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Effect of explants and growth regulators on the expression of Callogenesis, somatic embryogenesis and plantlets formation in Sugarcane (*Saccharum officinarum* L.)

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Key words: Tissue culture; 2, 4-D, Benzyl 6-amino purine, Callus, Somatic Embryos, Plant regeneration, Pakistan

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Abstract

The present research was done to standardize a protocol for hormone treatment and to determine the best type of explants for the induction of callus, somatic embryogenesis and regeneration response in sugarcane. The potential for callogenesis and somatic embryogenesis of different explants of sugarcane was explored. The influence of different concentrations and combinations of various phytohormones along with varying environmental conditions like temperature and light on in vitro callus induction and somatic embryo formation was also studied. Among three types of explants used in present study (leaf, shoot apical meristem and pith), the maximum response of callus induction and somatic embryogenesis was obtained from leaf explants. The maximum callus induction response obtained onto MS medium containing 3 mg/l of 2, 4- D was 92% from leaf, 72% from shoot apical meristem and 50% from pith explants after 14 days of inoculation. Direct and indirect somatic embryogenesis was observed and the best response was obtained on MS medium supplemented with 3 mg/l 2, 4-D + 0.5 mg/l BAP. MS media supplemented with 4mg/l 2, 4-D+ 0.25mg/l BAP was proved to be the best for regeneration of somatic embryos, where 90% plantlets formation was observed. Thus, leaf explants must be cultured onto MS medium with 2, 4 D / BAP corresponding to 4 mg/l / 0.25 mg/l to optimize somatic embryogenesis and plantlets regeneration.

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Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the most important cash crop of the world. The importance of sugarcane has increased in recent years because it is an important industrial raw material for sugar industries and the allied ones namely those producing alcohol, acetic acid, butanol, paper, plywood, industrial enzymes and animal feed. Another important use of sugarcane is the production of press mud, which is used as a source of organic matter and nutrients for crop production (Raja and Abbas, 2006, Ali *et al.* 2007).

Pakistan is the fifth largest sugarcane growing country of the world but our yield per hectare is lower than all over the world. There are many reasons for that. The most important of which is the non availability of disease free elite stock for seeding and lack of implementation of advance technologies in sugarcane propagation, development of new seed varieties and maintenance of current promising varieties. In Pakistan sugarcane is propagated by vegetative means and flowering has long been a seriously problem in sugarcane propagation and breeding. Although there are some areas where flowering occur but due to non synchronization of flowering breeding is not possible. Though seeds are produced but mostly they are not fertile.

In this context tissue culture studies have received considerable research attention because it is one of the most effective technique for the propagation of plant species that have problems in seed propagation and plants that produce non viable seeds. It has become an important tool in the study of basic areas of plant biology, biochemistry and biotechnology (Thorpe, 2007). The use of *in vitro* technology for improvement of sugarcane is an alternative to the conventional methods, because it is rapid, reliable and sustainable option (Khan *et al.,* 2008). It also allows the rejuvenation of plant material. Plant tissue culture techniques like callogenesis and somatic embryogenesis can be used for successful sugarcane propagation by controlling a lot of problems which are faced during conventional breeding practices (Jahangir *et al*., 2010).

Callus induction and somatic embryos production appears a new approach in sugarcane propagation and maintaining disease free stock by producing a high frequency of somatic embryos expressing somaclonal variations. Plant regeneration from somatic embryos is a viable alternative for large scale production of plants and offers a possibility for sugarcane crop improvement (Biradar *et al*., 2009; Behra and Sahoo, 2009; Sengar *et al*., 2011). It can be employed as an efficient way of propagation with the goal of enhancing the rate of multiplication of desired genotypes and commercial micropropagation (Helal, 2011).

Gene transfer, selection and regeneration of transformants are also employed by the plant tissue culture techniques (Shah *et al*., 2009). Biotechnological advancement not only provided alternative methods for *in vitro* preservation of tropical fruits and recalcitrant seeds but also provides tools for disease free germplasm conservation, lower labor cost and limiting disease transfer (Khan *et al.,* 2008; Alves *et al.,* 2011). Furthermore, this *in vitro* culture method is useful not only for plant breeding and genetic studies, but also for studying the tolerance of sugarcane plants to abiotic stresses, in particular, soil salinity, which is generally detrimental to plant growth and productivity in Pakistan and worldwide. The present study was conducted to establish the callogenesis response, induction of somatic embryogenesis and to develop a reproducible protocol for efficient regeneration of sugarcane variety NSG-59 through somatic embryogenesis.

Materials and methods

Plant materials collection, surface sterilization, preparation of callus induction and proliferation media

The explants of sugarcane variety known as NSG-59 were obtained from Shakar Ganj Research Institute, Jhang, Pakistan. The present research work was carried out in Plant cell tissue & organ culture lab, Department of Botany University of Sargodha, Pakistan. Three type of explants namely leaf, shoot apical meristem and pith were collected from sugarcane plants.

The plant material collected in field was washed several times with tap water with a few drops of liquid soap. The outer old leaf-base coverings were removed carefully without damaging the inner young and delicate tissue. The explants were then surface sterilized with ethanol 70 % for 15 minutes to avoid contamination and were inoculated to obtain callus and somatic embryos.

Callus is a mass of undifferentiated group of cells which can be obtained from any portion of the plant body like leaf, shoot, node, internodes, pith etc. Nevertheless, the callus induction is highly influenced by type of explants, temperature, light and type of medium used. Leaf tissues of 3.0-6.0 mm were excised and inoculated onto MS (Murashige and Skoog, 1962) basal medium supplemented with different ratios of auxins and cytokinins either singly or in combinations to identify the appropriate combinations of media for regeneration of sugarcane somatic embryos through callogenesis and somatic embryogenesis as mentioned in Table 1.

MS media consisted of MS basal salts (Macro, micronutrients, Iron EDTA and vitamins) with 3% sucrose, pH was adjusted to 5.5-5.7 and autoclaved at 120°C for 20 min at 105 kPa. Difco bacto agar 6% per liter was used as gelling agent. Explants were incubated at 23±2°C under 16-18 h light period (from fluorescent light tubes) with light intensity of 2000-3000 lux and 6- 8 h dark period.

Potential of Different Explants for Callus Induction, Somatic Embryogenesis and Regeneration of Plantlets from Somatic Embryos

In present study different type of explants (leaves, shoot apical meristem and pith) were used to check their potential for callus formation and proliferation. Among different type of explants used it was noticed that leaf explants were having maximum potential for efficient callus induction and proliferation followed by shoot apical meristem and pith explants were least potent for callus formation and proliferation. Callus induction was observed within two week after inoculation from explants on modified MS medium containing different concentrations of auxins and combinations of auxin- cytokinins (Table 1).

Somatic embryogenesis was obtained both by direct and indirect method. For somatic embryos MS medium was supplemented with different concentration and combinations of different plant growth regulators. For obtaining maximum proliferation of plantlets, regenerated plants were shifted to basal media and combinations of BAP and kinetin for further growth (Table 4).

Statistical analysis

Collected data were subjected to analysis of variance incorporating means separation according to Duncan's New Multiple Range tests at 5% threshold.

Results and discussion

Effect of Different Medium Composition for Callus Induction and Proliferation in Sugarcane

It was evident that the callus induction and proliferation was triggered on all concentrations, nonetheless, the best callus induction and proliferation was observed on MS medium supplemented with 3 mg/l 2, 4-D (Fig. 5) from leaf, shoot apical meristem and pith explants which were 92%, 72% and 50% respectively.

Similar results were obtained for maximum callus induction and proliferation in sugarcane using 3 mg/l of 2, 4-D was reported by many scientists (Naz *et al.*, 2008; Ather *et al.*, 2009; Jahangir *et al.*, 2010). Jahangir *et al.* (2010) reported that MS medium supplemented with 3-4 mg/l 2, 4-D alone induces callus induction and embryogenic callus was produced when 3 mg/l 2, 4-D was used alone in MS medium. Different combinations of auxin-cytokinin interaction were also used for callus induction and proliferation. MS medium supplemented with 4.0 mg/l, 2, 4-D with 0.25 mg/l, BAP the rate of callus induction was 70% from leaf, 62% from shoot apical meristem and 42% from pith explants (Fig. 6A & B). Similar results were also reported by Santosa *et al.* (2004) who obtained embryogenic callus in the presence of a combination of auxins and cytokinins.

Table 1. Effect of medium composition on callus formation and proliferation in sugarcane SAM*: Shoot apical meristem, Age of cultures = 2 week.

Tr.	Media	Conc. (mg/l)	No. of explants cultured	No. of test tubes showing callus induction		
				Leaf	SAM*	Pith
C_1	MS + 2,4-D	1	10	6.4±0.219^{c}	4.4±0.219^{ef}	3.8 ±0.334^{bc}
C_2		2	10	8.2±0.334^{ab}	6.0±0.282^{bcd}	4.2±0.334^{abc}
C_3		3	10	9.2±0.334^{a}	7.2±0.334^{a}	5.0±0.282^{a}
C_4		4	10	7.4±0.219^{bc}	5.0±0.282^{cdef}	4.8±0.334^{ab}
C_5	MS + 2,4-D + BAP	2.0 +0.5	10	4.6±0.219^{de}	3.4±0.219^{gh}	2.6±0.357^{de}
C_6		3.0 +0.5	10	7.2±0.334^{bc}	5.0±0.282^{cdef}	3.2±0.334^{cd}
C_7		3.0 +0.25	10	6.6±0.456^{c}	4.8±0.334^{def}	3.4±0.219^{cd}
C_8		4.0 +0.25	10	7.0±0.894^{bc}	6.2±0.334^{abc}	4.2±0.334^{abc}
C_9	MS + 2,4-D + Kin	2.0 +0.25	10	3.8±0.334^{e}	2.8±0.334^{h}	2.6±0.219^{de}
C_{10}		3.0 +0.25	10	6.6±0.456^{c}	4.4±0.219^{efg}	2.0±0.282^{e}
C_{11}		3.0 +0.5	10	7.0±0.894^{bc}	6.4±0.456^{ab}	4.2±0.334^{abc}
C_{12}		4.0 +0.5	10	6.8±0.334^{c}	5.0±0.894^{cdef}	3.6±0.219^{cd}
C_{13}	MS+ NAA+ BAP	1.0 +0.5	10	5.0±0.282^{d}	3.8±0.334^{fgh}	2.0±0.282^{e}
C_{14}		2.0 +1.0	10	6.4±0.456^{c}	4.4±0.456^{efg}	3.8±0.334^{bc}
C_{15}		3.0 +0.25	10	7.0±0.282^{bc}	5.0±0.282^{cdef}	4.8±0.334^{ab}
C_{16}		4.0 +0.25	10	6.8±0.334^{c}	5.4±0.456^{bcde}	3.2±0.334^{cd}
LSD				1.0942	1.0618	0.9735

Mean followed by different letters in the same column differ significantly at p= 0.05 according to Duncan's new multiple range test.

Table 2. Effect of different hormones on direct somatic embryo formation in sugarcane SAM*: Shoot apical meristem, Age of cultures = 2 week.

Tr.	Media	Conc. (mg/l)	No. of explants cultured	No. of test tubes showing callus induction		
				Leaf	SAM*	Pith
E_1	MS + 2,4-D	1	10	2.8±0.334^{f}	1.4±0.219^{f}	1.6±0.219^{cd}
E_2		2	10	5.6±0.357^{bc}	3.2±0.282^{cd}	1.8±0.334^{bcd}
E_3		3	10	7.4±0.456^{a}	4.8±0.334^{a}	2.0±0.282^{abc}
E_4		4	10	5.8±0.334^{bc}	4.4±0.282^{ab}	2.4±0.219^{abd}
E_5	MS + 2,4-D + BAP	2.0 +0.5	10	3.2±0.334^{cf}	2.8±0.334^{de}	2.6 ±0.357^{bc}
E_6		3.0 +0.5	10	7.4±0.456^{a}	5.0±0.282^{a}	3.0±0.282^{abc}
E_7		4.0 +0.25	10	6.8±0.334^{ab}	3.4±0.456^{bcd}	2.4±0.219^{abd}
E_8		4.0 +0.5	10	5.2±0.334^{cd}	4.0±0.282^{abc}	2.8±0.334^{ab}
E_9	MS + 2,4-D + Kin	1.0 +0.5	10	2.8±0.334^{f}	1.8±0.334^{ef}	1.4 ±0.219^{bc}
E_{10}		2.0 +0.5	10	4.2±0.334^{de}	2.4±0.456^{def}	1.8±0.334^{abc}
E_{11}		3.0 +0.5	10	6.0±0.282^{bc}	4.0±0.282^{abc}	2.2±0.178^{a}
E_{12}		3.0 +1.0	10	7.2±0.334^{a}	3.2±0.334^{cd}	3.0±0.894^{ab}
LSD				1.1314	1.0511	0.9213

Mean followed by different letters in the same column differ significantly at p= 0.05 according to Duncan's new multiple range test.

The varying concentration of 2, 4-D and kinetin was also proved promotive for callus induction and the rate of callus induction was maximum in concentration 3.0 mg/l 2, 4-D with 0.5 mg/l kinetin (C_{11} medium). It was 70%, 64%, 42% for leaf, shoot apical meristem and pith explants. Different concentrations of NAA with BAP were also tested for callus induction and proliferation.

Best result in this combination was obtained when 3.0 mg/l NAA + 0.25 mg/l BAP were used in MS medium (C_{15} medium). At this the rate of callus formation was 70%, 50%, 48% from leaf, shoot apical meristem and pith explants respectively (Table 1; Fig 1). The findings of Behra and Sahoo (2009) also support our results, who reported that the concentrations NAA at 2.0 and 3.0 mg/l produced small amount (20%-30%) of callus.

Table 3. Effect of different hormones on indirect somatic embryo formation in sugarcane SAM*: Shoot apical meristem, Age of cultures = 2 week.

Tr.	Media	Conc. (mg/l)	No. of explants cultured	No. of test tubes showing callus induction		
				Leaf	SAM*	Pith
S_1	MS + 2,4-D	1	10	8.6±0.456^{ab}	7.4±0.456^{a}	4.0±0.282^{def}
S_2		2	10	7.2±0.334^{cde}	6.8±0.334^{ab}	5.2±0.521^{abc}
S_3		3	10	6.4±0.456^{def}	5.2±0.334^{cde}	3.6±0.219^{fgh}
S_4		4	10	4.8±0.334^{ghijk}	3.4±0.456^{gijk}	3.8±0.334^{efg}
S_5	MS + 2,4-D + BAP	1.0 +0.25	10	9.2±0.334^{a}	7.4±0.456^{a}	6.2 ±0.334^{a}
S_6		1.0 +0.5	10	8.4±0.456^{abc}	6.8±0.334^{a}	5.4±0.456^{abc}
S_7		1.0 +1.0	10	7.6±0.357^{bcd}	6.0±0.282^{bc}	4.6±0.368^{bcd}
S_8		2.0 +1.5	10	4.2±0.334^{hijk}	3.4±0.219^{ghijk}	2.2±0.334^{ijk}
S_9	MS + 2,4-D + Kin	1.0 +0.25	10	6.0±0.282^{efg}	5.0±0.28^{cdef}	4.2±0.334^{cde}
S_{10}		1.0 +0.5	10	5.6±0.456^{fgh}	5.2±0.334^{bcd}	3.4±0.456^{fgh}
S_{11}		1.0 +1.0	10	3.4±0.456^{ki}	2.4±0.219^{k}	2.6±0.357^{hij}
S_{12}		2.0 +1.5	10	2.2±0.334^{i}	2.8±0.334^{jk}	1.6±0.219^{k}
S_{13}	MS+ NAA+ BAP	0.25 +0.25	10	4.0±0.282^{ijk}	3.4±0.456^{ghijk}	2.2±0.334^{ijk}
S_{14}		0.5 +0.25	10	6.2±0.334^{efg}	5.2±0.334^{cde}	5.6±0.456^{ab}
S_{15}		1.0 +0.25	10	5.4±0.456^{fghi}	5.4±0.219^{cde}	4.4±0.456^{bcd}
S_{16}		2.0 +1.5	10	4.2±0.334^{hijk}	3.6±0.456^{ghijk}	2.0±0.282^{jk}
S_{17}	MS+ BAP+ Kin	0.25 +0.25	10	6.4±0.456^{def}	5.2±0.334^{cde}	5.0±0.632^{ab}
S_{18}		0.5 +0.25	10	5.0±0.894^{fghij}	4.4±0.219^{efgh}	4.0±0.282^{def}
S_{19}		1.0 +0.25	10	5.2±0.334^{fghij}	4.6±0.219^{defg}	3.6±0.357^{fgh}
S_{20}		1.0 +0.5	10	4.4±0.456^{hijk}	3.8±0.334^{fghi}	3.8±0.334^{ef}
S_{21}	MS+BAP+ IAA	1.5 +1.0	10	4.2±0.334^{hijk}	3.2±0.521^{hijk}	3.2±0.178^{gh}
S_{22}		1.5 +0.5	10	5.0±0.282^{fghij}	4.2±0.334^{efghi}	3.4±0.219^{fgh}
S_{23}		2.0 +1.0	10	3.4±0.456^{ki}	6.4±0.456^{ab}	2.2±0.334^{ijk}
S_{24}		2.0 +0.5	10	3.8±0.521^{jk}	3.0±0.282^{ijk}	2.6±0.219^{hij}
LSD				1.228	1.11	1.134

Mean followed by different letters in the same column differ significantly at p= 0.05 according to Duncan's new multiple range test.

Table 4. Effect of media composition on regeneration of plantlets from somatic embryos No. of cultures examined = 10, Age of cultures = 8 week

Tr. No	Media	Conc. mg/l	Frequency (%age) of regeneration of somatic embryos into plantlets
M_1	Basal		50
M_2	MS + 2,4-D + BAP	4.0 + 0.25	90
M_3	MS + BAP	1.0 mg/l	70
M_4	MS + BAP + Kinetin	1.0 + 0.25	80

Effect of Different Concentrations of Auxins and Cytokinins on Direct Somatic Embryogenesis in Sugarcane

Somatic or asexual embryogenesis is the regeneration of embryo-like structures from somatic cells without gametic fusion (Tabassum *et al.*, 2010). Somatic embryogenesis is a potential tool in the genetic engineering of plants (Helal, 2011). MS medium with varying concentration of auxins and cytokinins were supplemented for initiation of direct somatic embryogenesis from all type of explants in sugarcane.

Among different concentrations and combinations for direct somatic embryogenesis, best performance was noticed on MS medium supplemented with 3.0 mg/l 2, 4-D. At this concentration the rate of somatic embryo induction was 74% from leaf, 48% from shoot apical meristem and 20% from pith explants. As for as combination of 2,4-D and BAP is concerned, the concentration 3.0 mg/l 2, 4-D + 0.5 mg/l BAP (E_6 medium) showed good result for somatic embryogenesis from all kind of explants used (Fig. 7C & D).

At this concentration somatic embryogenesis was 74% from leaf, 50% from shoot apical meristem and 30% from pith explants. Desai *et al.* (2004) reported direct embryogenesis in sugarcane using a combination of auxins and cytokinins. Similarly Ali *et al.* (2007) reported the embryo development in sugarcane using a concentration of 2, 4-D and BAP.

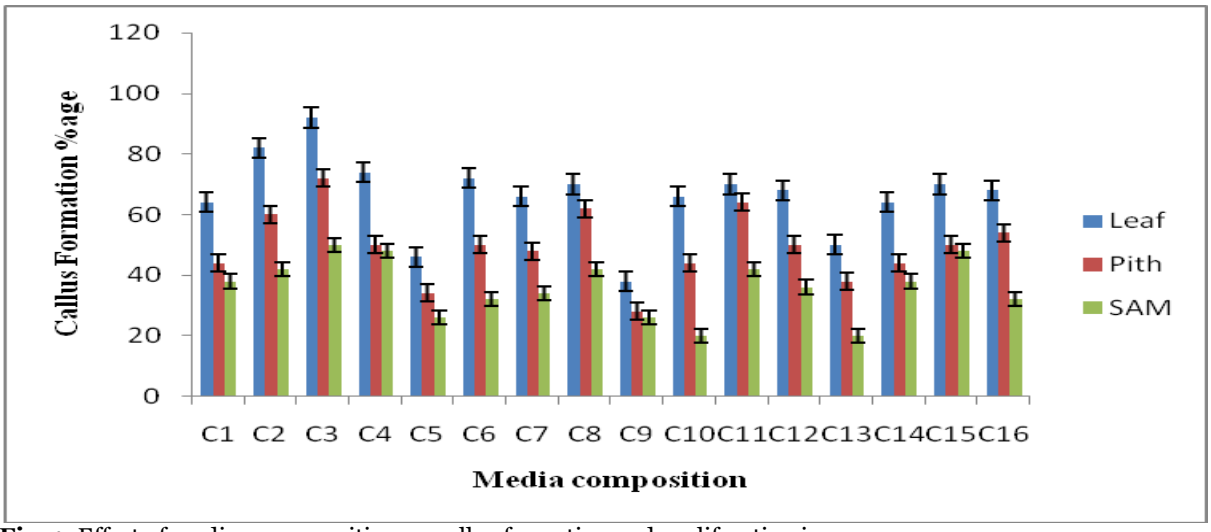

Fig. 1. Effect of medium composition on callus formation and proliferation in sugarcane.

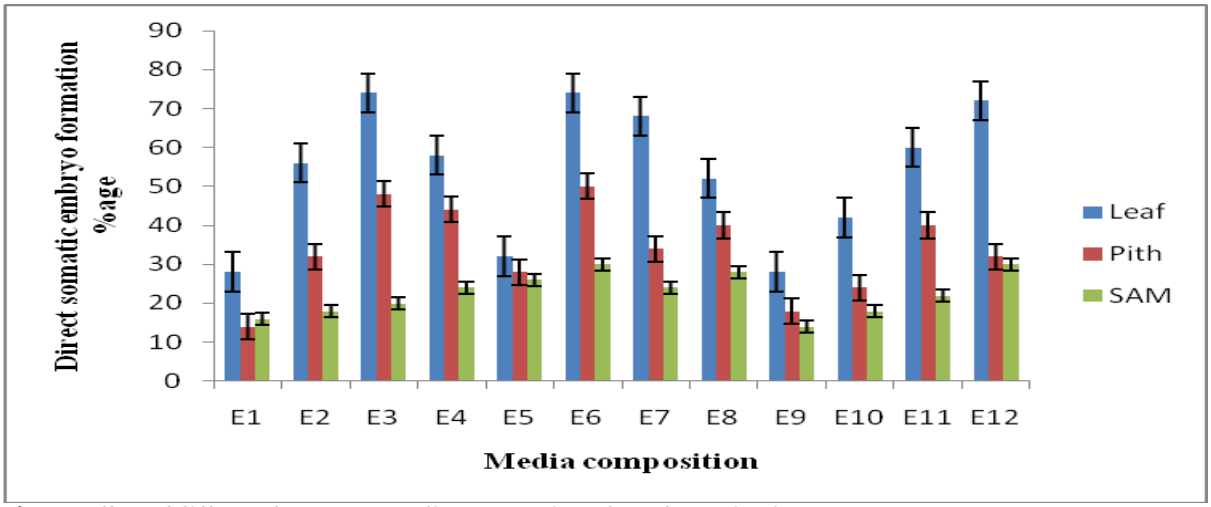

Fig. 2. Effect of different hormones on direct somatic embryo formation in sugarcane.

Varying concentrations of 2, 4-D and kinetin were also supplemented in MS medium for somatic embryogenesis (Table 2; Fig 2). Any increase or decrease in concentration of 2, 4-D and kinetin showed different result for somatic embryogenesis.

Effect of Different Concentrations of Auxins, Auxins-Cytokinins and Cytokinin- Cytokinin Combination on Indirect Somatic Embryogenesis in Sugarcane

MS medium supplemented with different concentrations and combinations of auxins, auxins-cytokinins and cytokinin-cytokinin was used for initiation of indirect somatic embryogenesis in sugarcane.

Among various concentrations of 2, 4-D supplemented in MS medium for indirect somatic embryo induction 1 mg/l 2, 4-D (S_1 medium) provided maximum results. At this concentration the rate of somatic embryo induction was 86%, 74% and 40% from callus obtained from leaf, shoot apical meristem and pith explants. MS medium with various concentration of 2, 4-D + BAP was used, the concentration 1.0 mg/l 2,4-D + 0.25 mg/l BAP (S_5 medium) showed 92%, 74%, and 62% embryo induction from leaf, shoot apical meristem and pith explants (Table 3; Fig 3).

The findings of Jahangir *et al.* (2010) also demonstrate our study who reported that auxin-cytokinin combination like 2, 4-D with BAP found very effective for somatic embryo induction.

MS medium with 2, 4-D and Kinetin was also used and the concentration 1.0 mg/l 2, 4-D and 0.25 mg/l Kinetin (S_9 medium) showed 60%, 50% and 42% embryo induction from leaf, apical shoot meristem and pith explants.

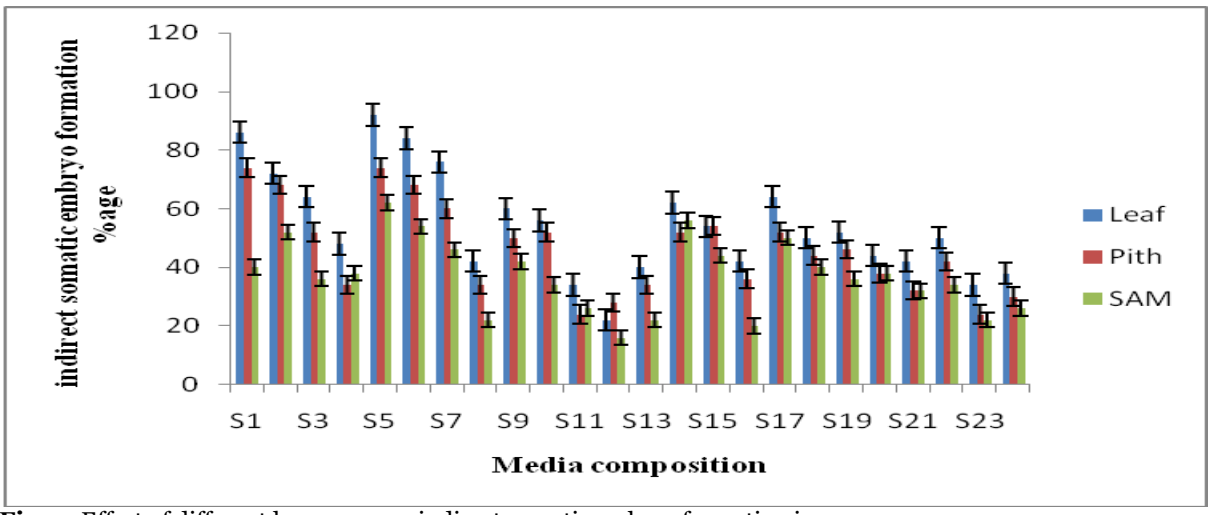

Fig. 3. Effect of different hormones on indirect somatic embryo formation in sugarcane.

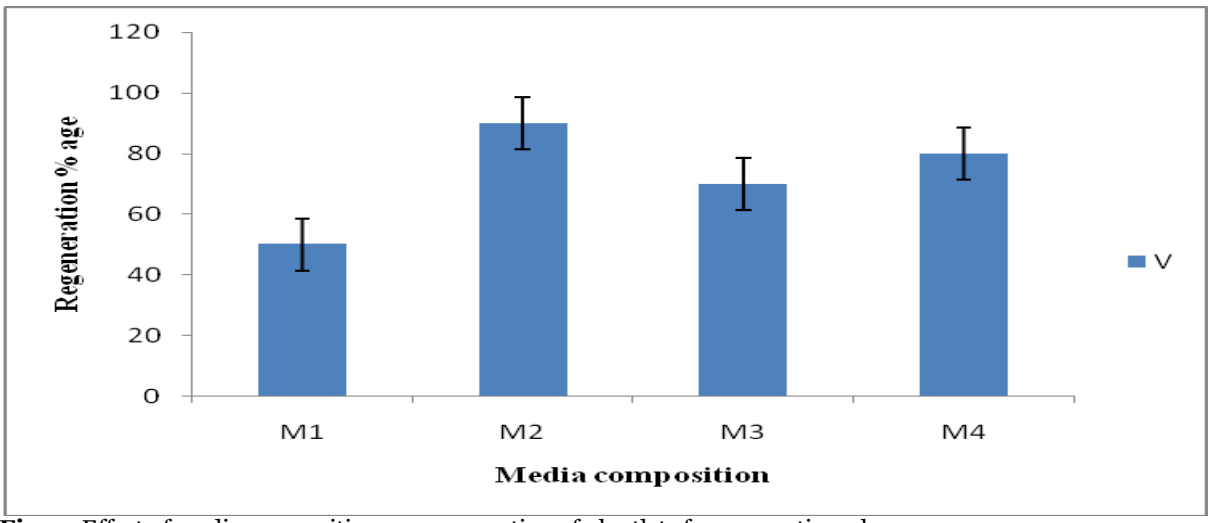

Fig. 4. Effect of media composition on regeneration of plantlets from somatic embryos.

Two types of cytokinin (BAP + Kinetin) were also used to study their effect on indirect somatic embryogenesis (Table 3; Fig 3) and 0.25 mg/l BAP + 0.25 mg/l kinetin (S_{17} medium) provided satisfactory results.

At this concentration the response for embryo induction was 64% from leaf, 52% from shoot apical meristem and 50% from pith explants. Different concentrations of NAA and IAA combination with BAP were also tried for embryo induction from different explants used.

The concentrations of NAA + BAP showed good results but concentrations of IAA + BAP was failed to show good results.

Latif *et al.* (2007) also reported similar findings that 1AA and IBA were ineffective, and did not produce any embryogenic callus, NAA stimulated the formation of low frequency of embryogenic callus and addition of 2, 4-D was very much effective in the induction of embryogenic callus.

Fig. 5. Well developed friable callus formed in light on MS medium containing 3mg/l 2, 4-D after 14 days of inoculation. (5X).

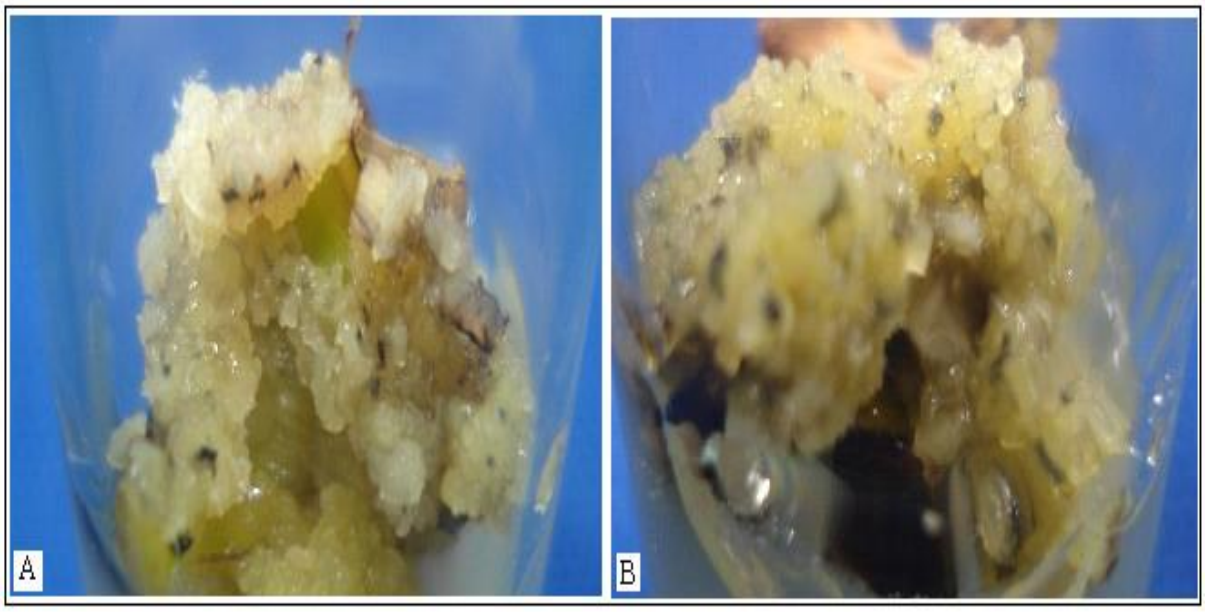

Fig. 6. (A & B). Well developed friable callus formed in dark on MS medium containing 4mg/l 2, 4-D+ 0.25mg/l BAP after 14 days of inoculation. (5X).

Effect of Media Composition on Regeneration of Plantlets from Somatic Embryos of Sugarcane

For regeneration of plantlets from somatic embryos, basal medium was used alone and along with different combinations of auxins and cytokines at different concentrations.

The number of regenerated plants was recorded per culture after eight-week of culture period. The best results for regeneration of somatic embryos were found on MS media supplemented with 4 mg/l 2, 4-D+ 0.25 mg/l BAP, where 90% plantlets formation was recorded (Table 4) (Fig. 8E & F).

Among different combinations of BAP and Kin better response was observed with M_4 medium i.e. MS medium supplemented with 1.0 mg/l of BAP and0.25 mg/l of Kinetin. Ali *et al.* (2010) reported regeneration of plants from somatic embryos.

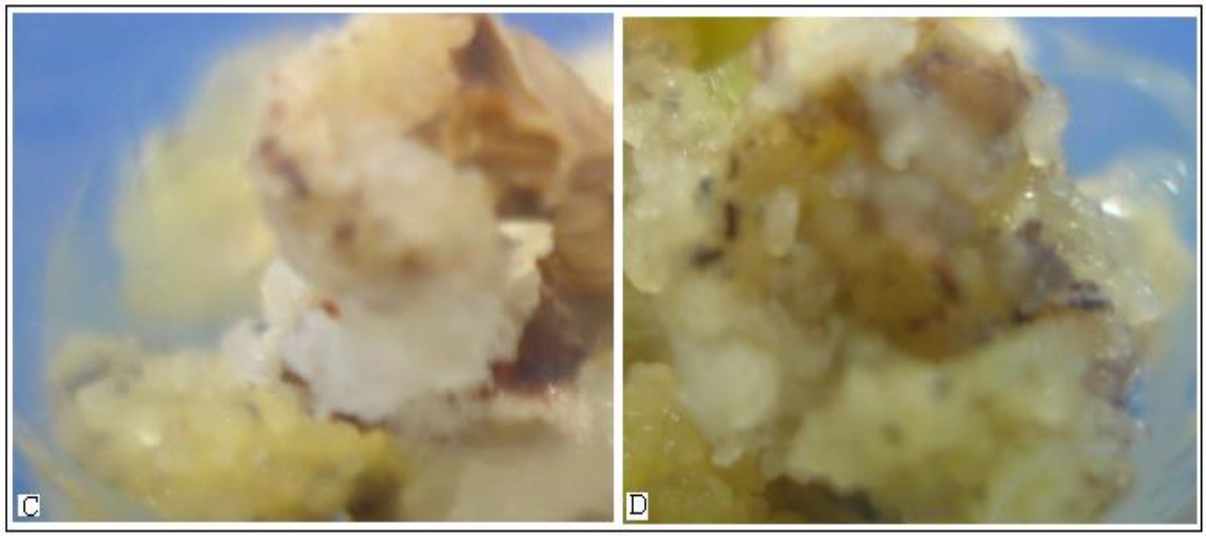

Fig. 7. (C & D). Initiation of proembryoids from well developed friable callus after 7 week of inoculation on MS medium containing 3mg/l 2,4-D+ 0.5mg/l BAP. (20X).

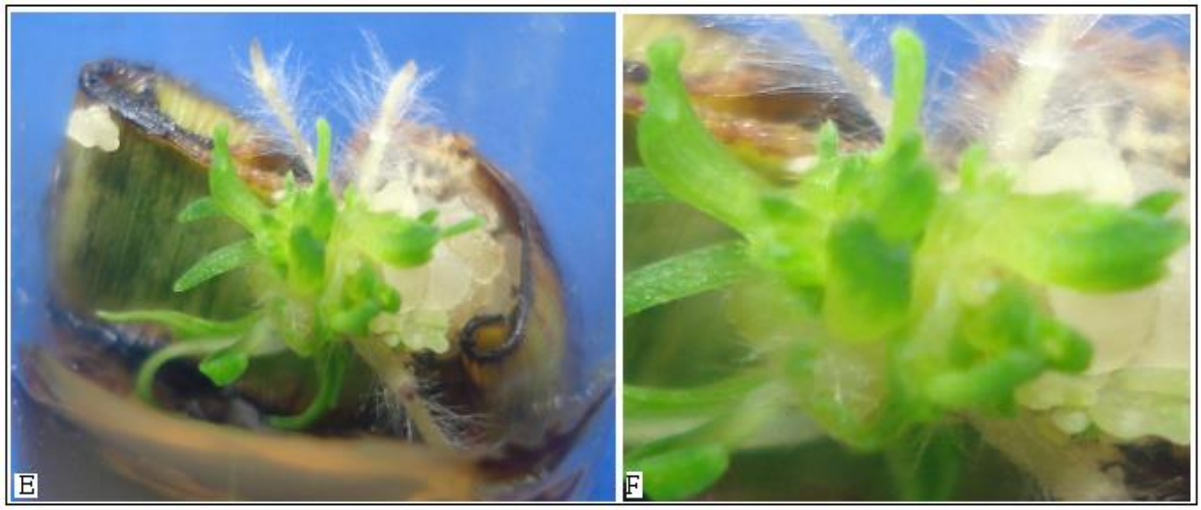

Fig. 8. (E & F).Plant regeneration from somatic embryos after 8 weeks of inoculation on MS medium containing 4mg/l 2, 4-D+ 0.25mg/l BAP. (10X).

Conclusion

Summarizing the main findings it is concluded that 2,4-D is more potent to callus initiation as compared to other hormonal combinations.

Direct somatic embryo formation was reported maximum in 2,4-D used alone or in combination with cytokinins. Indirect somatic embryos induction was obtained by sub culturing the callus on different media. The present study reported the high percentage of callus, somatic embryos and plantlet formation from different explants in sugarcane. The present established protocol can be used further for genetic improvement of sugarcane.

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