EFFECT OF MEDIUM COMPOSITION ON CALLOGENESIS AND SOMATIC EMBRYOGENESIS IN DIFFERENT VARIETIES OF SUGARCANE (*SACCHRUM OFFICINARUM* L.) I.E. S-2002-US-302, HSF-240 AND HSF-242

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ABSTRACT

Segments from inner young leaf whorls (of 3-9mm length) of sugarcane were cultured on MS growth medium supplemented with auxin alone i-e. 2,4-D (1-4 mg/l) and auxin-cytokinin combinations in different ratios. Leaf tissue explants had great potential for callus induction, somatic embryoid induction and supported plant regeneration. After inoculation embryogenic callus (of varied texture and color) was formed while in some cases direct embryoid emergence on cut edges of explant was also exhibited. Among MS media with auxin concentrations 2,4-D 3mg/l was more appropriate for callus and somatic embryoid induction as well as plant regeneration in all three varieties. Each variety showed different response to different ratios of auxin-cytokinin combinations.2,4-D+BAP (2+1, 2+2 & 2+3mg/l) and IAA+BAP (2+2 & 2+3mg/l) were more appropriate for callus and embryo induction and plant regeneration.
Abbreviations: BAP: 6-Benzyl aminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid, IAA: Indole acetic acid, MS: Murashige and Skoog's growth medium, EDTA: Ethyline diamine tetra acetate.

Key words: Sugarcane, *Saccharum officinarum*, Callogenesis, Somatic embryogenesis. Leaf whorls as explants

INTRODUCTION

Sugarcane is a member of family *Gramineae* and is commonly farmed, well known and important cash crop of Pakistan. It is cropped in Pakistan mainly for sugar production. It is second largest cash crop of Pakistan after cotton and the multibillion sugar industry of Pakistan entirely based on sugarcane crop.

Sugarcane is an important source of income and employment for the farming community of Pakistan along with sugar and sugary production. It also forms essential items for industries like sugar, chip board, paper, baggase, confectionary, and use in chemicals, plastics, paints, synthetics, fiber, insecticides and detergents (Alam & Khan, 2001).

It is cultivated on about 7.009 million hectares with 48.8 tons per hectare average yield and 8.33% sugar recovery. This is far below the world average of 63.7 tons per hectare yield and 10.6% sugar recovery (PSMA Annual report-2005). There may be many reasons for low cane production and sugar recovery but lack of extensive research studies in sugarcane crop technology & contamination of elite, transgenic varieties during multiplication by common methods are major causes. Yield decline due to pathogen is most threatening factor.

Production losses in cane crop due to plant diseases can be stopped or lowered by cropping sugarcane varieties with good agronomic characteristics i.e. tolerant to pests, diseases and

weather fluctuations. Disease tolerant or disease resistant varieties, produced by biotechnology technique can be rapidly multiplied (*in vitro* propagated) by plant tissue culture technique. Callogenesis, organogenesis and micropropagation techniques can be used to multiply transgenic elite species in very short time. These techniques also eliminate the threats of disease entry in a species during multiplication.

Sugarcane propagation by tissue culture technique is well appropriate because the main method of plant production is vegetative in nature by stem cuttings called 'setts'.

To get benefit from tissue culture technique, in this research work three varieties (S-2002-US-302, HSF-240 & HSF-242) of sugarcane were *in vitro* propagated through somatic embryoids via intervening callus phase.

MATERIALS AND METHODS

Plant material: The explants of three varieties S-2002-US-302, HSF-240 and HSF-242 were obtained from Ayub Agriculture Research Centre, Faisalabad. Inner whorls of leaves were used as explants. The field collected plant material was washed several times with tap water with a few drops of liquid soap followed by rinsing with autoclaved double distilled water. The outer mature leaves were removed carefully the explant was surface sterilized to escape the threat of contamination and innner leaf whorls of 3-9 mm were excised and inoculated on MS (Murashige and Skoog, 1962) medium supplemented with different ratios of auxins and cytokinins each singly and in combinations to find the optimum growth medium for *in vitro* propagation of sugarcane via callogenesis and somatic embryogenesis.

Media formulation: Growth medium was prepared on MS formulation (MS basal salts Macronutrients, Micronutrients, Iron EDTA and vitamins) with 3% sucrose and the media was gelled with 1% Difco-Bacto agar after adjusting the pH at 5.5 to 5.7. The media was autoclaved at 121°C for 20 min at 105 kPa and was inoculated with explants in complete aseptic cinditions. The cultures were incubated at 23±2°C under 16-18hr light period (from fluorescent light tubes) with 6-8hr dark period with light intensity of 3000-4000 lux.

Cultures were shifted to fresh media, regularly after three weeks, either with same supplementation or with different. Proliferation rate and percentage of callus formation and somatic embryoid formation was noted after each subculturing.

Callus induction: To study the sugarcane propagation via callogenesis, leaf tissue explants of all varieties were cultured on MS media supplemented with four different concentrations of auxin alone (in concentrations of 2,4-D 1- 4mg/l) and auxins in combination with cytokianins i-e. 2,4-D & BAP (1+1, 2+1, 2+2, 2+3 & 1+2 mg/l) and BAP & IAA (2+2 & 3+2 mg/l). Data about percentage of callus, form and color of callus was recorded after three weeks of incubation and the time (in days) to induce callus formation was noted for each medium separately.

Somatic embryoid induction and plantlet formation:

Direct somatic embryogenesis: In this study, leaf tissue explants were cultured on MS medium supplemented with different concentrations of auxin (2,4-D 1-4mg/l) and auxin-cytokinin combinations in different ratios i-e. 2,4-D & BAP (1+1, 2+1, 2+2 & 2+3 mg/l) and BAP & IAA (2+2 & 3+2 mg/l). Time taken (in days) to produce direct somatic embryos without intervening phase of callus was noted and the percentage of direct somatic embryo induction and plant regeneration from somatic embryos was calculated after six weeks of incubation in all three varieties.

Indirect somatic embryogenesis: However indirect somatic embryogenesis via intervening phase of embryogenic callus and number of regenerated plants were observed on the same type of culture medium and after shifting into new selected media with supplementations different from initial culture medium (auxin-cytokinin combinations). Percentage of indirect embryos induction and plant regeneration was calculated after ten weeks of incubation.

The embryos produced were germinated into roots and shoots after certain weeks of inoculation. To mediate plant regeneration through somatic embryos different media containing different concentrations of hormones and their combinations were used and percentage of plant regeneration was recorded.

Subculturing: Cultures were shifted to fresh media, regularly after three weeks, either with same supplementation or with different. Proliferation rate, percentage of callus and somatic embryoid formation was noted after each sub culturing.

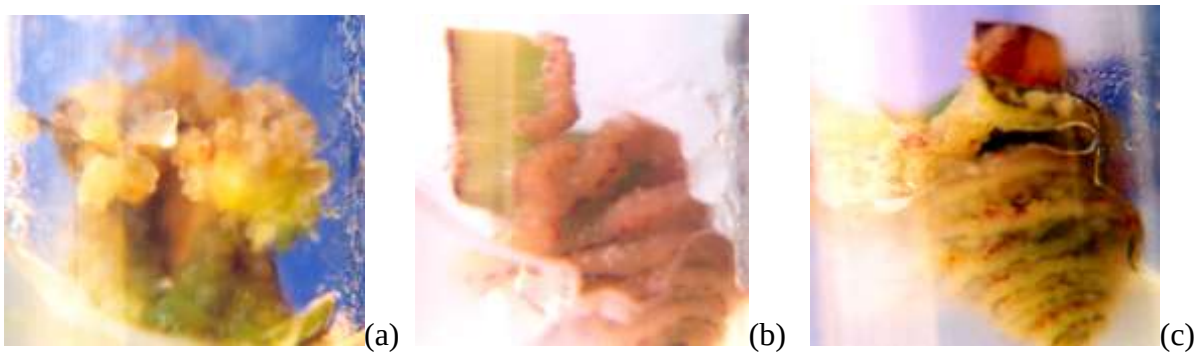
RESULTS AND DISCUSSION

Effect of Auxin alone and Auxin-Cytokinin combinations on callogenesis

In immature leaf segments callus induction was observed within first week of incubation. HSF-242 was observed to be latent in response to callus induction as compared to other two varieties; HSF-240 responded earlier than others with higher percentage of callus formation. Among four concentrations of auxin alone (2,4-D 1-4 mg/l) used, MS medium supplemented with 2,4-D 3mg/l proved to be the best for callus response with highest percentage of callus formation (100% in S-2002-US-302 & HSF-240 and 90% in HSF-242) with least time (5,3 & 9 days respectively) taken to induce callus formation (Table-1). 14 days old calli of S-2002-US-302, HSF-240 & HSF-242 in 2,4-D 3mg/l are shown in Fig-1(a) to (c).

Many researchers like Liu *et al.,* (1980), Ho and Vasil (1983) and Virupakshi *et al.,* (2002) have also used young leaf tissues of sugarcane as explant for callogenesis. Alam *et al*., (2003) used MS with supplementation of 2,4-D (1-4 mg/l) and found 2,4-D 3mg/l more appropriate for callus induction. Kharinarain *et al*., (1996) and Mannan & Amin (1999) found MS+2,4-D 3mg/l as best medium for callusing in sugarcane. Fitch and Moor (1990), Somashekhar *et al.,* (2000), Lal, (2003) and Gandonou *et al*., (2005) maintained callus on MS medium with 2,4-D at 3 mg/l and obtained good results. Gill *et al.,* (2006) reported swelling in cultured leaf segments of sugarcane before callus induction.

Two types of auxin-cytokinin combinations (2,4-D & BAP in concentration of 1+1, 2+1, 2+2 & 2+3 mg/l and BAP & IAA in 2+2 & 3+2 mg/l) were tested to check the potential of sugarcane for callogenesis. S-2002-US-302 responded better to



(a) (b) (c)

Fig.1 (a) callus of S-2002-US-302, (b) callus of HSF-240 & (c) callus of HSF-242 (all are 14 days old).

MS medium supplemented with IAA+BAP 2+3mg/l than any other combination. In HSF-240 & HSF-242 best callusing responses were exibited by 2,4-D & BAP in ratios of 2+2mg/l and 2+1mg/l.

Similar work was done by Kumari (2000) and has reported callus formation in sugarcane on MS basal medium with various concentrations and combinations of different auxins and cytokinins. Of the different media tested, the best response was found on MS with BAP + 2,4-D 1+2 mg/l for callus formation.

Significant variability was observed in the form and colour of callus even in viability (embryogenic calli produced plenty of plants through somatic embryoids) and proliferation rate (the rate with which explant turned into callus tissue) of calli of different varieties developed in growth medium with different supplementation as a result of hormonal action (detailed observations are given in (Table-1).

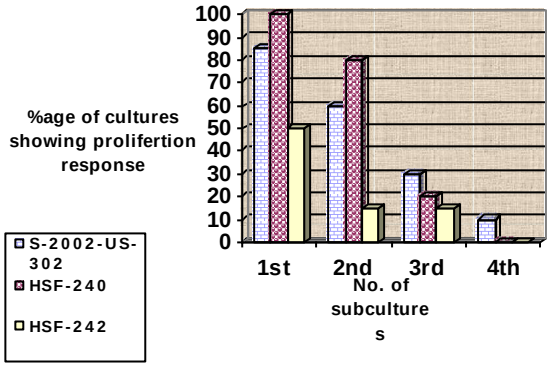
Many researchers have reported variety of callus colors and texture types in many varieties of sugarcane on different media from different explants i-e. Guiderdoni and Demarly (1988) found nodular and friable, Zhou *et al*., (1995) reported compact and granular, Escalona *et al.,* (1995) observed compact and nodular, Fitch and Moore (1990) noted white and green colored and Anbalagan *et al.,* (2000) reported (a) loose, friable and non embryogenic, and (b) compact, white, nodular and emryogenic calli.

Response to growth media with different supplementations lead to make two assumptions: first that the time taken by explant to induce callus formation goes on decreasing with the increase in concentration of auxin in medium upto specific concentration (3 mg/l) i-e, variety S-2002-US-302 took 7, 6, 5 and 9 days, variety HSF-240 took 9, 7, 3 and 5 days and variety HSF-242 took 15, 15, 9 and 11 days in 2,4-D 1mg/l, 2mg/l, 3mg/l & 4mg/l respectively (Table-1); second that auxins alone were more effective to produce fresh and viable calli as compared to auxins in

combination with cytokinins i-e, percentage of callus induction was higher in auxins than auxins+cytokinins (Table-1).

Effect of subculturing on callus proliferation rate

The calli formed from leaf tissue explant of all varieties, called as the main cultures, were subcultured on fresh MS medium with same supplementation as the main cultures, after every three weeks upto four subcultures and the data of observations was collected upto fifteen weeks of incubation. When the calli (the main cultures), formed in different media, were subcultured they proliferated at their maximum rate after first and second subculture but turned brown, at the end of third subculture and died after fourth subculture. The argument is supported with the percentage of cultures those showed positive response (proliferated better than before subcultured) i-e variety S-2002-US-302 showed 85, 60, 30 & 10%, variety HSF-240 showed 100, 80, 20 & 0%, and variety HSF-242 as 50, 15, 15 & 0% proliferation after first, second, third and fourth subculture respectively (Text fig.1).



Text fig-1 Percentage response of subculturing on callus proliferation in varieties S-2002-US-302, HSF-240 & HSF-242

Effect of Auxins alone and Auxin-Cytokinin combinations on direct somatic embryogenesis:

Initially the explants (young leaf segments) developed nodular outgrowths on the cut edges when cultured on MS medium, which transformed into pro-embryoids. These pro-embryoids developed into well-developed bipolar embryos within six weeks of incubation.

Direct embryogenic response was observed within 12-35 days of incubation in four different concentrations of 2,4-D. Pro-embryos were observed at cut edges of leaf tissue (Fig.2) in 2,4-D 3mg/l within 12 and 18 days after incubation with 40% and 20% embryo induction (Table-2) in varieties S-2002-US-302 and HSF-240 respectively. In other concentrations of 2,4-D embryos induced later than 2,4-D 3mg/l and didn't support plenty of plantlets. The same behaviour was true for both varieties. In variety HSF-242 no embryo induction was observed directly rather whole of the explant cultured turned into embryogenic callus.

Fig.2 Direct embryogenesis in S-2002-US-302 in 2,4-D 3mg/l.

Among hormonal combinations, embryo induction was observed within 16-39 days and 2,4-D+BAP 2+1 mg/l found most appropriate with 5% and 50% embryo induction within 39 and 16 days in S-2002-US-302 and HSF-240 respectively. 2,4-D+BAP 2+2 mg/l, induced embryoids within 22 and 37 days with 5% and 10% in S-2002-US-302 and HSF-240 respectively (Table-2). IAA+BAP combination didn't support direct somatic embryos at all in any variety.

Some other researchers have also used young leaf segments as explant for embryogenesis i-e. Gill *et al*., (2006) used young leaf segments. Manickavasagam and Ganapathi (1998) also used leaf segments and cultured on MS media supplemented with auxins alone (2,4-D 1-4mg/l) and in combination with 0.5, 1.0 & 2.0mg/l BAP. He found that 2,4-D+BAP 2+1mg/l among different combinations was best for direct embryogeesis.Niaz & Quraishi (2002) and Franklin *et al*., (2006) obtained considerable results in 3-mg/l 2,4-D for embryogenesis.

Effect of Auxin alone and Auxin-Cytokinin combinations on indirect somatic embryogenesis:

In variety S-2002-US-302 MS medium supplemented with 2,4-D 1- 4 mg/l was used to investigate the effect of auxins alone on indirect somatic embryogenesis. In all concentrations of 2,4-D, 1-4mg/l the calluses were observed to induce somatic embryos in main culture (before first sub-culture) within 16, 15, 14 & 20 days with 25, 50, 100 and 30% embryo induction respectively (Table-3) but these embryos died with passage of time in successive subcultures and showed no plant regeneration except in 2,4-D 3mg/l that seemed more appropriate and produced plenty of plants Fig.3 (a) shows brown colored somatic embryoids on yellowish green callus and root emergence is very clear and (b) shows shoot emergence through somatic embryoids.

Fig.3 (a) Somatic embryoids in 21 days old culture in S-2002-US-302 in 2,4-D 3mg/l (b) plant emergence through somatic embryoids in same medium in 40 days old culture.

Among diferent auxin-cytokinin combinations 2,4-D+BAP (2+3 mg/l) and IAA+ BAP (2+3mg/l) showed best results. In 2,4-D+ BAP (2 + 3 mg/l) embryo formation started after 14 days of incubation with 65% embryo formation (Table-3).

In HSF-240 2,4-D 3mg/l was more appropriate among 2,4-D 1-4mg/l for embryo induction (70 %) and took least time (among auxins alone) to induce somatic embryoids (second day after 1st subculture and 23 days of incubation) but these were not viable for plant regeneration. 2,4-D+BAP 2+1 and 2+2 mg/l media proved best with 90 and 100% induction of most viable embryoids within 20 & 18 days (Table-3), Fig.4 (a) shows yellow colored nodular embryos and Fig.4 (b) plantlet emergence through these embryoids. IAA+ BAP (1+2 and 2+2 mg/l) also showed good results.


(a)

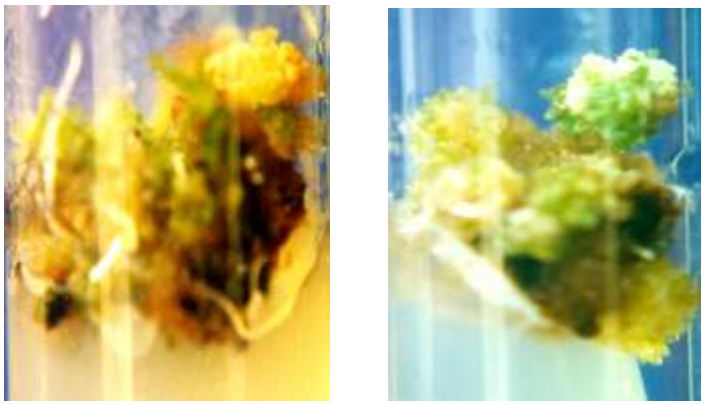

(b)

Fig. 4 (a) Somatic embryoids in 50 days old culture of HSF-240 in 2,4-D+BAP (2+2) mg/l, (b) plant emergence through somatic embryoids in same medium in 70 days old culture.

In HSF-242, in contrary to rate of callus formation the rate of embryo induction and shoot formation was too slow in different concentrations of 2,4-D as compared to auxin-cytokinin combinations. None of the culture was observed to induce somatic embryos before subculture (Table-3). 2,4-D+BAP (2+2 & 2+3 mg/l) combinations proved best for embryo induction (30 & 35% respectively, Table-3) and plant regeneration in this variety (Fig.5 a-b).

It was infered that the potential of leaf tissue explants for somatic embryo induction and plant regeneration was found to be very high in all varieties, cultured on MS medium supplemented with auxin-cytokinin combination and least in auxin alone except S-2002-US-302 which showed very good percentage, rate of embryo induction and plant regeneration in auxin (2,4-D) also. Direct embryogenesis showed rapid differentiation than that of indirect but the rate of survival was higher in plants generated through indirect somatic embryogenesis.

Some other researchers i-e. Niaz and Quraishi (2002), Gill *et al.*, (2004) and Ahloowalia & Maretzki (1983) have also found that among auxins alone 2,4-D 3mg/l is most appropriate medium for indirect somatic embyogenesis and plant regeneration in sugarcane.Ho and Vasil (1983) has used young leaves of sugarcane for somatic embryogenesis on MS medium with 2,4-D.



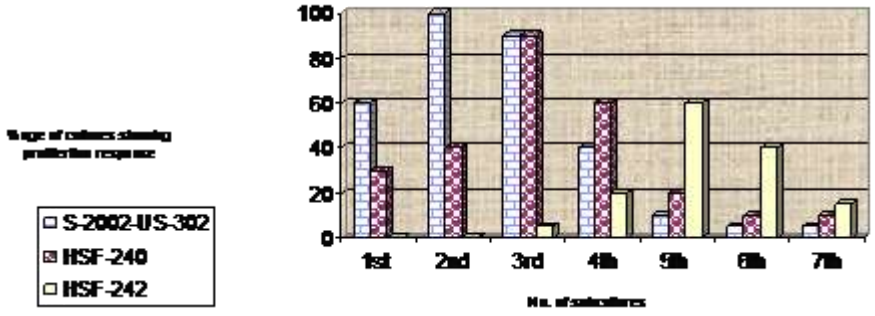
(b)

Fig.5 (a) Somatic embryoids in 105 days old culture of HSF-242 in 2,4-D+BAP (2+2) mg/l (b) plant emergence through somatic embryoids in 2,4-D+BAP (2+3) mg/l in 155 days old culture.

Ahloowalia and Maretzki (1983) reported somatic embryogenesis on MS medium with 3mg/l 2, 4-D after 10 weeks of culture. Effect of auxin-cytokinin combination on indirect somatic embryogenesis was studied by Manickavasagam and Ganapathi (1998) and they used 2, 4-D alone (1-4mg/l) and in combination with BAP (0.5, 1.0 & 2.0mg/l) and found best results with 2, 4-D+BAP (2+1 mg/l).

Effect of sub-culturing on somatic embryogenesis and plant regeneration

Sub-culturing strongly affects the somatic embryogenesis and rate of plant regeneration through somatic embryoids. Auxin (2,4-D) at the concentration of 3mg/l formed calli with highest rate of proliferation and good percentage but these calluses didn't induce any embryo or plantlet formation, even after several subcultures on medium of same supplementation. These calluses were sub-cultured and shifted to new media with different supplementations i.e. supplemented with auxin-cytokinin combinations. It was generally observed that sub-culturing favors embryo induction and plant regeneration especially when cultures were transferred from auxin alone (2,4-D) to auxin-cytokinin combination i.e. 2,4-D + BAP (1+1, 2+1, 2+2, 2+3mg/l) and IAA+BAP (2+2, 2+3 mg/l) in S-2002-US-302, 2,4-D + BAP (3+1, 1+1, 2+1, 2+2 mg/l)in HSF-240 and HSF-242. Two combinations, 2, 4-D + BAP (2 + 1, 2 + 2 & 2 + 3) and BAP + IAA (2 + 1 & 2 + 2) showed best results.



Text fig-2 Percentage response of sub-culturing on embryo induction and plant regeneration in varieties S-2002-US-302, HSF-240 & HSF-242

In S-2002-US-302 1st, 2nd and 3rd with 60, 100 and 90% response, in HSF-240 3rd and 4th with 90 & 60% (Fig.6 a & b) and in HSF-242 5th and 6th with 60 & 40% (Fig.7 a & b) subcultures were more effective to enhance the rate of embryogenesis and plant regeneration (Text fig.2) .


(a)

(b)

Fig.6 Effect of subculturing on somatic embryoids and plant formation in HSF-240 (a) Response to 3rd subculture 70 days old culture in 2,4-D+BAP 2+1mg/l (b) Response to 4th subculture 85 days old culture in same medium.


Fig.7 Effect of subculturing on somatic embryoids and plant formation in HSF-242 (a) Response to 5th subculture 120 days old culture in 2,4-D+BAP 2+2mg/l (b) Response to 6th subculture 145 days old culture in same medium.

Table-1 Effect of different concentrations of phytohormones and combinations on callus initiation and its development in variety S-2002-US-302, HSF-240 and HSF-242

Medium	Conc. mg/l	No. of explant cultured	Age of culture (weeks)	Days to callus initiation after incubation			%age of callus induction			Proliferation rate			Color of callus			Form of callus			Viability		
Varieties →				302	240	242	302	240	242	302	240	242	302	240	242	302	240	242	302	240	242
MS+2,4-D	1.0 2.0 3.0 4.0	20 20 20 20	3 3 3 3	7 6 5 9	9 7 3 5	 15 15 9 11	30 35 10 0 45	15 20 10 0 60	35 40 90 25	P E E G	P F E F	P P F G	B G Y B B	B D Y B Y Y	B Y B P Y Y	F-C LC LF LC	LF LF LF F	LC-LF LC-LF LF-NF NF	N V L V V V N V	N V L V V V L V	N V N V V L V
MS +2,4-D+ BAP	1+1 2+1 2+2 2+3 1+2	20 20 20 20 20	3 3 3 3 3	10 8 5 10 -	- 4 7 3 5	12 10 9 18 -	30 45 40 20 -	- 85 90 95 35	25 60 45 25 -	P F F F -	- G E F P	F P P P -	D G FG B- Y G Y -	- O Y FY Y LB	F Y F Y Y Y -	LF LF LF NF -	- L. F N. F F L. F	NF NF LC-NF LC-NF -	L V V V L V -	- V V V L V N V	N V V V V N V -
MS +IAA+ BAP	2+2 2+3	20 20	3 3	 14 20	- -	- -	40 90	- -	- -	F E	- -	- -	D B G Y	- -	- -	LF NF	- -	- -	V V V	- -	- -

Abbreviations:

Conc. concentration

B: brown DB: dark brown LC-LF: less compact to less friable

P: poor YB: yellowishbrown FG: fresh green

LF-NF: less friable to nodular friable F: fair PY: pale yellow

DG: dark green LC-NF: less copact to nodular friable G: good

Y: yellow OY: orange yellow NF: nodular friable

E: excellent FY: fresh yellow BY: brownish yellow

GY: greenish yellow B-Y: brown to yellow DY: dark yellow

BY: brownish yellow

Table-2 Effect of different concentrations of phytohormones and combinations on direct embryogenesis in variety S-2002-US-302, HSF-240 and HSF-242

Medium	Conc. mg/l	No. of explant cultured	Age of cultures (weeks)	Days to embryo induction after incubation			%age of embryo induction		
Varieties →				302	240	242	302	240	242
MS+2,4-D	1.0	20	6	18	25	-	5	5	-
	2.0	20	6	20	29	-	5	5	-
	3.0	20	6	12	18	-	40	20	-
	4.0	20	6	35	31	-	5	5	-
MS+2,4-D+BAP	1+1	20	6	-	-	-	-	-	-
	2+1	20	6	39	16	-	5	50	-
	2+2	20	6	22	37	-	5	10	-
	2+3	20	6	-	-	-	5	-	-
MS+IAA+BAP	2+2	20	6	-	-	-	-	-	-
	2+3	20	6	-	-	-	-	-	-

Table-3 Effect of different concentrations of phytohormones and combinations on callus initiation and its development in variety S-2002-US-302, HSF-240 and HSF-242

Medium	Conc. mg/l	No. of explant cultured	Age of culture (weeks)	Days to embryo induction after incubation			Days to embryo induction after transfer			%age of embryo induction			Color of callus		
Varieties →				302	240	242	302	240	242	302	240	242	302	240	242
MS+	1.0	20	10	16	-	-	-	-	-	25	5	-	+	-	-
2,4-D	2.0	20	10	15	(50)	-	-	8(2)	-	50	55	-	+	+	-
	3.0	20	10	14	(23)	-	-	2(1)	-	100	70	-	3+	2+	-
	4.0	20	10	20	(40)	-	-	19(1)	-	30	60	-	+	+	-
MS+	1+1	20	10	(44)	-	-	-	-	-	35	-	-	0	-	-
2,4-D+BAP	2+1	20	10	(37)	20	(150)	2(2)	-	3(7)	45	90	15	+	3+	+
	2+2	20	10	(25)	18	(110)	16(1)	-	15(5)	20	100	30	+	3+	2+
	2+3	20	10	14	-	(108)	4(1)	-	3(5)	65	-	35	3+	-	2+
	3+1	20	10	-	(50)	-	-	8(2)	-	-	40	-	-	0	-
MS+	1+1	20	10	-	(91)	-	-	13(4)	-	-	40	-	-	+	-
IAA+BAP	2+1	20	10	-	(96)	-	-	18(4)	-	-	40	-	-	+	-
	3+1	20	10	-	(106)	-	-	1(5)	-	-	20	-	-	+	-
	1+2	20	10	-	(31)	-	-	10(1)	-	-	70	-	-	2+	-
	2+2	20	10	(37)	(23)	-	16(1)	2(1)	-	55	90	-	2+	3+	-
	2+3	20	10	(27)	-	-	6(1)	-	-	70	-	-	3+	-	-

Abbreviations:0: less than previous +: same as before subculturing
2+: doubled than previous 3+: tripled than previous
Days to embryo induction after incubation: In the ( ) the age of cultures is given and represents induction after 1st subculture while without parenthesis represents before subculture.
Days to embryo induction after transfer: no. of subcultures is given in parenthesis.

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