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In-vitro Culture Response and Regeneration of Plantlets in Mesta

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Abstract: An attempt was made to develop a robust *in-vitro* regeneration protocol in mesta involving both the cultivated species-*H. sabdariffa* and *H. cannabinus*. The seed germination was found to be maximum (100%) in var. HS 4288 and minimum (80%) in var. HS 7910 in *H. sabdariffa* whereas in *H. cannabinus* maximum (90%) and minimum (76%) germination were observed in vars. MT 150 and AMC 108, respectively. Twenty three combinations of plant growth regulators (NAA, BAP, Kinetin, 2, 4-D, TDZ and IAA) in MS medium containing vitamins of B_5 medium were assessedin governing *in-vitro* culture response involving cotyledon and hypocotyl explants. The best callus induction was observed in case of cotyledon explant. The maximum callus proliferation (97.8%) was noticed *in-var*. HS 4288 on MS containing 2 mg L^{-1} 2, 4-D, 0.1 mg L^{-1} NAA and 2 mg L^{-1} Kinetin in *H. sabdariffa*. Whereas, in *H. cannabinus* maximum callus proliferation (66.8%) was observed in var. MT 150 on MS containing 0.8 mg L^{-1} IAA and 0.05 mg L^{-1} TDZ. The adjuvants showed good response in callus proliferation, however, no effect was observed on shootlet regeneration. The shootlet regeneration was found only in var. HS 4288 on MS medium containing 3.0 mg L^{-1} BA with 0.2 mg L^{-1} IAA and appreciable rooting on growth regulator free MS medium.

INTRODUCTION

Mesta is one of the popular lingo-cellulosic coarse bast fibre crops having discernible economic importance in India's economy. Yet, it is grown as an orphan crop on subsistence mode. Mesta grows best in well-drained sandy loam soil and requires a warm humid climate without excessive rainfall. There are >60 species of *Hibiscus* reported and in India two species are cultivated viz. *Hibiscus sabdariffa* and *Hibiscus cannabinus* belong to the family Malvaceae with chromosome number 2n = 36, 2n = 72, respectively. Mesta is grown in an area of about 1 lakh ha in India with average productivity of 10-12 q ha^{-1} summing total production amounts to 106 thousand tonnes (Source: Directorate of Economics, Statistics, DAC, GOI, Agricultural Statistics at a glance 2013). Besides fibre, mesta has also been found extensively used as an input for the manufacture of paper pulp. The fibres of mesta are conventionally mixed with jute to meet the requirements of textile and other ancillary industries. The mesta seed oil is also used for culinary purposes. The calyces of mesta are used in the preparation of jam, jellys and pickles. The major constraints of mesta are low seed viability, vulnerability to drought stress and

submergence, yields coarse fibre which is non remunerative. It is a rain fed, marginal crop grown virtually with no care, adoption of scientific and technical interventions viz. modern agronomic practices. In essence, mesta is an annual or biennial herbaceous plant (rarely a short-lived perennial) growing to 1.5-3.5 m tall with a woody base. The stem diameter ranges in between 1-2 cm, often branched. The leaves are 10-15 cm long, variable in shape with leaves near the base of the stems being deeply lobed (3-7 lobes) while leaves near the top of the stem are shallow lobed or un lobed, lanceolate. The flowers are of 8-15 cm diameter, white, yellow or purple when white or yellow, the centre is found to be dark purple. The fruit is a capsule of about 2 cm diameter, containing several seeds.

Cell technology in conjunction with genetic engineering has opened a new vista for plant genetic improvement. Development of an efficient *in-vitro* plant regeneration method is often considered as a prerequisite especially for exploitation of *in-vitro* culture induced variations or to dove tail transgene/s from diverse alien sources for transgenic development through diverse genetic transformation techniques for trait enrichment. There are number of reports on plantlet regeneration in certain species of *Hibiscus* which focused mainly on the propagation of ornamental species such as *H. syriacus*[1] and *H. rosasinensis*[2] and many species of the family Malvaceae such as *Gossypium hirsutum*[3], *Theobroma cacao*[4]. Tissue culture studies on *H. sabdariffa*and *H. cannabinus*are still now at infancy since inadequate efforts have been made and that too in limited laboratories. A direct regeneration protocol to develop transgenic plants was reported by Gassama*et al.*[5], interestingly transgenic stress tolerant roselleplants were found to be produced by a tissue culture independent method via. *Agrobacterium tumefaciens* using embryo axes with single cotyledon[6], however, the number of transgenic lines were found to be exceedingly low. Conversely, tissue culture studies on kenaf species have been widely reported from shoot apex[7-10]. Direct shootlet organogenesis from cotyledonary node[11, 12] and multiple shooting from young shoot explants of kenaf was also reported. Protoplast was isolated and cultured from leaf tissue of kenaf[13]. *In-vitro* micropropagation of kenaf was also successfully reported. *In vitro* propagation of kenaf on hormone free MS medium was earlier reported using shoot apex and nodal explants without any callus[14]. The present study describesa protocol for efficient plant regeneration from cotyledons in roselle *(H. sabdariffa).*

MATERIALS AND METHODS

Plant material: Healthy mature seeds of *Hibiscus sabdariffa* and *H. cannabinus*conserved in the Gene Bank, Division of Crop Improvement of Central Research for Jute and Allied Fibres, Nilganj, Barrackpore were employed in this study. Four varieties of *H. cannabinus*: JBM 2004, MT150, HC583, AMC108 and five varieties of *H. sabdariffa*: HS 4288, Amv1, Non Pons, HS 7910, Gr 27 were selected for the development of an efficient *in-vitro* culture protocol for plantlet regeneration.

Seed sterilization and germination: Matured healthy seeds were rinsed in tap water followed by dipping in water with 5% aqueous Teepol solution with 2 drops of Tween 20 (SRL, Cat. No. P9416) for 5 min. The seeds were washed and further soaked for 15 min in fungicide Bavistin (2% solution w/v, Carbendazim). Those seeds were subsequently washed with sterile water and surface sterilized in 70% (v/v) ethanol for 30 sec. Then the seeds were kept in 0.1% freshly prepared $HgCl_2$(Sigma-Aldrich, Cat. No. 429724) for 3 times of 10 min duration with intermittent shaking manually. Surface sterilized seeds were finally rinsed four times with distilled water to remove traces of $HgCl_2$. Those seeds were blot dried on sterile tissue paper and germinated on half-strength hormone free Murashige-Skoogmedium. The MS medium was supplemented with vitamins of B_5 medium, 2% sucrose (Sigma-Aldrich, Cat. No. 84097) and solidified with 0.8% (w/v) Gelrite (Sigma-AldrichCat. No. G1910). The pH of the medium was adjusted to 5.8 with 1NNaOH (Merck, Cat. No. 106482), before autoclaving at 15 lbs/sec pressure for 20 min at 121º C. The seeds were germinated in 50 ml capacity cotton-plugged culture tubes (Borosil, 22 cm dia, Cat. No. 9820U08) containing about 15 ml half-strength MS medium. The surface sterilized seeds were placed on the culture medium for 48 h in dark for 2 days in the culture room at 28±1º C when the seeds were found to sprout, on 3rd day the cultures were transferred to light under16/8-h photoperiod for 7 days. The light was provided from 4ft length fluorescent tube light (Philipp's make) and the intensity was kept at 130 µ Einstein m^{-2} sec^{-1}.

The data pertaining to seed germination and related parameters viz. number of seeds cultured, seed germination %, days taken to first seedlings emergence, speed of emergence, seedling vigour and plant height at 10 days (cm) was recorded systematically.

Explant preparation: Cotyledon and hypocotyl explants (1-2 cm long)were collected from 10-day old seedlings.

Callus initiation: The explants were cultured in radio sterile 90 mm petri plate (Tarson, Cat. No. 460091) containing callus induction MS medium. The plates were incubated in dark and kept in the culture room at 25±2 °C until discernible callus induction was observed. The medium was supplemented with different concentrations of plant growth regulators to study the synergistic effect

Table 1: Effect of different media components in governing *in vitro* culture response in mesta

Treatment (MS+B5 Vitamins)	Hormone
T1	0.5 mg L^{-1} 2,4-D+0.05 mg L^{-1} Kinetin
T2	1.0 mg L^{-1} 2,4-D+0.10 mg L^{-1} Kinetin
T3	1.5 mg L^{-1} 2,4-D+0.15 mg L^{-1} Kinetin
T4	2.0 mg L^{-1} 2,4-D+0.20 mg L^{-1} kinetin
T5	2.5 mg L^{-1} 2,4-D+0.25 mg L^{-1} Kinetin
T6	3.0 mg L^{-1} 2,4-D+0.30 mg L^{-1} Kinetin
T7	1.5 mg L^{-1} NAA+2.0 mg L^{-1} 6BAP
T8	2.0 mg L^{-1} NAA+2.5 mg L^{-1} 6BAP
T9	2.5 mg L^{-1} NAA+3.0 mg L^{-1} 6BAP
T10	3.0 mg L^{-1} NAA+3.5 mg L^{-1} 6BAP
T11	0.2 mg L^{-1} IAA+0.01 Kinetin
T12	0.4 mg L^{-1} IAA+0.03 Kinetin
T13	0.6 mg L^{-1} IAA+0.0 Kinetin
T14	0.8 mg L^{-1} IAA+0.10 Kinetin
T15	1.0 mg L^{-1} IAA+0.50 Kinetin
T16	0.2 mg L^{-1} IAA+0.01 TDZ
T17	0.4 mg L^{-1} IAA+0.02 TDZ
T18	0.6 mg L^{-1} IAA+0.03 TDZ
T19	0.8 mg L^{-1} IAA+0.05 TDZ
T20	2.0 mg L^{-1} 2,4_D+0.1 mg L^{-1} NAA+ 2.0 mg L^{-1} Kinetin
T21	2.0 mg L^{-1} 2,4-D+0.1 mg L^{-1} NAA+2.0 mg L^{-1} 6BAP
T22	0.5 mg L^{-1} NAA+0.2 mg L^{-1} IAA+0.5 mg L^{-1} 6BAP
T23	0.2 mg L^{-1} NAA+0.5 mg L^{-1} IAA+0.5 mg L^{-1} 6BAP

Table 2: Effect of different organic adjuvants in influencing *in-vitro* culture response in mesta

Treatments	Adjuvant (mg L^{-1})
MS+Vit B5+2 mg L^{-1} 2,4-D+0.2 mg L^{-1} NAA+with/without 3 mg L^{-1} kinetin (T20)	Caesin hydrolasate (250)
MS+Vit B5++1 mg L^{-1} NAA+1 mg L^{-1} IAA+with/without 3 mg L^{-1} 6 BAP (T23)	Yeast extract (250)
MS+Vit B5+2 mg L^{-1} 2, 4-D+ 0.1 mg L^{-1} NAA+with/without 3 mg L^{-1} 6 BAP (T21)	$AgNo_3$ (2.5)
MS+Vit B5+1.5 mg L^{-1} NAA+with/without 2 mg L^{-1} BAP (T7)	Casamino acid (250)
MS+Vit B5	Control

of different hormones in combination on callus induction. A total of 23 treatments were evaluated for callus induction which were supplemented with different combinations of plant growth regulators involving 2, 4-D (2, 4-dichlorophenoxy acetic acid, Sigma-Aldrich, Cat. No. D6679), NAA (Naphthalene acetic acid, Sigma-Aldrich, Cat. No. N0640), BAP (Benzyl amino purine, Sigma-Aldrich, Cat. No. B3408), IAA (Indole-3-acetic acid, Sigma-Aldrich, Cat. No. I5148), TDZ (Thidiazuron, Sigma-Aldrich, Cat. No. P6186) and kinetin (6-furfuryl amino purine) (Sigma-Aldrich, Cat. No. K0753) (Table 1). After 21 days of incubation callus induction was estimated. An array of ancillary data on callus health were also recorded.

Callus proliferation: Healthy calli from each treatment were selected for further sub culture after 21 days on callus proliferation medium with same constitution of callus induction medium but with half strength ofgrowth regulators initially used for callus induction for 3 weeks for further proliferation and maintenance of the calli. Only the best quality calli were cultured on MS medium supplemented with appropriate growth regulators. Brown, watery, mucilaginous/gelatinous and black dead calli were removed during each subculture. Friable, green callus more, less globular or round shaped were considered as putative embryogenic calli which were selected for plantlet regeneration.

Effect of adjuvants: The adjuvants are used to augment callus induction, callus growth rate, quality (texture, fragility) to produce robust healthy plantlet under *in-vitro* culture system. An experiment was carried out to study the effect of different adjuvants like yeast extract (Himedia, Cat. No. RM027), casein hydrolysate (Himedia, Cat. No. M200A), casamino acids (Amresco, Cat. No. J851) and $AgNO_3$ (Sigma-Aldrich, Cat. No. S8157) on callus proliferation and plantlet regeneration. In total nine treatments including control set were assessed in respect of *in-vitro* culture response. Based on the callus induction performance on 23 diverse treatments, four treatments were found to be promising. The healthy calli were transferred on to proliferation medium containing MS medium supplemented with synthetic plant growth regulators along with and without adjuvants for 21 days (Table 2). The cultures were kept under 16/8 h light, (130 µ Einstein m^{-2} sec^{-1}) dark condition cycle). For each treatment 15 culture tubes were deployed and the observations were recorded for % callus proliferation, % plantlet regeneration following the equation:

$$\text{Plantlet regeneration}\,\% = \frac{\text{Number of calli regenerated for regeneration}}{\text{Total calli deployed}} \times 100$$

Shootlet regeneration: The proliferated calli were transferred to regeneration medium containing MS medium supplemented with IAA (0.2-0.5 mg L^{-1}) and BA (2-5 mg L^{-1}) along with and without adjuvants until shootlet regeneration. The individual elongated shootlets of 3-4 cm in length were separated and transferred to MS supplemented with NAA (0.5-2 mg L^{-1} @ increment of 0.5 mg) and IBA (1-3 mg L^{-1} @ increment of 0.5 mg) and on growth regulator free MS medium for rooting.

RESULTS

Studies on seed parameters: Substantial difference in respect of mesta seeds germination was observed under *in-vitro* culture condition. The maximum seed germination was observed in HS 4288 (100%) in *H. sabdariffa* whereas the minimum value was observed in HS 7910 (80%) of the same species. In case of *H. cannabinus*, maximum (90%) and minimum (76%) seed germination was observed in MT 150 and AMC 108 varieties, respectively. In essence seeds from *H. sabdariffa* were found to be superior in respect of seed germination between the two species (Table 3). Seedling emergence, plant height and speed of emergence showed varied response between species and among varieties within some species.

In respect of days taken to first seedling emergence was found bit early in roselle than kenaf, similarly plant height was also found to be more in roselle than kenaf. But the speed of emergence of seedlings was found to be more in kenaf (Table 3).

Callus initiation: Based on the seed germination pattern, four best varieties of roselle viz. HS 4288, Amv 1, Gr 27, HS 7910 and three best varieties of kenaf viz. JBM 2004, Mt 150, HC 583, AMC 108 were assessed for *in vitro* culture response using diverse explant for callus induction on MS medium containing B5 vitamins supplemented with different combinations of hormones (23 treatments). After 21 days of callus induction, callus health (1-9 scale)* etc. were recorded (Table 4). Four best callus induction treatments were identified out of 23 combinations of hormones viz. T20, T23, T21 and T7 (Table 5). Individual calli were weighed and finally the fresh weight data were pooled and average value was worked out by adopting the formula to calculate the rate of callus proliferation which has been expressed in percentage as below: Final weight (mg)-Initial weight (mg)/Initial weight (mg)×100.

The maximum calli proliferation (97.8%) was observed *in-var*. HS 4288 on MS containing 2 mg L^{-1}

Table 3: Variation and comparative assessment of seed parameters within different and between genotypes of two cultivated species of mesta for selection of *in-vitro* culture response varieties

	Species								
	Sabdariffa					*Cannabinus*			
Varieties	HS 4288	Amv 1	Non pions	HS 7910	Gr 27	JBM 2004	MT 150	HC 583	AMC 108
No of seeds cultured	50	50	50	50	50	50	50	50	50
No of seeds germinated	50	45	-	40	48	42	45	40	38
Days taken to first seedlings emergence	15	17	-	20	16	12	7	8	15
Plant height at 10 days(cm*)	9.3	8.4	-	6.7	7.8	8.0	8.2	6.0	4.2
Speed of emergence #	0.62	0.49	-	0.33	0.48	0.66	1.17	0.75	0.28

Table 4: *In-vitro* culture response of different explants obtained from different explants in mesta

	Species													
	Sabdariffa								*Cannabinus*					
Varieties	HS 4288		Amv 1		HS 7910		Gr 27		JBM 2004		MT 150		HC 583	
Explant	H[#]	CL[@]	H	CL	H	CL	H	CL	H	CL	H	CL	H	CL
No of explants cultured	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Callus initiation (%)	80	95	75	85	85	90	70	85	60	70	80	70	70	90
Initiation of callus on callus induction medium (days)	14th	12th	15th	14th	19th	13th	15th	13th	16th	12th	16th	12th	15th	15th
Embryogenic callus induction	Y	Y	N	Y	N	N	N	Y	N	N	N	Y	N	Y
Sub culture day	21 days		21 days		21 days		21 days		21 days		21 days		21 days	
Colour and type of calli	Pale yellow, compact		Greenish, soft		Greenish, soft		Brownish, friable		White, soft		Greenish, compact		Greenish, soft	
Callus health Scale (1-9)*	1		2		3		6		5		2		3	
Shoot regeneration	Y	Y	N	N	N	N	N	Y	N	N	N	N	N	Y
Root regeneration	N	Y	N	N	N	N	N	N	N	N	N	N	N	N

[#]H- Hypocotyl; [@]CL- Cotyeldonary leaf; Y- Yes; N-No.; *Scale 1-9: 1 (Pale yellow,) = excellent; 2-3 (Greenish) = very good; 4-5 (white) = good; 6-7 (brownish) = poor; 8-9 (blackish) = dark brown poor, % Callus induction= No. of explants that showed callus induction/ Total No. of explants

Table 5: Effect of different growth regulators in governing *in vitro* culture response in two cultivated species of mesta

							Species					
	Growth regulator (mg L^{-1})						*H. sabdariffa*			*H. cannabinus*		
T*	----------Auxin----------			---------Cytokinin----------			----------Genotype----------			----------Genotype----------		
	2,4D	NAA	IAA	6BAP	KIN[#]	TDZ[$]	Hs 4288	Amv-1	Gr-27	JBM 2004	HC-583	Mt-150
1	0.5	-	-	-	0.05	-	77.2 ±0.7	18.8±0.6	NA	59.8±0.7	1.93±0.4	NA
2	1.0	-	-	-	0.10	-	51.5 ±0.7	3.3±0.7	NA	23.6±0.3	5.6±0.6	NA
3	1.5	-	-	-	0.15	-	0.8 ±0.3	31.6±0.6	NA	63.8±0.5	45.5±0.3	NA
4	2.0	-	-	-	0.20	-	46.5±0.6	23.0±0.6	NA	20.3±0.6	24.1±0.7	57.4±0.4
5	2.5	-	-	-	0.25	-	34.6±0.5	12.3±0.4	NA	44.3±0.6	34.5±0.6	52.3±0.7
6	3.0	-	-	-	0.30	-	53.6±0.3	8.9±0.3	6.43±0.6	11.3±0.5	31.5±0.6	65.1±0.3
7	-	1.5	-	2.0	-	-	41.1 ±0.5	4.3±0.5	23.8±0.6	59.2±0.8	9.7±0.8	57.4±0.7
8	-	2.0	-	2.5	-	-	0.6 ±0.7	0.7±0.3	52.4±0.7	25.1±0.6	9.1±0.7	52.3±0.7
9	-	2.5	-	3.0	-	-	46.2 ±0.6	8.3±0.6	42.6±0.7	23.6±0.7	1.9±0.5	NA
10	-	3.0	-	3.5	-	-	55.1 ±0.6	18.8±0.3	57.4±0.6	59.8±0.7	25.6±0.7	NA
11	-	-	0.2	-	0.01	-	3.8 ±0.7	3.3±0.7	52.3±0.4	3.0±0.9	6.9±0.0	NA
12	-	-	0.4	-	0.03	-	12.8 ±0.3	31.6±0.4	65.1±0.7	43.8±0.8	54.9±0.6	NA
13	-	-	0.6	-	0.0	-	77.2 ±0.6	53.0±0.4	57.4±0.3	20.3±0.7	6.9±0.6	NA
14	-	-	0.8	-	0.10	-	25.4±0.5	12.3±0.4	4.8±0.5	32.7±0.6	45.9±0.2	NA
15	-	-	1.0	-	0.50	-	57.4±0.3	8.9±0.3	5.8±0.7	23.8±0.9	56.3±0.5	32.1±0.6
16	-	-	0.2	-	-	0.01	52.3±0.9	24.4±0.7	29.4±0.4	43.9±0.8	34.8±0.3	29.7±0.7
17	-	-	0.4	-	-	0.02	65.1±0.7	83.7±0.5	29.3±0.2	57.4±0.7	56.3±0.3	29.9±0.4
18	-	-	0.6	-	-	0.03	55.7±0.7	28.3±0.6	48.5±0.3	52.3±0.4	45.7±0.7	30.7±0.4
19	-	-	0.8	-	-	0.05	45.8±0.8	48.8±0.6	69.3±.08	65.1±0.5	48.9±0.5	66.8±0.3
20	2.0	0.1	-	-	2.0	-	97.8±0.7	3.3±0.6	57.4±0.6	55.7±0.4	53.7±0.5	53.6±0.6
21	2.0	0.1	-	2.0	-	-	40.0±0.8	31.6±0.7	52.3±0.8	57.4±0.7	64.8±0.2	46.8±0.5
22	-	0.5	0.2	0.5	-	-	94.7±0.7	23.0±0.5	65.1±0.6	52.3±0.4	51.2±0.6	29.9±0.4
23	-	0.2	0.5	0.5	-	-	47.2±0.7	12.3±0.6	45.2±0.3	65.1±0.6	45.8±0.7	NA

T*: Treatments, [#]Kin: Kinetin, [$]TDZ: Thidiozuran cultured ×100

2,4-D, 0.1 mg L^{-1} NAA and 2mg L^{-1} kinetin followed by 83.7% *in-var*. AMV1 on MS containing 0.4 mg L^{-1} IAA and 0.02 mg L^{-1} TDZ and 69.3% in GR 27 on MS containing 0.8 mg L^{-1} IAA and 0.05 mg L^{-1} TDZ. To the contrary in kenaf maximum callus proliferation (66.8%) was recorded *in-var*. MT 150 on MS containing 0.8 mg L^{-1} IAA, 0.05 mg L^{-1} TDZ followed by 65.1% *in-var*. JBM 2004 on MS 0.2 mg L^{-1} NAA and 0.5 mg L^{-1} of IAA and 0.5 mg L^{-1} 6 BAP and 64.8% *in-var*. HC 583 on MS 2 mg L^{-1} 2,4-D, 0.1 mg L^{-1} NAA and 2 mg L^{-1} 6 BAP (Table 5)

To study the effect of different adjuvants on callus proliferation and plantlet regeneration four different adjuvants viz. casein hydrolysate, yeast extract, cassamino acid and $AgNO_3$ along with control were used. Maximum callus proliferation was observed in *H. sabdariffa*var. HS 4288, followed by AMV 1 and GR 27. The same var. (HS 4288) showed maximum response on media supplemented with casein hydrolase whereas varieties viz. AMV 1 and GR27 showed highest response oncasamino acid supplemented medium. *H. cannabinus* var. MT 150 produced highest callus proliferation on caesin hydrolysae supplemented medium, followed by HC 583 with yeast extract. JBM 2004 showed the best performance in respect of callus proliferation in the control set. It reflects adjuvants play no role in callus proliferation in this variety.

It is to be mentioned that among the varieties and between the two species, *H. sabdariffa* genotype HS 4288 and among all the adjuvants casein hydrolase showed the best performance in augmenting callus proliferation under *in-vitro* culture condition (Table 6).

Shootlet regeneration:

It was found that the shootlet formation showed substantial differential response in respect of enhanced concentration of BAP whereas increase in the concentration of IAA did not make significant difference. Beyond 3.0 mg L^{-1} BAP and 0.2 mg L^{-1} IAA, no enhancement in shootlet formation was observed. Other varieties of both the species did not respond to different concentrations of BAP and IAA. By increasing the BAP concentration up to 3.0 mg L^{-1} along with 0.2 mg L^{-1} IAA, highest numbers of shootlets were found to be induced in HS 4288 without any adjuvant mentioned earlier.

The shootlets were transferred to the rooting medium for root induction with varied concentrations of rooting growth regulators. Roots started emerging from the cut end of the shootlets within 10 days after transfer to the rooting medium containing MS supplemented with 1.0 mg L^{-1} NAA and 2 mg L^{-1} IBA. However, root development was found within 7 days on hormone free MS medium which were more profuse and healthy in comparison to the former. The elongated shootlets with roots (8-10 cm) were detached and adhered agar was washed and grown in Hoagland solution under culture room condition covered with polyethylene bag to retain required relative humidity. A few whole plantlets could survive by transferring rooted shootlets to soil, sand and

Table 6: Effect of different organic adjuvants in influencing *in vitro* culture response with special reference to callus proliferation and plantlet regeneration

H. sabdariffa	Organic adjuvant	Callus proliferation (%)	Plantlet regeneration (%)	Callus proliferation (%) (without adjuvants)	Plantlet regeneration (%)	*H. cannabinus*	Callus proliferation (%)	Plantlet regeneration (%)	Callus proliferation (%) (without adjuvants)	Plantlet regeneration (%)
HS 4288	C*	60.0	-	50.0	-	JBM 2004	46.6	-	32.5	-
	CH[@]	66.6	-	72.2	1		40.0	-	13.2	-
	CA[#]	40.0	-	36.5	-		16.6	-	18.4	-
	SN[$]	20.0	-	10	-		10.0	-	8.0	-
	YE[!]	16.6	-	12.3	-		0.0	-	0.0	-
AMV1	C*	33.3	-	38	-	MT 150	20.0	-	45.6	-
	CH[@]	16.6	-	23.7	-		53.3	-	53.2	-
	CA[#]	56.6	-	62.4	-		13.3	-	27.8	-
	SN[$]	33.3	-	17.3	-		50.0	-	63.9	-
	YE[!]	40.0	-	23.4	-		60.0	-	34.0	-
GR 27	C*	33.3	-	38.3	-	HC 583				
	0	-	12.8	-						
	CH[@]	0.0	-	12.6	-		56.6	-	3.0	-
	CA[#]	50.0	-	55	-		6.6	-	4.2	-
	SN[$]	20.0	-	58.4	-		6.6	-	23.2	-
	YE[!]	40.0	-	20.5	-		63.3	-	57.9	-

[!]YE- Yeast extract; [$]SN- Silver nitrate; [#]CA- Cassamino acid; [@]CH- Casein hydrolase; *C- Control, No of calli used for each treatment = 30

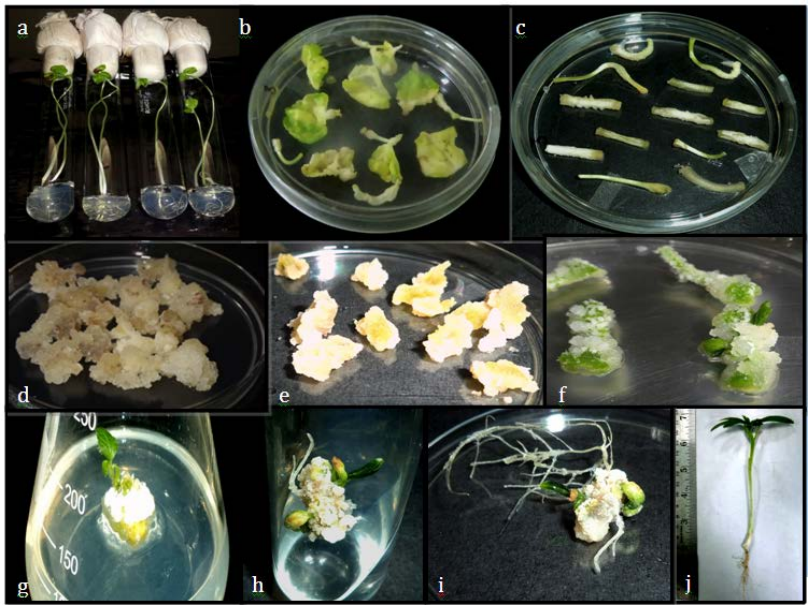

Fig 1(a-j): Different steps of *in-vitro* cultures in mesta *Hibisuss sabdariffa* var. Hs 4288, legends, (a) Emerging seedlings (10 days old) under *in vitro* culture conditions on seed germination medium containing full MS with no hormones, (b) Surface sterilized leaf explants cultured on callus induction medium, (c) Hypocotyl explants placed on callus induction medium, (d) and (e) Fast growing induced callus on proliferating media containing half dose of hormones as used in callus induction medium, (f) aAppearance of greenish colour on callus indicating initiation ofchlorophyll biogenesis andproliferation of callus displaying intense green colour before regeneration and induction of shootlet on the surface of nodal explants at very early stage, (g) Developing shootlets from the calli on regeneration medium, (h) Developing roots from the same calli indicates concurrent rhizogenesis, (i) Close view of developing shootlets and roots from different locations of the same calli and (j) Shootlets with induced roots taken out from the culture vessel ready for hardening and transfer to green house

vermiculite (1:1:1, v/v/v) mixture. Those plants were finally transferred to glasshouse for hardening and grown in soil filled cement pots upto maturity. The detailed *in-vitro* culture protocol in mesta for plantlet regeneration is presented in Fig 1.

DISCUSSION

In the present study, appreciable difference in respect of seeds germination and *in vitro* culture response under *in-vitro* culture condition was observed among different

genotypes within same species and also between two species indicates stringent genotype specificity. The var. HS 4288 in case of roselle and var. MT 150 in kenaf showed maximum [100% and 90%] seed germination, respectively (Table 3). In essence seeds from roselle were found to be superior among the two species. The reason plausibly behind this might be that the seeds of the former specie possess high seed viability and endowed with genetic superiority for more seed germination. Characters like biochemical constituents including an array of enzymes involved in the seed germination grid which is actually determined by the genetic architecture of the variety, extent of lipid per oxidation and electrolytic leakage of the cell membrane and response to the free radical during the process of seed germination etc. However, this needs to be confirmed in future with elaborate experimentation.

In respect of days taken to first seedling emergence, roselle response more quickly than kenaf. This might be owing to relatively thick seed coat of the kenaf which prevents the permeability of water mobilization into the seed at a slower speed that delayed the activation of the enzymes governing the process of seed germination. However, this requires further confirmation in the days to come involving more precise and leak proof experiments. Similarly plant height found to be more in roselle than kenaf. But the speed of emergence was found to be more in kenaf. It is a complex phenomenon which not only depends upon the quantum of reserve food in the seed but also depends upon the pace of activity of the enzymes involved in seed germination metabolic grid particularly the α-amylase activity. The plant which had more height, makes the plant to be a superior competitor than the neighboring entries in respect of nutrient uptake as well to perform better owing to its genetic supremacy and enhanced capability to harness the benefit of the influence of the growing environment. However, the present set of data is considered to be worthy since it provides benchmark information for selection of suitable varieties to embark upon in conducting more elaborate experiments in future in relation to *in-vitro* culture in mesta.

In the present study both the species were found to be *in-vitro* culture responsive at varying rate. Callus induction was found to be prolific and excellent especially from leaf discs in both species. Combinations of 2,4-D, NAA and Kinetin and IAA and TDZ displayed high percentage of callus induction and multiplication in *H. sabdariffa* and *H. cannabinus*, respectively. Maximum calli proliferation was observed to be 97.8% and 66.8% in var. HS 4288 and var. MT 150, respectively (Table 5). In nutshell it was observed that the callus proliferation rate was found to be superior in *sabdariffa* compared to *cannabinus* which may provide advantage to the former for its more use in callus induction, proliferation and plantlet regeneration either through organogenesis or somatic embryogenesis.

In plant tissue culture a large number of diverse materials both of organic and inorganic nature have been reported to be used to augment *in-vitro* tissue culture response in respect of callus induction, plantlet regeneration either through organogenesis or somatic embryogenesis pathway. For example $AgNO_3$ is deemed to interrupt the signal transduction pathway during *in-vitro* culture[15], casein hydrolysate supplies a completely hydrolyzed protein nitrogen to the plant for enhanced metabolism that encourages prolific plantlet regeneration. Yeast extract as source of amino acids and vitamins, especially inositol and thiamine[16, 17] were found to enhance *in-vitro* culture response by reducing the lag phase that ultimately led to very prompt regeneration of plantlets.

Our result showedhighest callus proliferation in var. HS 4288 in roselle and in var. MT 150 in kenaf with casein hydrolase; varieties viz. AMV 1 and GR27 with cassamino acid in roselle; var. HC 583 in kenaf with yeast extract and JBM 2004 in the control set i.e., with adjuvants. It reflects adjuvants play no role in callus proliferation in this variety. It is to be mentioned that among the varieties and between the two species *H. sabdariffa* var. HS 4288 and among all the adjuvants casein hydrolase showed the best performance in augmenting callus proliferation under *in-vitro* culture condition. However, they did not show any positive response in redifferentiation of calli into plantlets, indicating no role of adjuvants on plantlet regeneration in case of mesta. Many calli stay green but did not differentiated into plantlets which needs further research for efficient plantlet regeneration.

HS 4288 showedhighest number of shootlet formation on MS medium containing 3.0 mg L^{-1} BAP in combination with 0.2 mg L^{-1} IAA. None of the other varieties nor any other concentration of hormone combinations and adjuvant responded positively and failed to produce any shootlets. Rooting was observed on MS medium supplemented with 1 mg L^{-1} IAA with 2 mg L^{-1} IBA which was not satisfactory or healthy, however high numbers of roots were observed on hormone free media emerging from basal end of separated shootlets. Similarly both root as well as shootlet initiation was observed (Fig. 1) from different locations of the same calli, indicating high presence of endogenous level of auxins in mesta which is sufficient for root induction and there is no need of application of exogenous synthetic hormone in the medium for rooting.

CONCLUSION

It is concluded that plantlet regeneration via organogenesis and finally whole plant regeneration was established through execution of this elaborate experiment. HS 4288 of *H. sabdariffa* emerged as the best variety to be used for *in vitro* culture of mesta. Cotyledons were found to be the best explant when cultured on MS medium containing 2 mg L^{-1} 2,4-D,0.1 mg L^{-1} NAA and

2 mg L^{-1} kinetin in *H. sabdariffa* and 0.8 mg L^{-1} IAA and 0.05 mg L^{-1} TDZ in *H. cannabinus* for maximum callus induction and proliferation. The best shootlet regeneration was observed on MS with 3.0 mg L^{-1} BAP with 0.2 mg L^{-1} IAA and rooting on hormone free MS medium. However the efficiency of plantlet regeneration was found to be very low. Fine-tuning is essential for development of prolific callus induction and for development of a robust platform for plantlet generations either through organogenesis or somatic embryogenesis with ease and confidence to conduct diverse cell technological experiment especially for transgenic development for added value in the days to come.

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