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Int. J. Biotech Biosci, **ISSN 2231-0304**
Vol 4 (3) : 210-217 (2014)

DEVELOPMENT OF GENETIC VARIABILITY THROUGH BIOTECHNOLOGICAL INTERVENTION IN PROSO MILLET (*PANICUM MILIACEUM* L.)

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Abstract
Callus induction and in-vitro *plantlet regeneration was tried in six genotypes of proso millet through mature embryo axes as a source of explant. MS and B_5 medium supplemented with various growth regulators in different concentration and combinations were used in the present study. The callus initiation was found to be better in MS media supplemented with 2 mg/l 2,4-D alongwith 0.5 mg/l BAP followed by 3 mg/l NAA with 0.5 mg/l Kinetin. All genotypes produced friable, white and yellow colour callus in MS and B_5 media supplemented with 2, 4-D whereas in the same media supplemented with NAA produced loose light yellow and loose white colour callus. Regeneration of multiple shoots was readily achieved by transferring callus onto MS media supplemented with 4 mg/l BAP and 4 mg/l Kinetin in all genotypes. These regenerated plantlets were showed rooting on MS supplemented with 0.2 mg/l IBA alongwith 0.1 mg/l BAP and 2 per cent sucrose. Better survival percentage was observed in potting mixture of Soil, Sand and vermiculite in the ratio of 1:1:1.*

Key words: *Proso millet, Mature embryo axes, callus induction, In vitro regeneration.*

INTRODUCTION

Proso millet was the 'milium' of the Romans and the true millet of history. It contains large proportion of carbohydrate (72.9%) and thus provides bulk of energy in diets (378 kcal). It also contains fat (4.2%), protein (11%) and dietary fiber (8.5%). Apart from the major nutrients, each 100 grams of prosomillet consist of calcium 8 mg, magnesium 114 mg, phosphorous 285 mg, iron 3.0 mg etc. The essential amino-acid composition per 100 grams of edible portion is tryptophan 119 mg, methionine 221 mg, leucine 1400 mg and isoleucine 465 mg. (Anonymous, 2004).

The grains are also used for making beer, brandy and feed for animals, including pigs, fowls and cage-birds. The plant is used as forage. Starch from the grains has been used for sizing textiles (Kaume 2006).

Proso millet has high nutritional value which could be well exploited for bio-fortification of tribal, the society where maximum malnutrition and under nutrition noticed. Inspite of these added advantages this crop is overlooked because of its low productivity and non-availability of better genotypes. Local genotypes are poor yielders, having narrow genetic base and less variability. Attempt on improvement of proso millet is so far restricted to selection only due to its complicated floral biology.

Tissue culture approach has been proved effective gaining phenotypic variation in regenerated plants such as shorter plants and increased number of tillers (Eapen & George 1990; Kavi et al, 1992) In-*vitro* regeneration of proso millet is possible on Murashige and Skoog(1962) medium, using mature embryos, immature embryos and immature inflorescence (Jain et al 2001). Hence, there is enormous scope for providing improved cultivars of proso millet through biotechnological intervention. Keeping this in view the present investigation was carried out for standardization of *in vitro* callus derived regeneration and development of somaclones with variable characters.

MATERIALS AND METHODS

The six genotypes of proso millet namely, Sakharoli, Asond, Sukdhar, Gavhe, Kalsuli and Vari No.10 available at Department of Agril Botany, Dr. B. S. Konakn Krishi Vidyapeeth, Dapoli were used in the present study. Callus induction and *in-vitro* regeneration was tried by using mature embryo axes as explant. The experiments were carried out aseptically under well-defined conditions at Plant Biotechnology Center, Dr. B. S. Konkan Krishi Vidyapeeth, Dapoli- 415 712 (MS).

MS and B_5 medium supplemented with various concentration of 2, 4-D, NAA, BAP, Kinetin and IBA. Seeds were surface sterilized by 0.1 % Teepol for ten minutes followed by washing several times under running tap water. After that, seeds were quickly dipped in 70% ethanol and further immersed in 1% mercuric chloride ($HgCl_2$) for 15 minutes, followed by several washings with double distilled water. Treated seeds were soaked for six hours in double distilled water. Seeds (mature embryo axes) were inoculated on callus induction media. Observations were recorded on; Callus induction response (%), days to initiation of callus, weight of callus after 4 weeks, nature of callus, regeneration response (%), days to shooting, number of shoots per explant, days to rooting and number of roots per plantlet.

Plantlets with healthy root systems were washed (especially the root portions) by double distilled water to clear off the entire residual agar medium so as to check the chances of fungal contamination in soil. The root portions were dipped for about 5-10 minutes in an antifungal solution (0.1% (w/v) Bavistin. These rooted plantlets were transplanted to the potting mixture of soil, sand and vermiculite (1:1:1).

Factorial Completely Randomized Design (FCRD) was employed for the experiment. Data of explants from different media combinations obtained from similar experiments were pooled. The pooled data was analyzed in SASs (Statistical Analysis System software V. 9.1).

RESULTS AND DISCUSSION

Effect of genotypes

Genotype can influenced both frequency of callus induction and frequency of regeneration (Larkin and Scowcroft, 1981). Significant difference was observed for callus induction amongst the genotypes (Table 1). The highest callus induction response was recorded in Gavhe 37.08% followed by Asond 34.58% and sakhroli 32.08%. The lowest callus induction response was observed in kalsuli 31.62%. These results are in accordance with Cummings et al (1976).

Effect of growth regulator

The mean values for callus induction response in mature embryo axes are presented in Table 1. It ranged from 31.62 to 37.07 per cent. The maximum response for callusing was recorded in 2mg/l 2, 4-D concentration (63.66%) followed by 1mg/l 2, 4-D concentration (46.94%) and 0.5mg/l 2, 4-D concentration (20.55%) in all genotypes (Plate I).

The genotype Gavhe showed highest callus induction (81.66%) in 2mg/l 2,4-D concentration whereas; lower response (20.00%) was recorded in genotype Asond, Gavhe, Sakhroli, and Vari no.10 in 0.5mg/l 2,4-D. All the genotypes recorded White friable and hard creamy embryogenic callus in these media (Plate II). Eapen and George(1990); Heyser, and Nabors, 1982; Patil et al (2009)recorded somatic embryogenesis and plant differentiation in media supplemented with 2, 4-D. Characteristics of callus in terms of its colour and appearance plays an important role in determining regeneration (Mohanty et al 1985).

Similarly, maximum response for callusing was re-

corded in 3mg/l NAA (38.33%) followed by 4mg/l NAA (37.10%) and 2mg/l NAA (30.77%). The lowest (27.77%) response for callusing was recorded by 1mg/l NAA. The per cent callus induction response ranged from 23.33% to 31.66%, 23.00% to 38.33%, 31.66% to 45.00% and 33.33% to 41.61% with NAA concentration of 1, 2, 3 and 4mg/l NAA, respectively.The callus induced in NAA was loose white and loose light yellow in colour (Plate II). The similar type of results are obtained by Eapen and George (1990); Patil et al (2009).

The data regarding response to callus induction in B_5 media with 2, 4-D and NAA along with mature embryo axes explant is presented in Table 1. Mean values for callus induction response ranged from 18.12% to 21.45%. Among the different media, B_5 enriched with 2mg/l 2, 4-D recorded maximum callus induction response (35.27%) followed by 1mg/l (32.49%) and 0.5mg/l 2, 4-D (16.10%). No response for callusing was recorded when media supplemented with 0.1mg/1 2, 4-D. The per cent callus induction ranged from 13.33% to 16.66%, 30.00% to 40.00% and 33.33% to 41.66% in 0.5mg/l, 1mg/l and 2mg/l 2, 4-D, respectively. All the genotypes recorded hard creamy embryogenic callus in these media (Plate II).The similar types of results are obtained by Patil et al (2009).

As like 2, 4-D, B_5 media supplemented with various concentrations of NAA the maximum callus induction (40.27%) was observed in 2mg/l NAA concentration followed by 1mg/l concentration (33.32%). No response for callusing was recorded when media supplemented with 3mg/1 and 4mg/l 2, 4-D. Per cent callus induction response ranged from 31.66% to 36.66%, and 31.66% to 46.66% in 1mg/l and 2mg/l NAA, respectively. The callus induced in B_5 media with NAA are loose white in colour (Plate II).

Effect of genotype on days to callus induction

Genotype Gavhe took 5.68 days for callusing while genotype kalsuli took 7.69 days for callusing when mature embryo axes used as explants. Mean response for days to callus initiation ranged 5.68 to 7.69 days in both media tried. When MS media was used for recording the observation for number of days required for callusing it was observed that genotype Sakhroli took minimum 6.13 days and maximum 8.43 days were required for genotype Kalsuli. However, when B_5 media was used Gavhe showed quickest response 5.12 days and Kalsuli was least in responding taking 6.95 days for callusing. Similar observations were recorded by Patil et al (2009) in finger millet.

Effect of growth regulator on plantlet regeneration

Different growth regulators at varying concentrations resulted in causing variability in plantlet regeneration. The levels of cytokinin were significant for plantlet regeneration (Table 2). Highest response (60.41%) was observed in media containing BAP, while lowest response was observed (27.08%) in media enriched with kinetin. The range for regeneration response in BAP was 0.00 to 60.41% and that of media supplemented with kinetin was 0.00 to 47.91%. Different levels of BAP and Kinetin reported variability in regeneration response and suggesting that the presence of required level of hormones is a pre-requiste for the initiation of organogenesis *in-vitro* (Thiru and Ram 1980). The highest plantlet regeneration response (60.41%) was observed in Sakhroli while, lowest plantlet regeneration response (31.25%) was observed in Sukdhar in media containing BAP.

Similarly, Media supplemented with 4mg/l Kinetin only showed plantlet regeneration. The highest plantlet regeneration response (47.91%) was observed in Asond while, lowest plantlet regeneration response (27.08%) was observed in Vari No.10 in media containing Kinetin. Mean value for regeneration response ranged from 11.45 to 17.70%. (Plate III - Somatic embryogenesis and plantlet regeneration).Similar types of results were obtained by Patil et al (2009) and Jha et al (2009).

Effect of growth regulators on plantlet production per callus

The effect of growth regulators on plantlet production reveals that the genotype Gavhe were produced maximum (8.58) number of plantlets followed by Asond (6.50) and Sakhroli (6.33) in 4mg/l BAP concentration while, Sukdhar produced minimum (2.91) number of plantlets. Similarly, the genotypes Gavhe were produced maximum (7.91) number of plantlets followed by Asond (5.75) and Sakhroli (5.75) in 4mg/l Kinetin concentration while, Sukdhar produced minimum

(2.66) number of plantlets (Table 2).

Effect of growth regulator on Days to rooting

Supply and regulation of endogenous auxin may play a crucial role in root initiation; similarly exogenous auxins are essential for rooting (Wakizuka and Yamaguchi, 1987). In some species it is reported that reduced sugar levels (10-20 g/l) have a role in root formation (Mohanty et al 1985)

Data regarding total number of days required for rooting in different growth regulators is depicted in Fig 1. Minimum mean days (7.02) were required by media supplemented with IBA as compared to NAA (8.89) days. Mean value for days to rooting ranges from 6.81 to 8.62days. The most suitable combination which required only 5.37days to rooting was seen when IBA 0.2mg/l + 0.1mg/l BAP + 2% sucrose was supplemented whereas; 7.38days to rooting were required when ½ MS was supplemented with 0.1 IBA+ 0.1mg/l BAP +3% sucrose and 8.31days when ½ MS was supplemented with 0.1 IBA+ 0.1mg/l BAP +2% sucrose.

Similarly, NAA concentration of 2.00 mg/l required minimum days to rooting (7.13 days) followed by 1.0 mg/l NAA (9.02 days) and ½ MS + 0.5mg/l Kinetin + 3% sucrose (10.52days).These results are in conformity with Patil et al (2009) (Plate IV).

Hardening and establishment:

Rooted plants were transferred to the potting mixture of soil, sand and vermiculite 1:1:1. Maximum survival was recorded (74.17%) in Sakhroli while lower percentage of survival was observed (17.90%) in Sukdhar. Such type of genotypic variability is also recorded by Patil et al (2009).

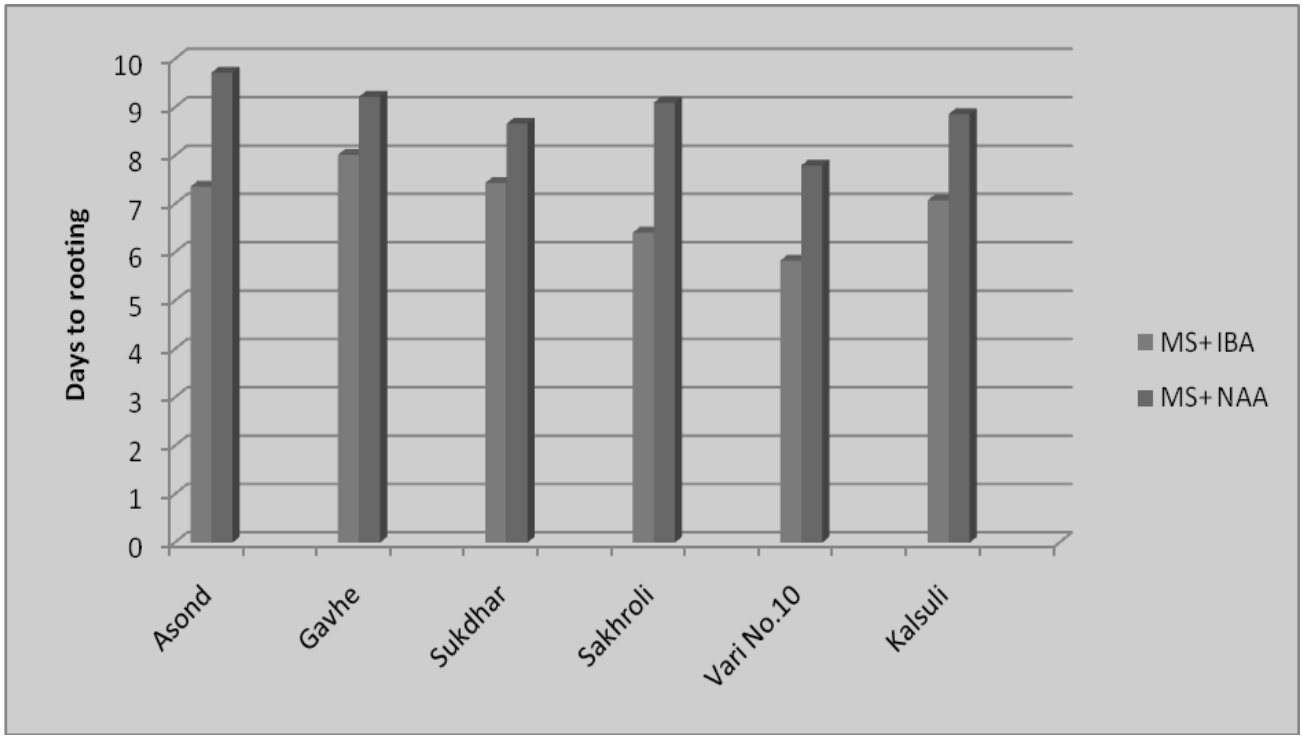


Fig 1. Genotypic variability in relation to Auxin levels (mg/l) for days to rooting

Table 1 . Genotypic variability in relation to auxin levels (mg/l) for callus induction response (%) in MS and B_5 medium**.**

Sr. No.	Genotype	MS											B_5										
		2,4-D (Mg/l)				Mean	NAA (mg/gl)				Mean	Total Mean	2,4-D (Mg/l)				Mean	NAA (mg/gl)				Mean	Total Mean
		0.1	0.5	1.00	2.00		1.00	2.00	3.00	4.00			0.1	0.5	1.00	2.00		1.00	2.00	3.00	4.00		
1	Asond	0.00	20.00	51.66	75.00	36.66	25.00	28.33	35.00	41.66	32.49	34.58	0.00	15.00	30.00	33.33	19.58	33.33	46.66	0.00	0.00	19.99	19.79
		(0.00)	(26.45)	(45.96)	(60.07)	(33.12)	(29.74)	(32.02)	(36.24)	(40.20)	(34.55)	(33.83)	(0.00)	(22.60)	(33.16)	(35.25)	(22.75)	(35.17)	(43.09)	(0.00)	(0.00)	(19.56)	(21.16)
2	Gavhe	0.00	20.00	56.66	81.66	39.58	26.66	36.66	36.66	38.33	34.57	37.07	0.00	13.33	30.00	33.33	19.16	31.66	43.33	0.00	0.00	18.74	18.95
		(0.00)	(26.45)	(48.85)	(64.69)	(34.99)	(30.76)	(37.26)	(37.26)	(38.22)	(35.87)	(35.44)	(0.00)	(21.14)	(33.16)	(35.17)	(22.36)	(33.97)	(41.16)	(0.00)	(0.00)	(18.78)	(20.58)
3	Sukdhar	0.00	21.66	40.00	58.33	29.99	31.66	23.33	45.00	33.33	33.33	31.66	0.00	18.33	31.66	35.00	21.24	36.66	31.66	0.00	0.00	17.08	19.16
		(0.00)	(27.60)	(39.21)	(49.80)	(29.15)	(34.23)	(28.86)	(42.12)	(35.22)	(35.10)	(32.13)	(0.00)	(24.81)	(34.23)	(36.24)	(23.82)	(37.20)	(34.15)	(0.00)	(0.00)	(17.83)	(20.83)
4	Sakhroli	0.00	20.00	48.33	55.33	30.91	23.33	38.33	36.66	35.00	33.33	32.12	0.00	15.00	33.33	41.66	22.49	35.00	46.66	0.00	0.00	20.41	21.45
		(0.00)	(26.45)	(44.04)	(47.88)	(29.59)	(28.67)	(38.24)	(37.20)	(36.24)	(35.08)	(32.34)	(0.00)	(22.60)	(35.17)	(40.20)	(24.49)	(36.24)	(43.09)	(0.00)	(0.00)	(19.83)	(22.16)
5	Vari No.10	0.00	20.00	45.00	53.33	29.58	28.33	35.00	31.66	41.00	33.99	31.79	0.00	18.33	40.00	35.00	23.33	31.66	40.00	0.00	0.00	17.91	20.62
		(0.00)	(26.45)	(42.12)	(46.92)	(28.87)	(31.74)	(36.27)	(34.23)	(40.20)	(35.61)	(32.24)	(0.00)	(25.00)	(39.21)	(36.18)	(25.09)	(34.02)	(39.21)	(0.00)	(0.00)	(18.30)	(27.70)
6	Kalsuli	0.00	21.66	40.00	58.33	29.99	31.66	23.00	45.00	33.33	33.24	31.62	0.00	16.66	30.00	33.33	19.99	31.66	33.33	0.00	0.00	16.24	18.12
		(0.00)	(27.60)	(39.21)	(49.80)	(29.15)	(34.23)	(28.86)	(42.12)	(35.22)	(35.10)	(32.13)	(0.00)	(23.74)	(33.16)	(35.25)	(23.03)	(34.15)	(35.11)	(0.00)	(0.00)	(17.31)	(20.18)
	Mean	0.00	20.55	46.94	63.66	32.78	27.77	30.77	38.33	37.10	33.49	33.14	0.00	16.10	32.49	35.27	20.96	33.32	40.27	0.00	0.00	18.39	19.68
		(0.00)	(26.83)	(43.23)	(53.20)	(30.81)	(31.56)	(33.58)	(38.20)	(37.55)	(35.22)	(33.02)	(0.00)	(23.31)	(34.68)	(36.38)	(23.59)	(35.12)	(39.30)	(0.00)	(0.00)	(18.60)	(21.10)

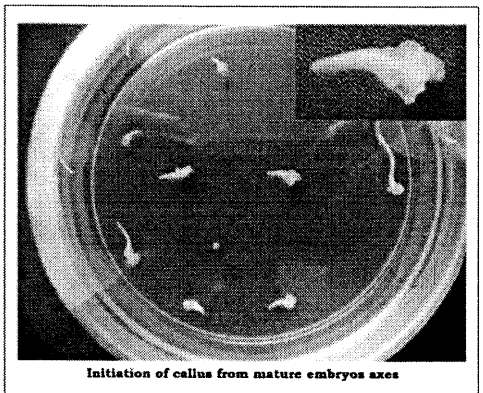
	Genotype	Auxin	Genotype X Auxin		Genotype	Auxin	Genotype X Auxin
SEm±	0.57	0.66	1.63	SEm±	0.60	0.70	1.72
C. D. at 1%	2.13	2.46	6.03	C. D. at 1%	2.25	2.60	6.37

Table 2. Genotypic variability in relation to cytokinin levels (mg/l) for regeneration response (%) and Number of plantlets per callus in mature embryo axes explant.

Sr. No.	Genotype	Regeneration response (%)								Total Mean	Number of plantlets per callus								Total Mean
		BAP (mg/l)			Mean	Kinetin (mg/l)			Mean		BAP (mg/l)			Mean	Kinetin (mg/l)			Mean	
		1	2	4		1	2	4			1	2	4		1	2	4		
1	Asond	0.00	0.00	56.25	18.75	0.00	0.00	47.91	15.97	17.36	0.00	0.00	6.50	2.16	0.00	0.00	5.75	1.91	2.04
		(0.00)	(0.00)	(48.80)	(16.26)	(0.00)	(0.00)	(43.80)	(14.60)	(15.43)									
2	Gavhe	0.00	0.00	56.25	18.75	0.00	0.00	41.66	13.88	16.31	0.00	0.00	8.58	2.86	0.00	0.00	7.91	2.63	2.74
		(0.00)	(0.00)	(48.61)	(16.20)	(0.00)	(0.00)	(40.00)	(13.33)	(14.77)									
3	Sukdhar	0.00	0.00	31.25	10.41	0.00	0.00	37.50	12.50	11.45	0.00	0.00	2.91	0.97	0.00	0.00	2.66	0.88	0.92
		(0.00)	(0.00)	(33.92)	(11.30)	(0.00)	(0.00)	(37.59)	(12.53)	(11.32)									
4	Sakhroli	0.00	0.00	60.41	20.13	0.00	0.00	45.83	15.27	17.70	0.00	0.00	6.33	2.11	0.00	0.00	5.75	1.91	2.01
		(0.00)	(0.00)	(51.20)	(17.06)	(0.00)	(0.00)	(42.59)	(14.19)	(15.63)									
5	Vari No.10	0.00	0.00	58.33	19.44	0.00	0.00	27.08	9.02	14.23	0.00	0.00	4.66	1.55	0.00	0.00	4.33	1.44	1.49
		(0.00)	(0.00)	(50.00)	(16.66)	(0.00)	(0.00)	(31.14)	(10.38)	(13.52)									
6	Kalsuli	0.00	0.00	39.58	13.19	0.00	0.00	45.83	15.27	14.23	0.00	0.00	5.50	1.83	0.00	0.00	5.08	1.69	1.76
		(0.00)	(0.00)	(38.80)	(12.93)	(0.00)	(0.00)	(42.61)	(14.20)	(13.57)									
	Mean	0.00	0.00	50.34	16.78	0.00	0.00	40.96	13.65	15.21	0.00	0.00	5.74	1.91	0.00	0.00	5.24	1.74	1.83
		(0.00)	(0.00)	(45.22)	(15.07)	(0.00)	(0.00)	(39.62)	(13.20)	(14.14)									

	Genotype	Cytokinin	Genotype X Cytokinin		Genotype	Cytokinin	Genotype X Cytokinin
SEm±	0.83	0.83	2.03	SEm±	0.07	0.07	0.19
C. D. at 1%	3.09	3.09	7.58	C. D. at 1%	0.22	0.22	0.54

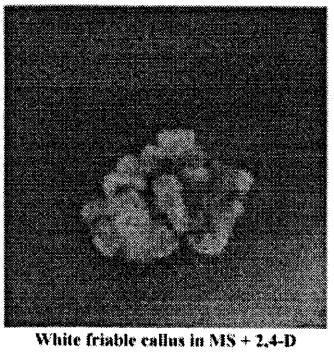
Plate I: Callus initiation in Proso millet



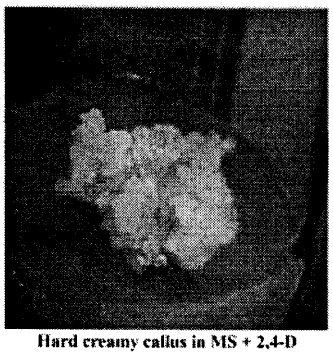
Initiation of callus from mature embryos axes

Well organized white friable callus in MS + 2 mg/l 2,4 D

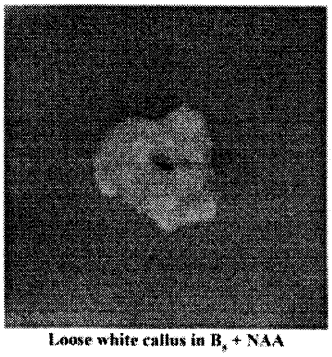
Plate II: Nature of callus



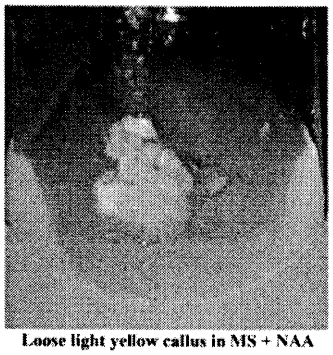
White friable callus in MS + 2,4-D



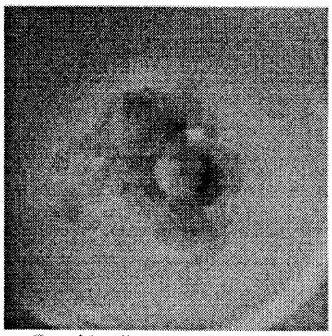
Hard creamy callus in MS + 2,4-D



Loose white callus in B_5 + NAA

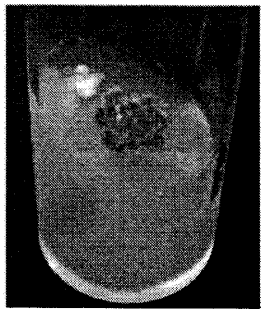


Loose light yellow callus in MS + NAA



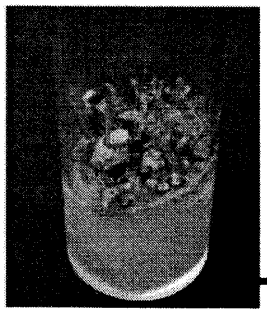
Greenish yellow callus in B_5 + 2,4-D

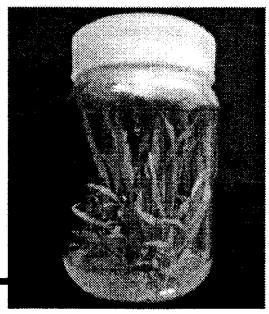
Plate III: Plantlet regeneration



Somatic embryogenisis

Initiation of plantlet





Multiple shooting

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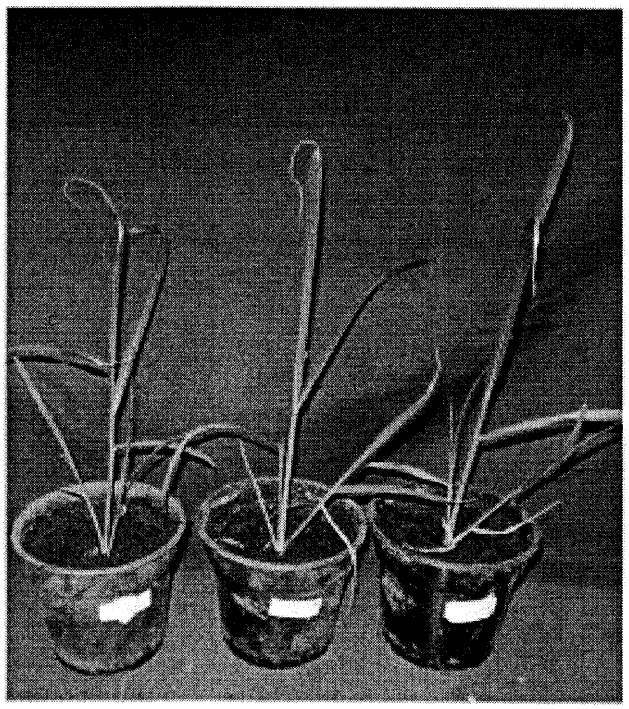
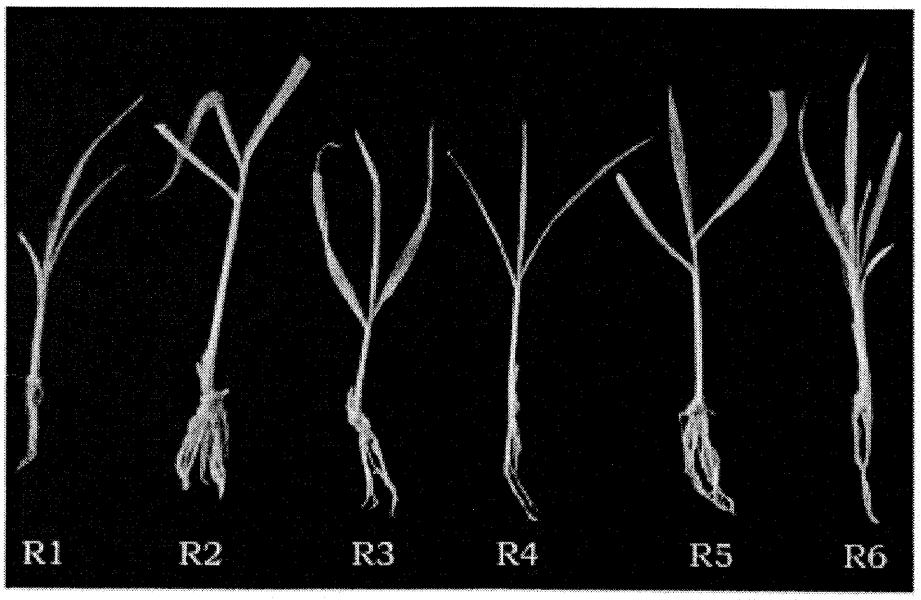
Plant IV: Rooting and hardening

In vitro rooting



Established plants of proso millet



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