2,4-D on days to callus initiation. Simple MS medium was used as control where no explant was developed into callus. Different concentrations of 2,4-D (2–4 mg/l) were used, where 2.0 mg/l induced callus in a short period of time, while 3–4 mg/l comparatively produced callus in longer period of time

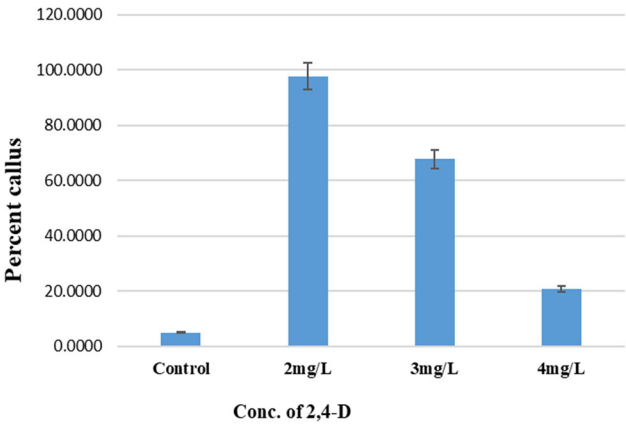

Fig. 4 Effect of different concentration of 2,4-D on callus induction. Among different concentration of 2,4-D (2–4 mg/l), 2.0 mg/l produced calli with 100% frequency where all explants developed callus

highest on 2.0 mg/l 2,4-D, and the least was recorded on 4.0 mg/l supplemented medium (Fig. 5). That is with further increase in the concentrations of the auxins, the relative growth rate of callus was found decreased. The highest growth of the fresh callus (98 ± 0.8 mg) and dry callus (72.8 ± 0.8 mg) was observed on MS supplemented with 2.0 mg/l of 2, 4-D as shown in Table 1.

Callus Types

The calli produced by the TCLs on MS media were observed for the compactness and appearance. Most of the calli were seen from the innermost whorl/part of the explants with yellowish and compact stature. Outer whorls of the explants also produced callus but in less percentage. The MS media added with the 2.0 mg/l 2,4-D caused

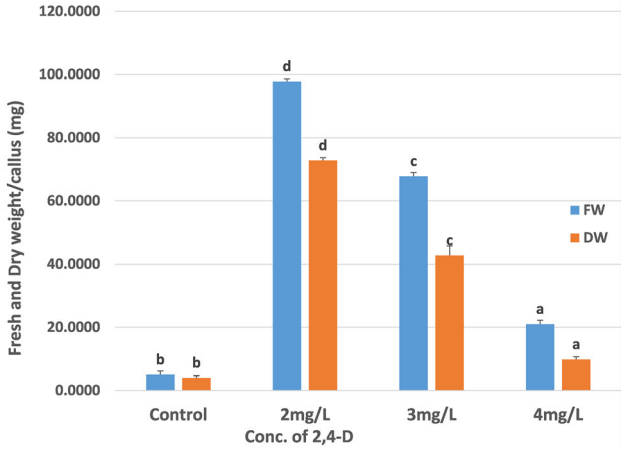

Fig. 5 Effect of different concentrations of 2,4-D on sugarcane fresh and dry weight of callus. Highest fresh weight (97.8 ± 0.8) and dry weight (72.8 ± 0.8) were recorded on 2.0 mg/l of 2,4-D supplemented MS media

production of the callus on most of the explants (as shown in Table 1) followed by 3.0 and 4.0 mg/l of 2,4-D.

Callus initiation could be seen from the cut surface of young inner layers in all treatments after three weeks of culture. Callus produced was found compact and bright yellow (Fig. 2).

Organogenesis/Plantlet Regeneration

Callus-mediated regeneration has been reported in sugarcane (Rao and Arjun 2015; Jamil et al. 2017; Mustafa and Khan 2015; Jabeen and Rao 2013; Khamrit 2012; Tesfa and Ftwi 2018). Therefore, callus was evaluated for regeneration on MS medium along with cytokinins like BAP in different combinations with NAA (Table 1). It was noticed that BAP 2.0 mg/l with NAA 0.20 mg/l induced multiple shoots (25 ± 1.6) with the highest shoot length (6 ± 0.5 cm) with a frequency of 100% (Fig. 6a), while BAP 2.0 mg/l with NAA 2.0 mg/l did not increase the frequency of shoot induction and shoot length (Fig. 6b, c).

Rhizogenesis

In vitro developed plantlets of 5–8 cm height were transferred to MS medium supplemented with different concentrations of NAA for root induction (Fig. 7a). The highest frequency (100%) and maximum number of thick and healthy roots per plantlet (28.2 ± 0.8) and root length (2.8 + 0.09 cm) were obtained on medium containing 4.0 mg/l NAA. The frequency of root induction remained increasing at increasing concentration of NAA (2.0–4.0 mg/l) with less number of roots on 2.0 mg/l NAA (Fig. 7b, c).

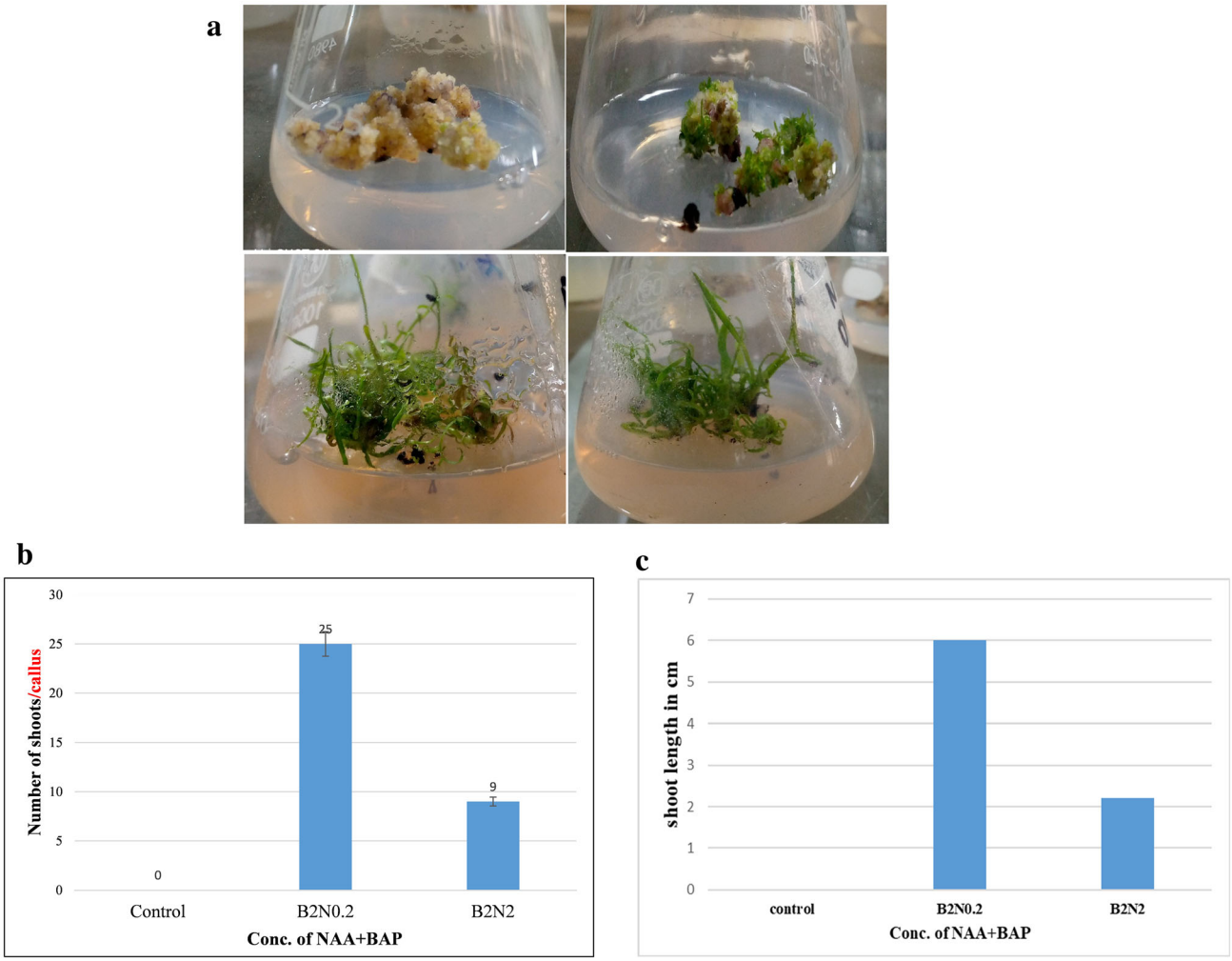

Fig. 6 Regeneration of sugarcane callus into shoots. a Callus regenerated into shoots. Maximum number of shoots regenerated per callus mass in flasks containing BAP + NAA in 2 + 0.2 mg/l concentration. **b**, **c** BAP 2.0 mg/l with NAA 0.20 mg/l induced multiple shoots (25 ± 1.6) with highest shoot length (6 ± 0.5 cm) and a frequency of 100%

Discussion

Sugarcane varieties are vegetatively multiplied by stem cutting in sugarcane breeding program. Serious threats include the lack of appropriate multiplication procedure, as sugarcane is highly heterogeneous species (Lal and Singh 1994). Establishment of efficient callus induction and regeneration protocol prior to transformation is an essential step.

The callus produced by the TCLs in the present research work was subjected to regeneration media having different combinations of PGRs (Table 1) which resulted in optimization of efficient regeneration system in sugarcane cv. Israr Shaheed.

The present investigation demonstrated efficient callus and shoot formation in sugarcane on MS medium with different combinations of PGRs using tTCL explants. During the study, the type of media for callus initiation and regeneration was optimized for better response in shoots and roots formation along with selection of suitable explants to achieve higher multiplication rate under in vitro condition. In our findings, tTCL explants gave promising response, which produced good quality callus and then highest number of shoots with better growth on full strength solid MS medium supplemented with PGRs. TCL technology is also a solution to many of the issues currently hindering the efficient progress of forest tree improvement, since it resolves problems at the first stage, i.e., regeneration by using the most basic developmental building blocks, cells, and tissues. For example, in Iris plants, TCL was used to regenerate the whole plant through somatic embryogenesis using tTCLs (Tan et al. 2006). Shoot regeneration has also been observed in Amaranthus using transverse TCLs of seedlings, only after 1 week, in MS media containing 3 µM TDZ (Jain et al. 2010). Similar results were also reported for some other industrial and

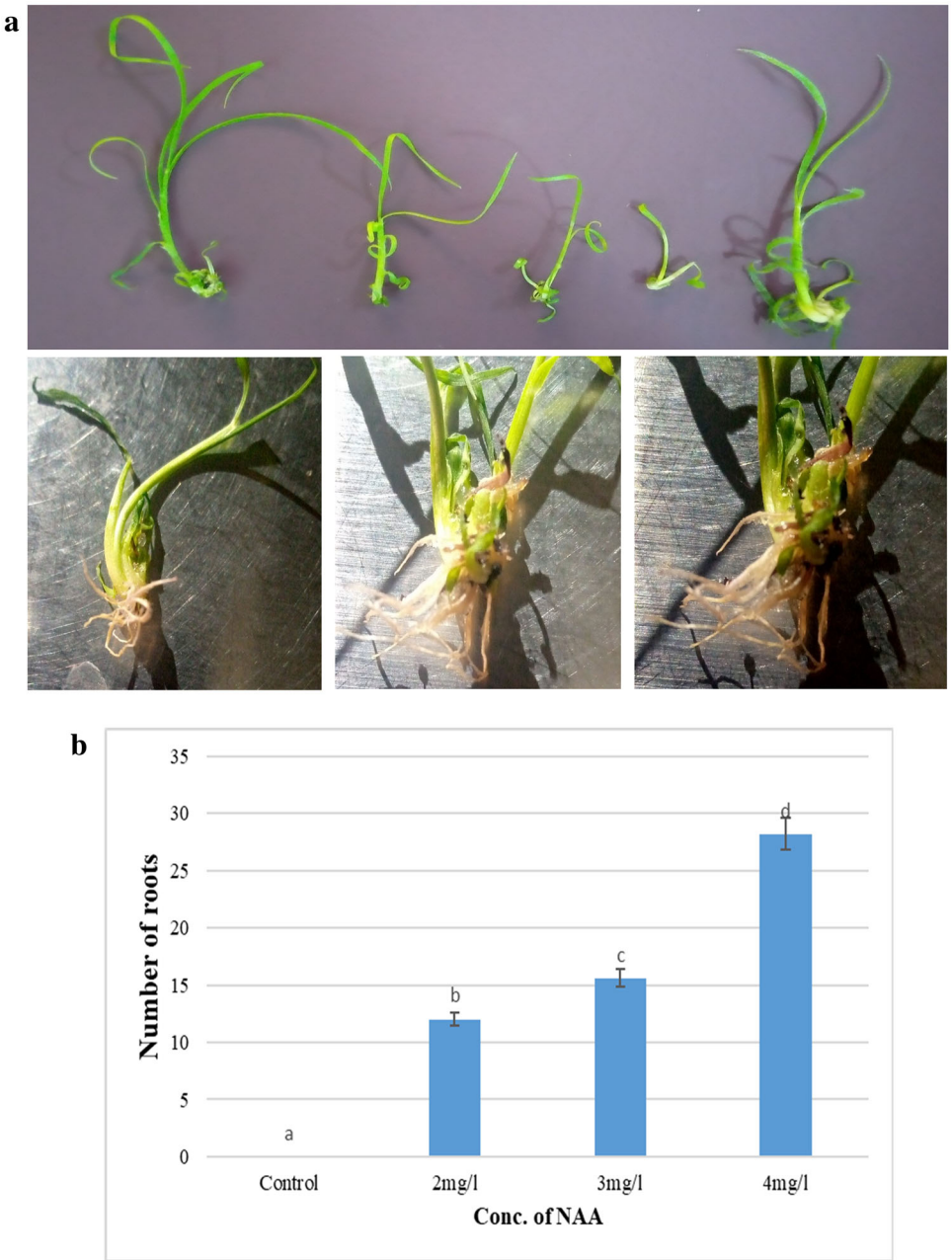

Fig. 7 Root formation by the regenerated shoots. a Regenerated shoots produced roots on medium containing NAA. **b**, **c** Highest frequency (100%) and maximum number of thick and healthy roots per plantlet (28.2 ± 0.8) and root length (2.8 + 0.09 cm) was obtained on medium containing 4.0 mg/l NAA

economic plants using TCL as the best explants for in vitro propagation like *Jatropha curcas* (Nhut et al. 2013), *Ceropegia bulbosa* (Dhir and Shekhawat 2014), *Bacopa monnieri* (Croom et al. 2016), *Vanilla planifolia* (Jing 2016), *Withania coagulans* (Tripathi et al. 2018), and *Dendrobium aqueum* (Parthibha et al. 2018). On the basis of plant growth correction factor (GCF) and geometric factor (GF), Teixeira da Silva and Dobránszki (2011, 2015) reported that TCL explants have higher producing capability than all other traditionally used explants. TCL system has been used mainly for difficult to regenerate plants such as grasses (Nhut et al. 2003c). Through the use of TCLs, the regeneration of specific organs may be effectively manipulated and, together with specific controlled in vitro conditions and exogenously applied PGRs, many problems hindering the improvement in in vitro plant systems are potentially removed, as has been reviewed (Nhut et al. 2006; Teixeira da Silva et al. 2007; Malabadi and Teixeira da Silva 2008, 2009, 2011; Teixeira da Silva and Dobránszki 2014, 2015).

In the present investigations, it was noticed that tTCLs system produced good quality callus from the leaf sheaths on MS media containing 2,4-D (2.0 mg/l). The auxin 2,4-D is an important hormone for callus induction when

compared to other auxins. However, Gallo-Meagher (2000) and Khan et al. (2008) reported that picloram is better than 2,4-D for callus initiation and proliferation in the sugarcane cultivars NIA98, NIA204, and BL4. Such contradicting results may be attributed to different genotypes used in these studies. Sugarcane is known to be highly genotype dependent for its response to callus induction (Gandonou et al. 2005). Callus induction is also highly dependent on the type of explants. Explants from the inner young leaves of sugarcane were found good for induction of callus through TCL. Other studies conducted on callus production by Gandonou et al. (2005) and Nawaz et al. (2013) in sugarcane show similarity with results achieved in the present research.

Cytokinin in combination with auxin has been used to regenerate shoots in sugarcane (Rao and Arjun 2015). In our findings, BAP along with NAA induced multiple shoots from callus and led to rapid multiplication and elongation.

Geetha and Padmanabhan (2001) suggested that different concentration of auxins used in the culture media for callogenesis showed variable regeneration capacity and proliferation. Efficient callogenesis on MS medium with 2,4-D and the highest tendency and ability for regeneration on MS with NAA have been reported (Baksha et al. 2003). In our findings, good-quality callus was induced by using only 2,4-D at lower concentration of 2.0 mg/l, and then, high concentration of NAA led to better root development. Both NAA and IBA (Behara and Sahoo 2009; Gopitha et al. 2010) are reported to be useful in root induction in sugarcane; however, rooting on medium devoid of growth regulators but only at high concentrations of sucrose (6–8%) has also been reported (Meretzaki and Hiraki 1980).

Conclusion and Future Prospects

From the present investigation and results of the earlier literature, it can be concluded that tTCL is a good choice of explant for sugarcane. The callus induction mainly depends on the type of explants, the concentrations, and combination of growth regulators used. Callus induction requires auxins especially 2,4-D in lower concentration. Media supplemented with BAP and NAA played significant role for regeneration through callus requires low concentrations of cytokinins (BAP) for efficient regeneration and induction of maximum number of multiple shoots, while NAA in higher concentration led to the formation of multiple roots. The most obvious future expansion of the use of TCLs is for establishing a regeneration system for a plant that has only displayed a weak, or no, regeneration potential in vitro. A second possibility is to use this sensitive cellular/tissue system for medicinally, commercially, or ornamentally important plants for which no in vitro regeneration system exists. A third possibility for better exploiting the potential of TCL as a regeneration system is to utilize explants of different origin, i.e., from organ or tissue types that have absolutely not yet been used or that were used for organogenesis or embryogenesis in a given species but only displayed low efficacy.

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Declarations

Conflict of interest All the authors of this article have no conflict of interest.

Consent to Participate Not applicable

Consent for Publication All the authors have their own contribution in writing of this article.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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