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Standardization and Development of Improved Tissue Culture Techniques for Genotype CO 94012 in Sugarcane

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Abstract

Sugarcane is a major cash crop in India and is affected by various pests and diseases. Also, it requires more irrigation as compared to other crops to grow. The genetic improvement by conventional breeding is still a challenge due to long crop cycle and poor genetic diversity. To overcome these constraints, genetic transformation and CRISPR mediated gene editing in plants can prove as beneficial tools. To develop traits in sugarcane using these technologies requires highly reproducible and very efficient somatic embryo based protocol in popular cultivars. In this study, we aimed to develop high efficiency, reliable and reproducible somatic embryos based tissue culture system amenable for Agrobacterium or biolistic-mediated genetic transformation in popular cultivars of sugarcane. The improved protocol can be used for generation of transgenic or genome edited plants in desired numbers. In the present investigation, we used high yielding sugarcane var. Co 94012 for development of high efficiency callus based regeneration protocol. Leaf disc from two months old shoots were used to induce the callus. Embryogenic calli were obtained using initial boost of 3mg/lit, 2, 4 D followed by subsequent subculture at 2 mg/lit 2, 4 D. Regeneration response was 100 percent on MS media with 1 mg/lit BAP. These regenerated shoots were elongated on MS media enriched with 0.2 mg/lit BAP for two to three weeks. Furthermore, Cv. (cultivar) Co 94012 showed 100 percent rooting on MS media with 1 mg/lit NAA. Root initiation was observed within 8 to 10 days. 100 percent survival of transplanted plants was observed. This is the first report of sugarcane regeneration and development of plants through callus using var. (variety) Co 94012.

Keywords: Standardization, Tissue culture, Co 94012, Sugarcane.

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Introduction

Sugarcane (*Saccharum spp*.) is major cash crop in India and cultivated in 48.57 Lac ha. India contributed 19.07 percent to the world area under sugarcane cultivation with production of 399.25 million tons). Maharashtra is the second highest state in area and production of sugarcane in India. (E&S Est.-2020-21). In India sugar is produce 100 percent from sugarcane while in worldwide percentage varies from 70 percent to 80 percent. (Thorat et al., 2017). Sugarcane is affected by various biotic and abiotic stresses resulting considerable economic losses (Khaliq et al. 2005). Due to such stresses, sugarcane yield is decreasing or remained stagnant. (Jalaja. et. al 2006) Varieties of sugarcane are highly heterogeneous and are generally multiplied vegetatively by stem cutting (Behera and Sahoo 2009). Genetic improvement in sugarcane is hampered by the narrow genetic variability available in the present day clones (Jackson, 2005). To address these and many more issues, tissue culture articulated with TALENs and CRISPR/ Cas editing tools is playing very important role in crop improvement. With these new plant breeding technologies scientists can insert desired traits (without inserting foreign gene) very instantly and precisely than conventional breeding. (Parkhi.et.al.2021). An advantage of tissue culture amongst many other approaches is the production of high quality and uniform planting material that can be multiplied under disease free conditions (Merkle et al., 1990). Unlike other crops sugarcane have very few parts available for tissue culture and plant propagation and for varietal improvement through in- vitro techniques it required efficient protocol to improve plant regeneration through more calli production. (Shobha kumari 2010)

Callus is unorganized plant cell structure results of different growth hormones in culture media. (Shah et al 2003). Concentration and types of growth hormone changes according to crop to crop and species to species. (Charriere et al 1999). The present study we have focused popularly known genotype Co 94012. This is the first variety produced by soma clonal variation through tissue culture of genotype Co C 671. (Jalaja. et. al 2006). Co 94012 variety is having better traits than Co C 671 like more sucrose content and moderately resistant to red rot and smut disease. We have developed efficient and simple tissue culture reproduction techniques through callus regeneration and plant development.

Abbreviations

1) 2-4-D : 2,4 Dichlorophenoxy acetic acid
2) BAP : 6- Benzylamino purine
3) NAA: Naphthalen acetic acid
4) MS media : Murashige and Skoog medium

Material and Method

Explant Surface Sterilization

Collected stem tops from green house grown sugarcane plants of verities viz. Co 94012. Expanded leaves were removed and washed under running tap water. Outer leaves were removed to isolate immature leaf rolls (about 1 to 1.5 cm diameter). Rinsed explants in 70percent ethanol for 30 second and washed with sterile water for three times. Surface sterilized the explants with 2 percent Sodium hypochlorite and Tween twenty (10 ul/ 10 ml) for 5 minutes. Gave vigorous five washes with sterile distilled water and peeled out outer layer of leaf whorl. Excise the leaf whorl transversally with thickness approximately 2 mm.

Callus Induction

Inoculated these explants on callus induction MS media (Murashige and Skoog 1962) enriched with four treatments of Auxin concentration of 2-4-D viz. 1 mg (CI-2), 2 mg (CI-3), 3 mg (CI-4) per liter and no 2-4-D (CI-1), (Patel et al 2015). Ten explants were placed par Petri plate. The Petri plate or glass plate dimension was 10 cm in diameter and 2 cm in height. Plates were filled with 25ml of media and sealed with micro pore tape. Total sixty explants per treatment were maintained. But for treatment 3mg/lit, 2-4-D (CI-4) used 120 explants. These plates were incubated at 28^{o}C (degree Celsius) in dark for two weeks. All these callusing explants were sub cultured again on respective media except 60 explants from CI-4 to CI-3 media and maintained for six weeks with regular 14 days interval sub culturing on respective same fresh media. During each subculture nodular calli obtained were again transferred on fresh media.

Shoot Regeneration & Elongation

Induced nodular embryogenic calli (approximately 250 mg/stack) were transferred on shoot regeneration MS media containing different concentration of BAP 0.5 mg (SR-2), 1 mg (SR-3), 1.5 mg (SR-4) and no BAP (SR-1) (Shobha kumari 2010). These cultures were incubated at 25^{o} C with 16 hrs.(hours) light /8

hrs. dark. Regenerated shoots were divided in to three types such as rosette shoot, normal shoot and multiple shoot. Multiple shoots obtained were transferred to shoot elongation MS media with BAP 0.1 mg/lit (E-1). These shoots were sub cultured once or twice on same media with 15 days interval to obtain 3 to 5 cm long shoots. Cultures were incubated at 25° C with 16 hrs. light/ 8 hrs dark.

Rooting

Elongated shoots were separated and transfer on rooting MS media enriched with different concentration of NAA viz. 0.5 mg (RT-2), 1 mg (RT-3), 1.5 mg (RT-4), 2 mg (RT-5) and no NAA (RT-1) . In each bottle 6 shoots were maintained. Cultures were incubated at 25° C with 16 hrs. light/ 8 hrs. dark. These shoots were sub cultured twice on same media with 14 days interval till to produce profuse roots. Developed roots were categorized in to three category such as fibrous or hairy roots, thin roots and thick roots.

Hardening

Well rooted plants were hardened in plastic cups containing autoclaved soil and soil rite with proportion 1:1 ratio and covered these plants with polythene bags. These plants were maintained in growth chamber at 25° C with 16 hrs. light/ 8 hrs. dark. Hardened plants were exposed to environment gradually by cutting polythene bag at edges. Polythene bag was removed in one week and transplanted these plants in green house.

Results and Discussion

Callus Induction: In genotype Co-94012 callus started producing within 7 to 8 days in all the treatment except CI-1.In this treatment no callus developed within two months. Two types of callus induced on the surface of leaf whorl. Initially explants produced fluffy white callus, afterword creamy nodular and compact embryogenic callus developed over it. As mentioned in Table No.2 explants shown highest callus induction (80percent) and rooting of callus (20percent) when kept for 15 days on CI-4 media and then subculture on CI-3 media for two months. Whereas explants continued on CI-4 media showed 73percent callus induction with 28percent root induction on callus tissues. This shows that as we reduced 2-4-D after giving booster dose of 3 mg/lit for 15 days to 2 mg/lit, more nodular callus develops with reducing root induction from explants. In

genotype Co 86032 and Co 265 (Parmar Rina D et.al 2017) observed profuse and good quality callus in 3 mg/lit. Averagely 300 mg of nodular callus was produced during each sub culturing. Also it was observed that callus growth as well as callus inducing explants increases with increase in 2,4- D concentration (Shobha kumari 2010).

Shoot Regeneration:

Shoot regeneration from callus tissues were observed within 6 days by producing green bud primordia. As shown in Table No: 3. SR-1 media with no BAP, calli produced 76 percent normal shoots with frequent fibrous roots. Whereas on media SR-4 only 70 percent calli were regenerated with rosette types of shoots. On SR-3 media 100percent callus regenerated with approximately 2 cm long multiple shoots and average number of shoots per callus observed more (16 shoots/ calli) in SR-3 media. This shows that 1 mg/lit BAP produced 100percent callus regeneration with well differentiated multiple shoots. But in genotype Co 86032 and Co 94012 (Shobha kumari 2010) claimed maximum shoot regeneration on 0.5 mg/lit BAP. While in genotype Co 86032 (Salokhe S.2021) found multiple shoots on media enriched with BAP 0.2 mg/lit and kinetin 0.1 mg/lit.

Shoot Elongation:

Shoots differentiation and elongation were started. The shoots were elongated on E-1 media within 15 to 20 days to attain the height 3 to 5 cm.

Rooting:

In this genotype elongated shoots started rooting within 15 to 18 days in all the treatments. Root color was white in all the treatment. Without NAA in MS media (RT-1), 80percent shoots produced fibrous roots with 5 cm long. Whereas media RT-3 showed 100 percent rooting with 9 cm long and average 9 thick roots par plants. Surprisingly results were observed on media RT-4 and RT-5 root growth restricted drastically to 2cm with increased thickness. From these two treatments roots were thicker than RT-3. Salokhe S. (2021) found best rooting in sugarcane genotype Co-86032 in media MS containing 1 mg/lit IAA and 1 mg/lit NAAl. Lal and Singh (1999) reported best response in 1 mg/lit NAA in sugarcane spp. whereas low concentration of auxin induces best rooting. Cooke et.al (2002)

mentioned 3 mg/lit NAA was effective in production of roots. However high concentration of auxin stimulate cells to produce ethylene which affect root elongation. (Yi et al 2004). Which we observed same results in 1.5 mg/lit and 2 mg/lit concentration of NAA.

Hardening:

Well rooted plants of height 6 cm to 8 cm were hardened in soil mixed with soil rite with ratio 1:1. Plants were showing normal phenotypic growth after hardening and transplanting in soil.

Flow chart for callus regeneration and plant development in Co-94012

Leaf whorl surface sterilization with 70percent ethanol for 30 sec followed by 2percent sodium hypochlorite and tween twenty (10ul/10ml). Rinse with distil sterile water for 5 to 6 times.

Excise the leaf rolls about 2mm thickness and inoculate on MS media enriched with 2-4-D, 3 mg/lit. Incubate the cultures in dark at 28 ^{0}C for 14 days.

Transfer the callusing explant on MS media enriched with 2 mg/lit 2-4-D media. Subculture every two weeks newly produced callus on fresh media. Incubate the cultures in dark at 28 ^{0}C for 6 weeks.

Excise the nodular compact callus and inoculate on MS media enriched with 1 mg /lit B.A.P. Keep the cultures at 26 ^{0}C and light for 16 Hr. photoperiod.

Excise the growing multiple shoots and subculture on MS media enriched with 0.2 mg/lit B.A.P. for two to three weeks. Keep the cultures at 26 ^{0}C and light for 16 Hr. photoperiod.

Separate the elongated shoots and subculture on Ms media containing 1 mg/lit NAA

Keep the cultures at 26 ^{0}C and light for 16 Hr. photoperiod for three weeks.

Harden the well rooted plants in soil mixed with soilrite (1:1 ratio). And cover the plant with polythene. After 4th day cut slightly or punctured polythene at upper end. Next day give another cut at other side. Remove whole polythene subsequent day. Keep the plants at 26 ^{0}C and light for 16 Hr. photoperiod. Transplant these plants in soil.

Conclusion

In the present study we have developed simple and reliable reproducible protocol in genotype Co 94012. This genotype responded very well to tissue culture. Optimum doses for callus induction, Shoot regeneration and rooting were standardized with high frequency of regeneration. In our study we developed nodular and regenerative callus with modified doses of auxin 2-4-D. Initially we used 3 mg/lit 2-4-D to induce the callus and then reduced to 2 mg/lit to avoid root formation during callus induction and multiplication. When calli were subjected to BAP for shoot regeneration, 1 mg/lit BAP found optimum dose for higher (100percent) and normal shoot regeneration. These multiple shoots were elongated on 0.2 mg/lit BAP. Elongated shoots were rooted on NAA. We observed different concentration of NAA affects the root formation and root growth. 1 mg/lit NAA proved to be best dose to induce 100percent robust root induction.

This protocol will be more effective in micro propagation, genetic transformation and gene editing techniques in sugarcane.

Table 1 : Tissue Culture Media Composition

Sr. No	Stages/Media code	Media Composition
1	Callus Induction:	
	CI-1	MS salts, B5 vitamins, Sucrose 30 gm./lit, phytagel 0.3percent, pH 5.8
	CI-2	MS salts, B5 vitamins, 2-4-D 1 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
	CI-3	MS salts, B5 vitamins, 2-4-D 2 mg/lit, Sucrose 30 gm./lit, phytagel 0.3percent, pH 5.8

	CI-4	MS salts, B5 vitamins, 2-4-D 3 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
2	Shoot regeneration :	
	SR-1	MS salts, B5 vitamins, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
	SR-2	MS salts, B5 vitamins, BAP 0.5 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
	SR-3	MS salts, B5 vitamins, BAP 1 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
	SR-4	MS salts, B5 vitamins, BAP 1.5 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
4	Shoot elongation: E-1	MS salts, B5 vitamins, BAP 0.1 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
5	Rooting:	
	RT-1	MS salts, B5 vitamins, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
	RT-2	MS salts, B5 vitamins, NAA 0.5 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
	RT-3	MS salts, B5 vitamins, NAA 1 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
	RT-4	MS salts, B5 vitamins, NAA 1.5 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8
	RT-5	MS salts, B5 vitamins, NAA 2 mg/lit, Sucrose 30 gm/lit, phytagel 0.3percent, pH 5.8

Table No 2 : Callus induction response on different concentration of 2-4-D

Sr. No	Auxin 2-4-D Conc.	Media code	Media code	Explants used	Callusing explants	Callus induction	Callus induction Percentage	Explants producing roots	Callus rooting percentage
	mg/lit	(I)*	(II)**	No.	No.	Days	percent	No.	percent
1	0	CI-1	CI-1	60	00	00	0	0	0
2	1	CI-2	CI-2	60	25	13	42	2	3
3	2	CI-3	CI-3	60	34	12	56.6	6	10
4	3	CI-4	CI-3	60	48	12	80	10	16
5	3	CI-4	CI-4	60	44	12	73	17	28

Conc. : Concentration, (I)* : Callus induction media, (II)**: Callus proliferation media, No: Numbers

Table :3 Shoot regeneration response with different concentration of BAP.

Sr. No	B.A.P Conc.	Media code	Calli pieces	Shoots regenerated	Percentage	Average shoot par callus	Shoot type	Shoot size Average
	mg/lit		No.	No.	percent	No.		cm
							Normal	
1	0	SR-1	50	38	76	8.5	Normal	5 mm
2	0.5	SR-2	50	43	86	14	Multiple	8 mm
3	1	SR-3	50	50	100	16	Multiple	2 cm
4	1.5	SR-4	50	35	70	12	Rosette	1 cm

Conc. : Concentration, No: Numbers

Table 4 : Rooting of shoots on various concentration of NAA

Sr. No	N.A.A Con.	Media code	Single shoot	Roots induced Shoots	Average roots par shoot	Av. Length of root	Root type	Rooting Percent
	mg/lit		No.	No.	No.	cm		percent
1	0	RT-1	40	32	7	5	Fibrous	80
2	0.5	RT-2	40	37	7	7	Thin	92.5
3	1	RT-3	40	40	9	9	Thick	100
4	1.5	RT-4	40	15	2	2	Thick	37.5
5	2	RT-5	40	13	2	2	Thick	32.5

Conc. : Concentration, No: Numbers.

Figure 1: Initial stage of explants inoculated on media,

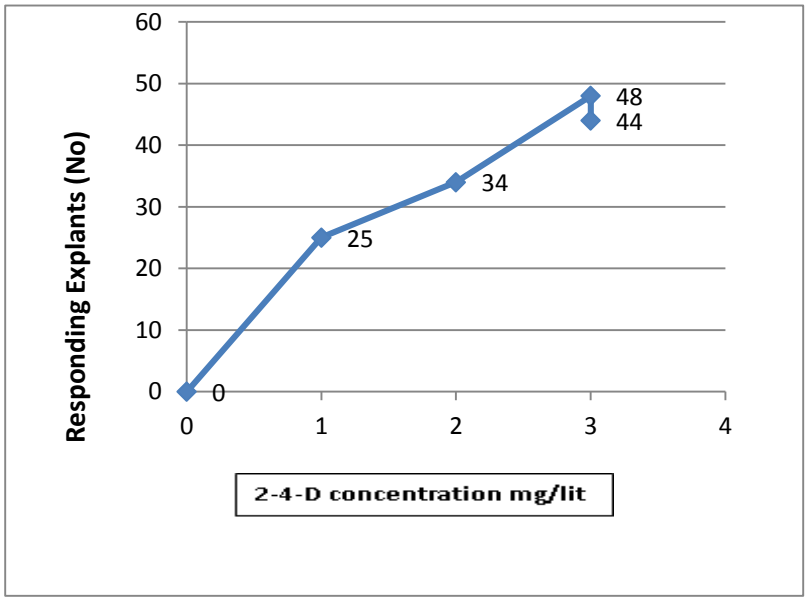

Figure 2 : Callus induction with respect to 2, 4 D concentration

Figure 3 : Explants producing callus on CI-4 media

Figure 4 : Explants producing nodular calli on CI-3 media.

Figure 5 : Explants producing roots on CI-4 media subculturing

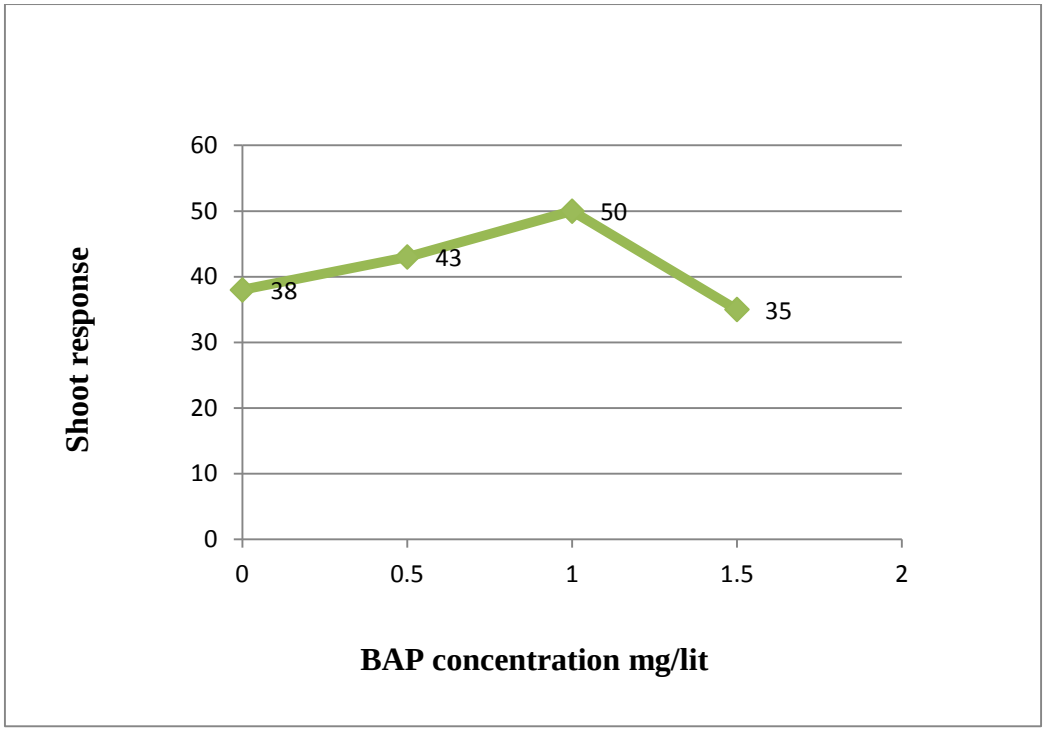

Figure 6: Graphical presentation of shoot induction with BAP concentration

Figure 7: All calli pieces showing shoot regeneration on SR-3 media

Figure 8: Multiple shoots elongating on E-1 media

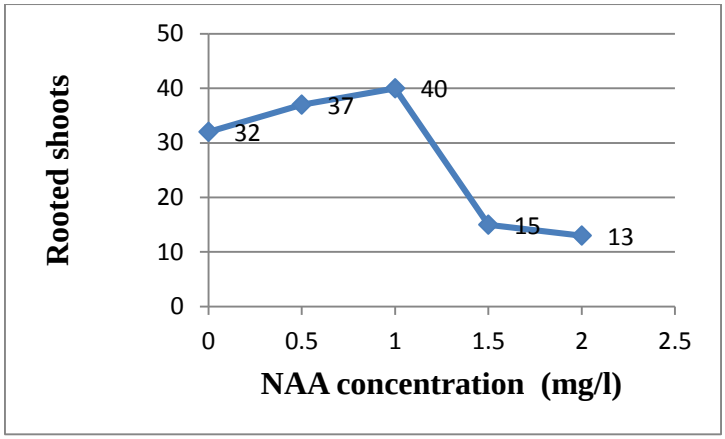

Figure 9 : Graph showing rooting of shoots with respect to NAA concentration

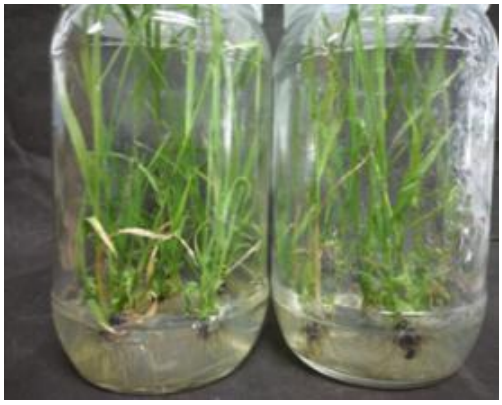

Figure: 10: All elongated shoots rooted on RT-3 media

Figure: 11 and 12: Rooted plants hardened in soil

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