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Improving in vitro leaf disk regeneration system of sugarcane (*Saccharum officinarum* L.) with concurrent shoot/root induction from somatic embryos

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Abstract: Genome engineering experiments are impeded by poor performance of regeneration systems. The present study was aimed at establishing a short and cost-effective in vitro regeneration system for elite sugarcane cultivars through simultaneous shoot/root induction. The innermost spindle leaf and shoot tip were used as explants. For callus induction, Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) was used and 5.0 mg/L of 2,4-D supported maximum callus induction (84.5%). Three-week-old calli were treated with different levels of benzylaminopurine (BAP) ranging from 0.00 to 3.5 mg/L in MS medium, where 2.5 mg/L BAP was proven to be the best level for regeneration. In a multiplication and root formation medium, 0.5 mg/L naphthalene acetic acid supported the maximum number of roots per plant. Finally, a direct somatic embryogenesis protocol was established, competent enough for simultaneous root/shoot induction. The results indicated that the plantlets were established within 12 weeks only. This in vitro regeneration protocol was fast and cost-effective and may be used for large-scale in vitro regeneration of sugarcane cultivars to save time and resources. The sugarcane cultivar SPF-234 remained the most responsive, followed by HSF-242 and CPF-246.

Key words: Sugarcane, somatic embryogenesis, fast regeneration

1. Introduction

Sugarcane (*Saccharum officinarum* L.), the major source of sugar and alcohol, is a crop of prime importance due to its high agroeconomic values (Naz, 2003). Keeping in view the prominent position of sugarcane in the agricultural industry, strenuous efforts are being made for the improvement of this crop for sustainable and better agronomic traits. Vegetative multiplication of this crop is common in many regions of the world. Its heterozygous and perennial nature, along with a lengthy juvenile period, is an inevitable hurdle in the fast genetic improvement of the crop through traditional breeding programs (Khan et al., 2008). Genetic engineering, being the alternative strategy, demands an established tissue culture and in vitro regeneration system (Arencibia et al., 2000).

Plant tissue culture techniques like micropropagation (Ali and Afghan, 2001; Okumuş et al., 2011; Verma et al., 2011) and somatic embryogenesis are being successfully used for the multiplication of disease-free and elite cultivars. Regeneration systems for different plant species such as pine (Finer et al., 1989), white mustard (Klóska et al., 2012), forage grass (Kumar and Bhat, 2012), wheat (Rashid et al., 2002), rice (Bano et al., 2000), maize (Ahmadabadi, 2007), and sugarcane (Snyman et al., 2011) have been established. Moreover, different parts of the plant with different ages have been exploited as explants for regeneration (Geetha and Padmanabhan, 2001). Considerable progress has been made in the past few years for improving the regeneration system of sugarcane cultivars/genotypes (Geetha and Padmanabhan, 2001; Naz, 2003; Snyman, 2011). The established regeneration systems for sugarcane are laborious and time-consuming, and they require large amounts of resources. Hence, there is still a dire need to improve the regeneration system of sugarcane in terms of chemical consumption and time period.

Agrobacterium tumefaciens-mediated gene transformation has been established both for dicot (Block et al., 1984) and monocot (Hiei, 1994) plants and is hence being used for different crops such as wheat (Jones, 2005),

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maize (Ahmadabadi, 2007), rice (Hiei et al., 1994), and sugarcane (Enriquez et al., 2000). Poor regeneration of transformants is still a major barrier in crop improvement through genetic engineering (Popelka and Altpeter, 2003). In addition, micropropagation of sugarcane also depends on the selection of the genotype. Hence, different sugarcane (*Saccharum officinarum* L.) cultivars need an established regeneration system to become a candidate for gene transformation and in practice regeneration processes are more laborious and time-consuming. There is an urgent need to improve the regeneration system for rapid shoot/root induction to save time and resources.

A large number of strategies are being used to improve the regeneration system of different genotypes of crop plants in terms of time and cost values (Desai et al., 2004). Among these, utilization of plant growth hormones in different combinations is generally considered very effective for efficient regeneration systems. The present study was conducted to assess the efficiency of plant growth regulators on direct shoot/root induction in somatic embryos of sugarcane (*Saccharum officinarum* L.). The objective of the study was to find an effective and more rapid in vitro regeneration system for elite sugarcane cultivars commercially grown in the Punjab area of Pakistan.

2. Materials and methods

Healthy leaves (innermost spindle leaf and shoot tips) of 3 elite sugarcane cultivars, CPF-246, SPF-234 and HSF-242, were used as experimental material. The cane seeds (vegetative parts) of these cultivars were obtained from the Agricultural Biotechnology Research Institute of Faisalabad, Pakistan. Plants were cut 20–30 cm below the uppermost part having approximately 10-cm spindle leaves along with the shoot tip. The upper leaf sheaths were removed and the remaining tissue was wiped with 90% ethanol for 45 s. These spindles and shoot tips were surface sterilized with 0.1% (W/V) mercuric chloride ($HgCl_2$) solution for 1 min and subsequently rinsed 4 times with double distilled deionized water.

The upper rolled leaves of the cylinders were removed and the innermost rolled leaf was sliced into small disks (2.00 mm) in a sterile environment. These leaf disks and small pieces of shoot tips were placed on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 200 mg/L of myo-inositol, 30 g/L of sucrose, and various concentrations (0–5 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D). Bacterial-grade agar was used for gelling purposes at the concentration of 1% (w/v). The pH of all the media was set at 5.8 using 1 N NaOH before autoclaving. Media sterilization was done at 15 psi (121 °C) for 20 min. All the cultures were grown at 25 ± 02 °C under 16-h white cool fluorescent lights.

The regeneration study was carried out in 3 steps. First, 3-week-old selected calli were subjected to regeneration media having different combinations of benzylaminopurine (BAP; 0.0, 0.5, 1.5, 2.5, and 3.5 mg/L) along with MS medium with vitamins (Phyto Technology Laboratories) and sucrose (30 g). Bouquets of shoots were separated in a sterile environment and were subjected to rooting media (1/2 MS and 0.5 mg/L naphthalene acetic acid [NAA]) after 3 weeks. The data were recorded in terms of regeneration percentage and average shoot number.

In the second step, a multiplication and root formation medium (MS medium with vitamins [Phyto Technology Laboratories], sucrose [30 g], and BAP [2.5 mg/L] with different concentrations of NAA [0.0, 0.5, 1.5, 2.00 and 2.5 mg/L]) was optimized and data were recorded for average number of roots, plant height, and average number of days for root induction.

In the third step, a direct somatic embryogenesis medium (MS with 5 mg/L of 2,4-D, 2.5 mg/L of BAP, and 0.5 mg/L of NAA) was used for rapid and efficient in vitro regeneration of cultivars studied (Figure 1) and data were recorded for average number of shoots and roots per plant. A completely randomized design was used with 5 treatment levels and 5 replications. The data collected were analyzed statistically using the Costat Computer Module (CoHort Software).

3. Results

Analysis of variance of data showed that different levels of 2,4-D have highly significant effects on callus induction, as shown in Table. The maximum callus induction (84.5%) was supported by 5.0 mg/L of 2,4-D. The 3 sugarcane cultivars under investigation showed highly significant differences for this variable. The sugarcane cultivar SPF-234 showed the highest (60.81%) while CPF-246 showed the lowest (50.11%) value for callus formation (Figure 2). The calli produced by SPF-234 and HSF-242 were creamy white, granular, and compact, whereas calli produced by CPF-246 were brownish, loose, and watery in nature.

Callus regeneration percentage was significantly influenced by varying levels of BAP when inner leaf roll was used for explants (Table). Callus regeneration percentage was minimum (33.3%) at 0.5 mg/L of BAP. The application of BAP at 2.5 mg/L showed the highest callus regeneration percentage (74.6%), followed by 1.5, 1, and 3.5 mg/L, respectively. The 3 sugarcane cultivars used in the present study also showed statistically highly significant differences for this variable. Sugarcane cultivar SPF-234 showed the highest callus regeneration percentage (68.4%), followed by HSF-242 (46.4%) and CPF-246 (38.4%). Different levels of BAP also had highly significant effects on callus regeneration percentage when shoot tip was used for explants. Minimum callus regeneration percentage (23%)

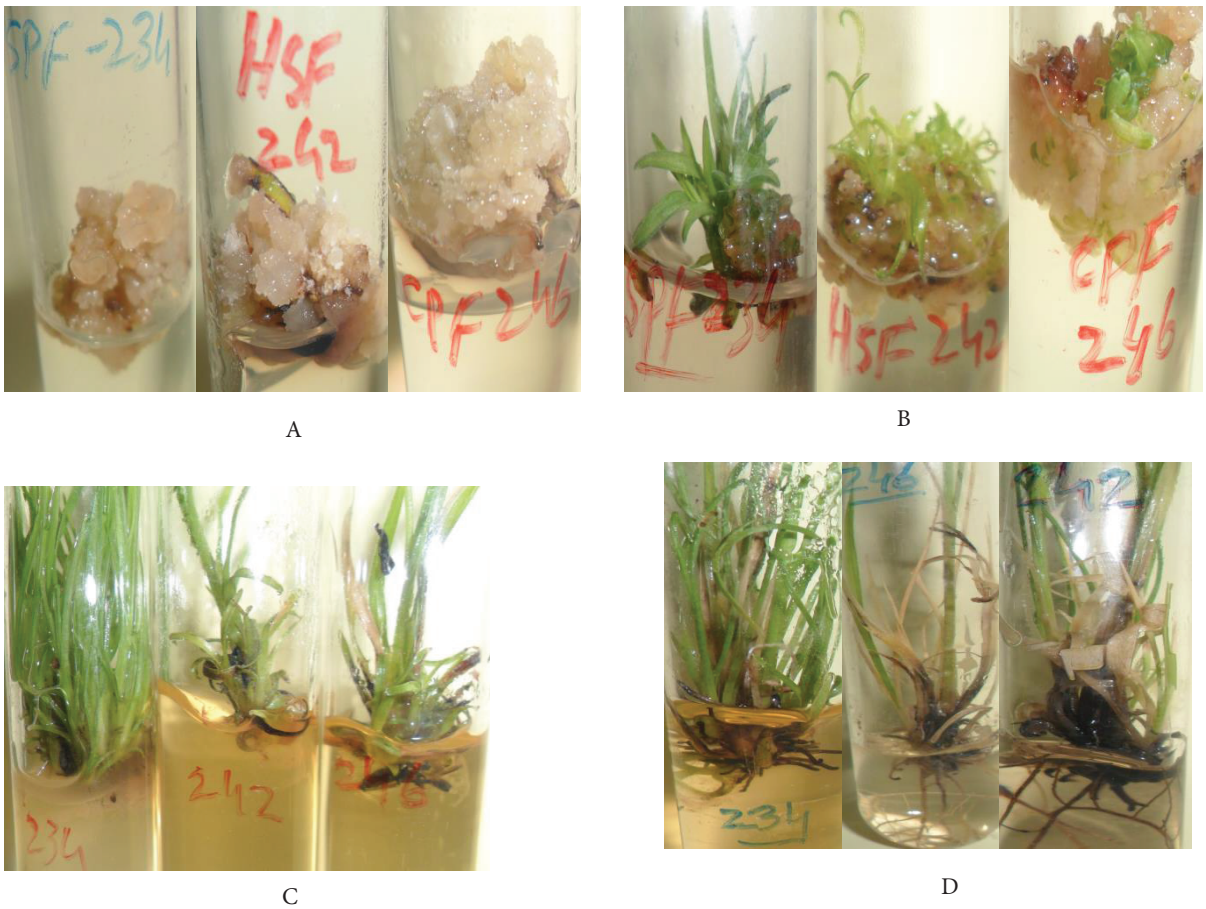

Figure 1. In vitro regeneration of 3 sugarcane cultivars (SPF-234, HSF-242, and CPF-246) through direct somatic embryogenesis (MS with 5 mg/L 2,4-D, 2.5 mg/L BAP, and 0.5 mg/L NAA). A: Direct somatic embryogenesis (3 weeks). B: Regeneration of somatic embryos (6 weeks). C: Shoot formation and elongation (8 weeks). D: Rooting and acclimatization (12 weeks).

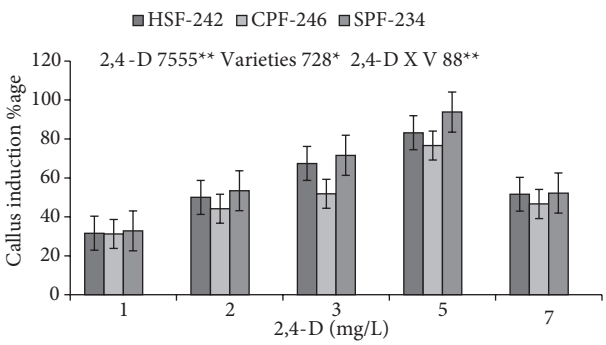

Figure 2. Callus induction of 3 elite sugarcane cultivars (SPF-234, HSF-242, and CPF-246) at different levels of 2,4-D.

was recorded at 0.5 mg/L while maximum (63.6%) was recorded at 2.5 mg/L BAP (Figure 3).

Different BAP levels had varying effects on average number of shoot inductions from embryogenic calli produced from leaf roll and shoot tip explants (Table). The average number of shoot inductions was minimum at 0.5 mg/L BAP from leaf roll, i.e. 7, and for shoot tip this value was 5.1. The use of BAP at 2.5 mg/L produced the highest average number of shoots (16.7 for leaf roll and 14.6 for shoot tip explants) (Figure 4). All the sugarcane cultivars showed highly significant differences for this variable. The sugarcane cultivar SPF-234 produced the highest average number of shoots (14.8 for leaf roll and 12.7 for shoot tip explants), followed by HSF-242 (10.81 for leaf roll and 8.9 for shoot tip explants) and CPF-246 (7.81 for leaf roll and 6.3 for shoot tip explants).

Analysis of the variance of root induction data revealed that different levels of NAA have significant effect on root induction of plantlets produced by regeneration media (Table). The average number of roots was maximum at 0.5 mg/L NAA (13.2), followed by 1.5 mg/L (9.3), 2.0 mg/L (7.8), and 2.5 mg/L (5.2), respectively. The minimum number of roots was produced at 0.0 mg/L NAA (2.61). Sugarcane cultivars used in this study also showed

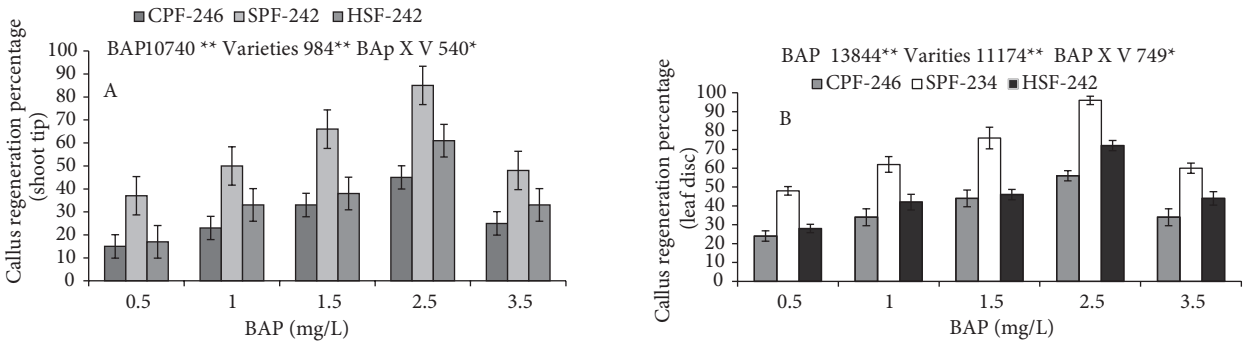

Figure 3. Callus regeneration from (A) shoot tip explants and (B) leaf disks of the 3 sugarcane cultivars (SPF-234, HSF-242, and CPF-246) using different levels of BAP.

Table. Average plant height and average number of days for root initiation of 3 elite sugarcane cultivars (HSF-242, CPF-246, and SPF-234) at different levels of NAA and at BAP level of 2.5 mg/L.

Attributes	Sugarcane varieties	NAA levels (mg L^{-1})					ANOVA		
		0	0.5	1.5	2.0	2.5	Source of variation	Degrees of freedom	MS value
Plant height (cm)	CPF-246	4.00 ± 0.77	13.08 ± 1.03	10.17 ± 0.10	7.5 ± 0.30	5.08 ± 0.20	NAA	4	597***
	HSF-242	2.33 ± 0.40	12.05 ± 0.35	9.5 ± 0.35	6.5 ± 0.00	3.25 ± 0.30	Varieties	2	11***
	SPF-232	3.00 ± 0.71	13.00 ± 0.35	9.5 ± 0.35	6.5 ± 0.00	3.25 ± 0.31	Error	30	13
Root initiation days	CPF-246	14.17 ± 1.48	10.33 ± 1.95	10.17 ± 1.02	10.33 ± 0.82	13.67 ± 2.41	NAA	4	288***
	HSF-242	17.5 ± 0.00	9.00 ± 1.95	11.00 ± 0.00	14.17 ± 2.04	13.5 ± 0.00	Varieties	2	18*
	SPF-232	17.5 ± 0.00	8.5 ± 0.00	11.00 ± 0.00	12.83 ± 0.41	15.83 ± 1.48	Error	30	80

ns = nonsignificant, *, *** = significant at 0.05, and 0.001 levels of probability, respectively.

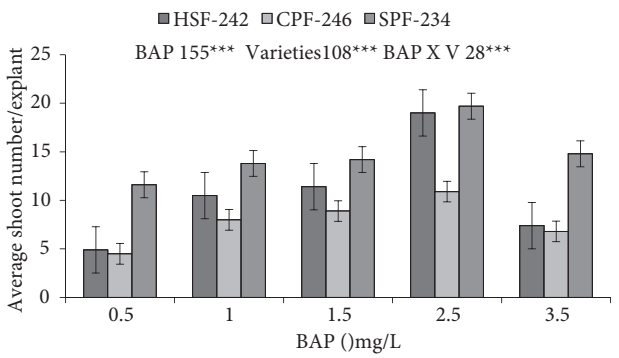

Figure 4. Average number of shoots per explants of 3 elite sugarcane cultivars (HSF-242, CPF-246, and SPF-234) at different levels of BAP.

significant differences for root formation. The sugarcane cultivars SPF-234, HSF-242, and CPF-246 produced 8.1, 7.7, and 7.7 average roots per plant, respectively (Figure 5). The interaction among different levels of NAA and cultivars studied was also significant.

Analysis of variance revealed that different NAA levels had varying effects on the average shoot height of

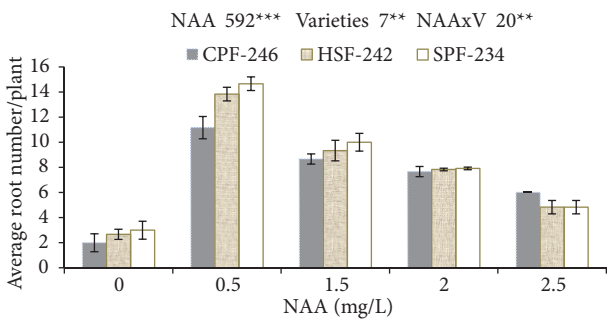

Figure 5. Average number of roots per plant of 3 elite sugarcane cultivars (HSF-242, CPF- 246, and SPF-234 at different NAA levels.

plantlets produced (Table). The minimum average shoot height (3.11 cm) was recorded at the 0.0 mg/L NAA level and maximum (12.91 cm) at 0.5 mg/L NAA, followed by 9.71 cm at 1.5 mg/L, 6.8 cm at 2.0 mg/L, and 3.86 cm at 2.5 mg/L. Sugarcane cultivars did not show significant differences for this variable.

A highly significant effect of different levels of NAA on root initiation time was recorded. Maximum time (16.4 days on average) was taken for root initiation when 0.0 mg/L NAA was used and root initiation time was recorded as minimum (9.2 days on average) at 0.5 mg/L NAA (Table).

Data for the third step of experimentation showed that different cultivars produced significantly varying number of roots/shoots when the explants (leaf roll) were put directly onto somatic embryogenesis media. The sugarcane cultivar SPF-234 produced 13.63, HSF-242 produced 10.16, and CPF-246 produced 8.8 roots per plant on average, while the number of shoots per explants on average was 20.73, 20.4, and 17.3 for SPF-234, HSF-242, and CPF-246, respectively (Figure 6). The innermost leaf roll disk was observed as the best explant in this new protocol of direct embryogenesis and simultaneous shoot/root development, while cultivar HSF-234 was the most responsive cultivar for direct regeneration.

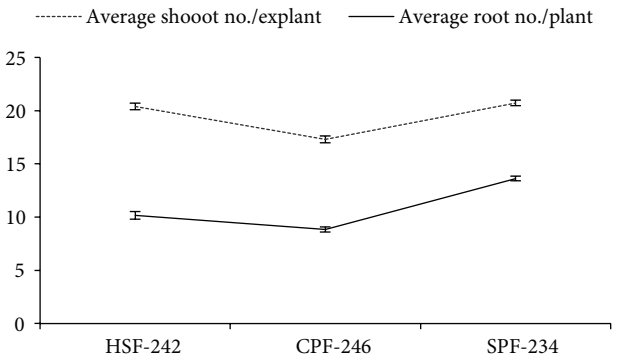

Figure 6. Average number of roots per plant and average shoot number per explant of the 3 sugarcane cultivars (HSF-242, CPF-246, and SPF-234) with NAA (0.5 mg/L), BAP (2.5 mg/L), and 2-4D (5 mg/L).

4. Discussion

An efficient and reproducible regeneration system is a fundamental need for producing transgenic plants through genetic engineering (Arencibia et al., 2000). Many regeneration systems have been reported in sugarcane using different explants, such as young leaves (Brisibe et al., 1994), immature inflorescences (Desai et al., 2004), and apical meristems (Ahloowalia and Maretzki, 1983). In the present study, 2 different tissues (innermost leaf roll and shoot tip) were used to record observations on in vitro regeneration potential of 3 elite sugarcane cultivars. Shoots were developed from somatic embryos in 3 weeks and 3 more weeks were taken by these plantlets to establish their roots. In this initial experiment, 2.5 mg/L BAP proved to be the best level for maximum shoot induction. Many other studies also indicated that the optimum level of BAP for sugarcane regeneration was near 2.5 mg/L. For instance, Xu et al. (2008) reported that 2.00 mg/L BAP was the best level for shoot induction with an average shoot number of 15.1 compared with our studies, where 16.7 and 14.6 were the average shoot numbers at 2.5 mg/L BAP from leaf roll and shoot tips experiments, respectively. Similarly, Eldessoky et al. (2011) reported that 2 mg/L kinetin + 1 mg/L BAP produced 7.66 elongated shoots on average. Results from the shoot growth studies indicated that addition of 2.5 mg/L BAP + 0.5 mg/L NAA in the growth medium produced maximum shoot growth (12.86 cm). In contrast to these results, Razi-ud-din et al. (2004) reported a very high concentration of BAP (5.0 mg/L + 1.00 mg/L GA_3) for average shoot growth (3.7 cm). This huge difference in the shoot growth as observed in the present study and the above-mentioned report may be due to the fact that different cultivars were used, show varying responses to different media.

In another study, smaller shoot length (4.5 ± 0.01 cm) was recorded on media having 2.0 mg/L BAP and 0.5 mg/L IBA (Baksha et al., 2002) as compared to the present study, where only BAP with MS media produced a greater number of average shoots, and when used with 0.5 mg/L NAA, more shoot length was recorded as compared to other reports (Chattha et al., 2001; Eldessoky et al., 2011). The regeneration potential of SPF-234, HSF-242, and CPF-246 from the innermost leaf roll explants was 16.2%, 9.5%, and 10.2% higher, respectively, than that of shoot tip explants. It was observed from this experiment that leaf roll was the best explant, as supported by many other studies (Brisibe et al., 1994; Chengalrayan and Gallo-Meagher, 2011; Malabadi et al., 2011), and 2.5 mg/L BAP was the best level for in vitro regeneration of the cultivars studied.

The maximum average number of roots (13.2) per plant using 0.5 mg/L NAA, as observed in the present study, had also been reported by some other workers using media having 2 mg/L NAA with 60 g sucrose/L (Ahloowalia and Maretzki, 1983). Lal (2003) reported that regenerated plants established their roots within 5 weeks when put on media having 0.2 mg/L NAA while, in our studies, roots were established in 3 weeks only. Some other workers used different media to induce the maximum number of roots. For instance, Khalil (2002) used 7 mg/L NAA with 60 g/L sucrose, and Kharinarain et al. (1996) used media having

6% sucrose with 5 mg/L NAA to produce the maximum number of roots. Similarly, Xu et al. (2008) reported that the average number of roots ranged from 10.8 to 15.0 when 2.00 mg/L NAA was used.

In the third step, direct somatic embryogenesis medium was used and the number of shoots per plant was 24.11% higher than in the previous BAP treatments while the number of roots per plant was 3.11% greater as compared with the previous NAA treatment (Table). In the initial study, we observed that approximately 15 weeks were required to develop an established plantlet with roots from explants, while, in our modified protocol of direct somatic embryogenesis, young plantlets were established with roots in 12 weeks only (Figure 7). The media used here excluded the use of coconut water and casein hydrolysate as reported in other such studies (Nasir et al., 2011). The protocol established during the present study may be employed in routine regeneration of elite cultivars. The culture time was reduced by 3 to 4 weeks. It was reported that direct somatic embryogenesis may be established with suitable combination by growth regulators (Desai et al., 2004). In contrast, the present study suggested that sugarcane regeneration protocol can be simplified and excessive use of chemicals can be avoided. Hence, the induction of direct somatic embryogenesis using the innermost leaf roll is cost-effective, efficient and time-saving. It could be used successfully in genetic transformation and rapid propagation of sugarcane cultivars in a short period of time.

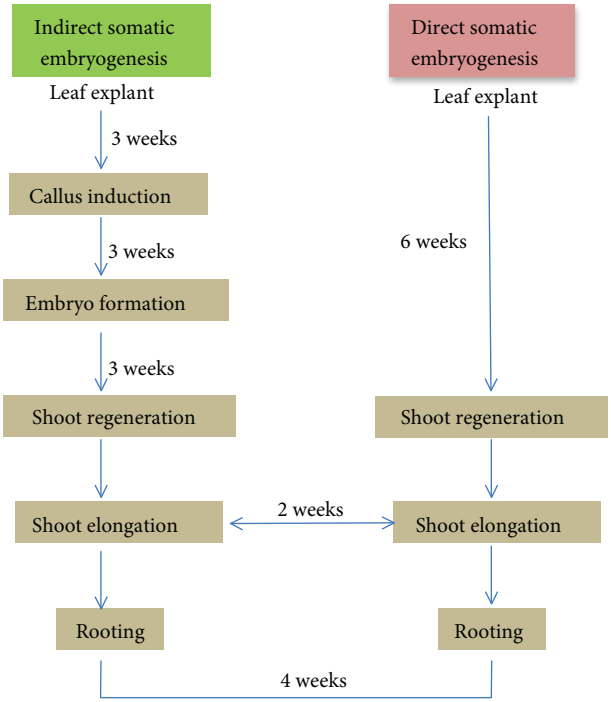

Figure 7. Comparison between indirect somatic embryogenesis and direct somatic embryogenesis of sugarcane elite cultivars (SPF-234, HSF-242, and CPF-246).

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