RESEARCH ARTICLE Legume Research- An International Journal, Volume 46 Issue 12: 1697-1705 (December 2023)

Development of Somaclones through Callus Culture in Fenugreek (*Trigonella foenum-graecum*) Variety Gujarat Methi-2

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10.18805/LR-4467

ABSTRACT

Background: Fenugreek (*Trigonella foenum-graecum*) a widespread aromatic herb, holds an important place in Indian traditional medicine system. A fenugreek or *methi* seed commonly used to flavour food contains various alkaloids, different saponins and fixed oil content. The steroidal nature of natural saponins has high market potential as alternative to *Dioscore* species, the current declining resource of commercial saponins. The current experiment designed to induce and identify somaclone variants of fenugreek resistant to powdery mildew, rich in saponins and fixed oil content with acceptable yield.

Methods: Seeds of fenugreek var. Gujarat Methi-2 (GM-2) were used for *in vitro* regeneration during 2019 laboratory experiment. Explants cultured on Murashige and Skoog (MS) media supplemented with different concentrations of 2,4-D and NAA. The induced calluses were further transferred in to the regenerated semi solid MS medium with various levels of BAP for the development of calliclones. For root induction, shoots were transferred in White's rooting media with different concentrations of Indole Acetic Acid.

Result: Our experimental findings shows induction and detection of somaclonal variants, as a possible source of genetic improvement in fenugreek with limited information on genetic diversity. This approach could generate new genotypes useful to pharmaceutical industries with competitive yield and resistance to powdery mildew.

Key words: Callus induction, Fenugreek, *In vitro*, Powdery mildew, Shoots regeneration, Somaclones.

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum*) a widely cultivated herb, belongs to the family Fabaceae (Balodi and Rao, 1991.) For centuries, it has grown wild in India, the Mediterranean and North Africa where it is mainly cultivated. In India it is known as "methi," in Moroccan "helba" and in Ethiopia "abesh."It was used by the ancient Egyptians to combat fever and grown in classical times as cattle fodder. In India, it is used medicinally and as a yellow dyestuff. Fenugreek shoots and leaves used into salads and its powder, add flavor to cuisine and chutneys.

The seeds and leaves of fenugreek have potential to reduce blood sugar and blood cholesterol levels in both animals and human (Acharya *et al*., 2007). The medicinal properties of crop are due to the presence of various phytochemicals, including trigonelline, diosgenin, fenugreekine and 4-Hydroxyisoleucine (Losso *et al.,* 2009a; Losso *et al.,* 2009b and Zandi *et al*., 2015). Thus, the demand of fenugreek in pharmaceutical and nutraceutical industries is quite high (Zandi *et al*., 2017). The production of fenugreek is limited by various aetiological agents among them, powdery mildew caused by *Erysiphe polygoni* DC is a potential threat (Petropoulos, 2002). It causes damage to all above ground plant parts. It usually appears during later stage of the crop growth and becomes serious during flowering and pod formation stage (Prakash and Saharan, 2002). In this disease, white powdery patches appear on the lower and upper surface of leaves and other parts of the plant. Powdery mildew affects both biomass and seed yield and can result in seed yield loss up to 50 per cent (Rathi *et al*., 2002). Disease resistance offers a sustainable solution to control plant disease due to economic and environmental factors (Komarek *et al*., 2010). However, limited data on genetic diversity of *Trigonella* species (Marzaugui *et al*., 2009), non-availability of resistance source for powdery mildew (Raje *et al.,* 2002) and lengthy conventional breeding procedures hinder the production of powdery mildew resistance varieties in fenugreek. Somaclonal variations can be used to generate new genetic diversity in existing genepool and to overcome the constraints of conventional resistance breeding approach.

Plethora of research has been conducted on *in vitro* regeneration, callus induction and production of secondary metabolites in fenugreek (El-Bahr, 1989, Qaderi *et al.,* 2016 and Valizadeh, 2018). Genetic variability have also been introduced through *in vitro* culture techniques to isolate fenugreek variants with superior yield and seed quality (Basu *et al.,* 2008), yet critical studies to screen somaclones resistant to powdery mildew have not been done in the past.

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How to cite this article: Patel, S.R., Abhishek, D., Patel, H.N., Patil, S.S. and Patel, S.G. (2023). Development of Somaclones through Callus Culture in Fenugreek (*Trigonella foenum-graecum*) Variety Gujarat Methi-2. Legume Research. 46(12): 1697-1705. doi: 10.18805/LR-4467.

Submitted: 23-07-2020 **Accepted:** 02-02-2021 **Online:** 05-04-2021

However, attempts were made by several workers to screen and isolate powdery mildew resistance fenugreek lines among the existing natural genetic diversity in the germplasm (Raje *et al*., 2003; Kumar, 2009 and Mulat, 2017). Since fenugreek has extensive adoption in industrial sectors and powdery mildew being an important disease on fenugreek, an extensive study was conducted to induce and isolate somaclones resistant to powdery mildew in popular variety Gujarat Methi-2.

MATERIALS AND METHODS

The experiment was conducted at Plant Biotechnology Laboratory, Department of Genetics and Plant Breeding, College of Agriculture, Navsari Agricultural University, Campus Bharuch in the year 2019. Healthy, bold and fresh crop seeds of fenugreek var. Gujarat Methi-2 (GM-2) released from Sardarkrushinagar Dantiwada Agricultural University (SDAU), S. K. Nagar, Gujarat in 2006 were used for regeneration of tender and disease-free leaves and shoots *in vitro.* The grains are bold, uniform in size, attractive and have more test weight. On an average GM-2 yielded 1920 kg/ha and showed its superiority by producing 10.03 and 20.32 per cent higher than the varieties GM-1 and Hisar Sonali, respectively

The seeds were treated by dipping in 1% teepol detergent solution for 5 minutes for removing dirt and dust particles adhered on the seed surface and thoroughly washed with double distilled water (DDW). Surface sterilization fenugreek seedswere done with 70% Alcohol for 30 seconds and thoroughly washed with DDW. Finally, the material was taken into Laminar Air Flow Cabinet for sterilization, rinsed with 0.1% $HgCl_2$ solution for 10 minutes to avoid all the contaminants from the seeds. Seven seeds were inoculated in each glass bottle with 5 repetitions in Agar Agar semi solid MS medium (Murashige and Skoog, 1962) with BAP 2.0 mg/l in addition to 1.0 mg/l Adenine Sulphate under aseptic condition for regeneration. The cultures were incubated at 25±1°C temperature with 2000 lux light intensity. The regenerated hypocotyl and cotyledonary segments were placed in callus media (semi solid MS medium with different concentration of 2,4-D and NAA *i.e.* 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) for callus induction. The induced calluses were further transferred in to the regenerated semi solid MS medium with various levels of BAP *i.e.* 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l with same concentration of Adenine sulphate *i.e*. 1.0 mg/l for development of calliclones. For root induction, shoots were transferred in White's rooting media with different concentrations of IAA such as 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l).

Regenerated tissue culture seedlings were planted for hardening process using low polythene tunnels. After hardening, seedlings were transplanted in the earthen pots in the screen house, all the planted material was labelled and necessary management practices were adopted. Potted plants were artificially inoculated by dusting conidia of *Erysiphe polygoni* DC. Powdery mildew severity was visually scored, as suggested by Kumar, (2009) on 0-5 scale and lines were categorized based on disease reaction, experimental material was further categorized based on percent disease intensity (Kumar, 2009).

Statistical analysis

Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Duncan's Multiple Range Test using SPSS for Windows computer program. Data given in percentages were subjected to arcsine (Vx) transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS AND DISCUSSION

Callus induction

The effects of plant growth regulators (2,4-D and NAA)in MS medium on explants (hypocotyl and cotyledonary nodal segments) and their comparison on initiation of callus, % callusing, callus weight (g), callus colour, callus texture and morphogenetic response showed in Table 1, Fig 1-4 and Chart 1A-1C.

The treatment MS medium containing 2.0 mg/l 2,4-D was significantly superior for callus initiation in hypocotyl (12.80) and cotyledon (13.40) segments had taken less number of days, while in the treatment MS medium containing 1.5 mg/l NAA taken less number of days for callus initiation in hypocotyl (13.20) and cotyledon (14.60) segments. The comparison of both the auxins, 2,4-D had given early response as compare to NAA for callus induction. The results obtained through experimentation were in accordance to EL Nour *et al.* (2013).

Callus weight is important character for regeneration of calliclones and development of more numbers of somaclones. The maximum callus formation in hypocotyl segment was obtained 2.0 mg/l 2,4-D (1.456 g) and it was significantly superior over all the treatments, whereas in cotyledon (1.322 g) in the same 2,4-D level. Similarly, callus weight was highest in 1.5 mg/l NAA in hypocotyl segments (1.268 g) as compare to cotyledon (1.144 g). The callus formation was increased from 0.5-2.0 mg/l 2,4-D and it was fall down after 2.5 mg/l 2,4-D. Similar trend was examined in NAA after 2.0 mg/l. Minimum callusing were recorded in hypocotyl and cotyledonary nodal segments by the concentration 0.5 mg/l 2,4-D *i.e.* 0.374 g and 0.292 g respectively and in NAA *i.e.* 0.312g and 0.276 g respectively (Bahram *et al.,* 2005). The various kinds of callus colour and callus textures and morphogenetic responses were observed during the experimentation mentioned in the Table 1, the yellowish green colour and friable texture callus reported good callus induction and gave high callus weight in hypocotyl and cotyledonary nodal segments. Our results revealed that 2,4-D gave high frequency of callus induction, these results agreed with finding of Rezaeian, (2011).

Shoot regeneration

The study presents the effect of cytokinin on frequency shoot regeneration (%), shoots/gm callus and shoot length (cm)

in fenugreek using cotyledonary nodes and hypocotyl explants were cultured on MS medium containing BAP *i.e.* 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l with same concentration of Adenine sulphate 1.0 mg/l depicted in Table 2, Fig 5 and Chart 2.

Result showed that hypocotyl segment responded better than cotyledon for shoot regeneration. The shoot regeneration frequency of hypocotyl and cotyledon segments ranged from 39.80% to 73.20% and 33.80% to 71.40%, respectively. In hypocotyl, maximum shoot regeneration frequency (73.20%) was found significantly superior over all the treatments in MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l Adenine sulphate, where as it was noticed highest (71.40%) in 2.0 mg/l BAP + 1.0 mg/l

Table 1: Influence of various levels of growth regulators *viz.,* 2,4-D and NAA on callus formation in hypocotyl and cotyledonary nodal segments of fenugreek cultured on MS medium.

Explant		Growth parameters					
		Days taken in initiation of callus	% callusing	Callus wt.(g)	Callus colour	Callus texture	Morphogenetic response
Concentrations of 2,4-D							
Hypocotyl	0.5	4.146 (17.20)	48.33 (55.80)	0.374	Yellow	C	C^{+}
	1.0	4.022 (16.20)	53.86 (65.20)	0.732	Yellow	SC	C^{++}
	1.5	3.891 (13.60)	59.51 (74.20)	0.912	White	F	C^{+++}
	2.0	3.685 (12.80)	68.75 (86.80)	1.456	White	F	C^{+++}
	2.5	3.974 (15.20)	39.46 (40.40)	0.846	Light brown	C	C^{+}
	S.Em.±	0.033	0.77	0.01	-	-	-
	C.D. at 5%	0.241	2.28	0.03	-	-	-
	C.V. %	4.730	3.20	2.60	-	-	-
Cotyledon	0.5	4.193 (17.60)	43.16 (46.80)	0.292	Yellow	C	C^{+}
	1.0	4.416 (17.20)	51.25 (60.80)	0.628	Yellow	SC	C^{++}
	1.5	3.895 (15.20)	57.18 (70.60)	1.112	White	F	C^{+++}
	2.0	3.655 (13.40)	66.66 (84.20)	1.322	White	F	C^{+++}
	2.5	4.020 (16.20)	36.02 (34.60)	0.714	Light brown	C	C^{+}
	S.Em.±	0.035	0.84	0.01	-	-	-
	C.D. at 5%	0.245	2.47	0.03	-	-	-
	C.V. %	4.668	3.69	2.86	-	-	-
Concentrations of NAA							
Hypocotyl	0.5	4.426 (19.60)	44.77 (49.60)	0.312	Yellow	C	C^{+}
	1.0	4.165 (17.40)	51.12 (60.60)	0.962	White	SC	C^{++}
	1.5	3.626 (13.20)	64.37 (81.20)	1.268	White	F	C^{+++}
	2.0	3.948 (15.60)	47.41 (54.20)	0.878	Light brown	F	C^{+++}
	2.5	4.072 (16.60)	33.58 (30.60)	0.7175	Dull brown	C	C^{+}
	S.Em.±	0.036	0.70	0.01	-	-	-
	C.D. at 5%	0.250	2.06	0.04	-	-	-
	C.V. %	4.690	3.24	3.62	-	-	-
Cotyledon	0.5	4.601 (21.20)	44.54 (49.20)	0.276	Yellow	C	C^{+}
	1.0	4.400 (19.40)	51.48 (61.20)	0.594	White	SC	C^{++}
	1.5	3.819 (14.60)	56.44 (69.40)	1.144	White	F	C^{+++}
	2.0	4.021 (16.20)	43.74 (47.80)	0.688	Light brown	F	C^{+++}
	2.5	4.288 (18.40)	32.93 (29.60)	0.478	Dull brown	C	C^{+}
	S.Em.±	0.034	0.84	0.01	-	-	-
	C.D. at 5%	0.242	2.47	0.04	-	-	-
	C.V. %	4.335	4.09	4.71	-	-	-

Note: Callus colour, texture and morphogenetic response were examined with eyes observation.

C= Compact, SC= Semi Compact and F= Friable; C^{+} = Low callus, C^{++} = Medium callus, C^{+++}= Profuse callus, (-) = No response; 5 Repetitions of each parameters.

Days taken in initiation of callus: Values in ( ) parenthesis represents original values whereas, outside values are square root transformed values.

% callusing: Values in ( ) parenthesis represents original values whereas, outside values are arc sine transformed values.

Adenine sulphate in cotyledon segment. The minimum shoot regeneration frequency, shoots/gm callus and shoot length (cm) was observed in 0.5 mg/l BAP + 1.0 mg/l Adenine sulphate in both cotyledonary nodes and hypocotyl segments.

In hypocotyl segments, maximum 9.40 shoots was found in MS medium supplemented with 1.5 mg/l BAP + 1.0 mg/l Adenine sulphate and this treatment was highly significant over remaining treatments, where as it was noticed that highest 8.20 shoots in 1.5 mg/l BAP + 1.0 mg/l Adenine sulphate in cotyledon segment. The less number of shoots (2.80) were found in the treatment 0.5 mg/l BAP+ 1.0 mg/l Adenine sulphate in hypocotyl explant. Thus, the shoot regeneration frequency reported more in 2.0 mg/l BAP, while numbers of shoots were achieved higher in 1.5 mg/l BAP. The number of shoots increasing was restricted after the BAP concentration 1.5 mg/l. The result was in accordance with the research finding of Aasim *et al*. (2010).

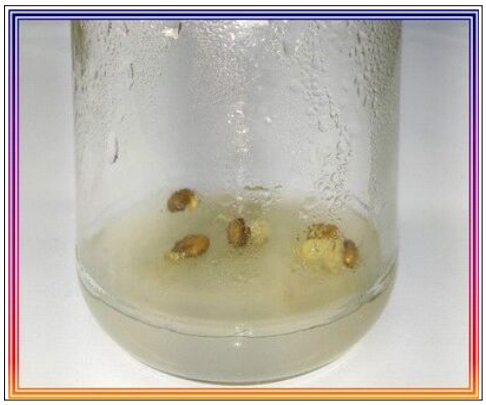

Fig 1: Healthy, bold and fresh crop seeds of fenugreek.



Fig 3: White callus induction from plant parts.



Fig 2: Development of hypocotyl and cotyledons from regeneration seeds.



Fig 4: Regeneration of calli-clones from photosynthetic callus.

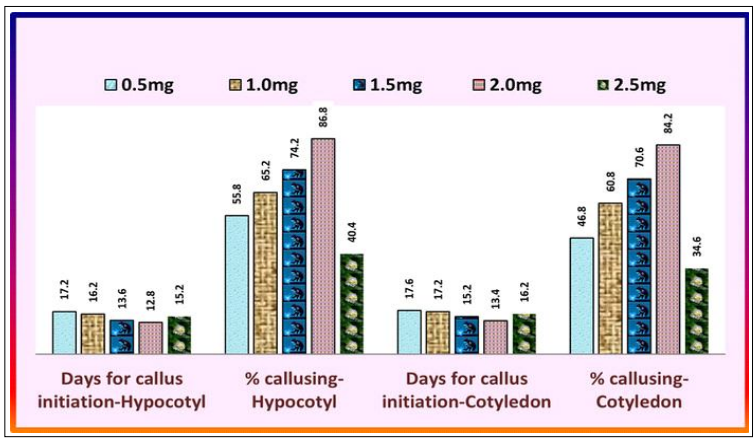

Chart 1A: Influence of various levels of 2,4-D on callus formation in hypocotyls and cotyledons.

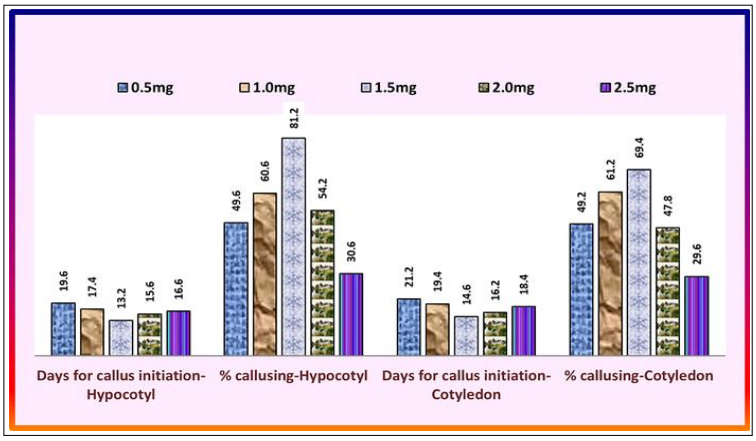

Chart 1B: Influence of various levels of NAA on callus formation in hypocotyls and Cotyledons.

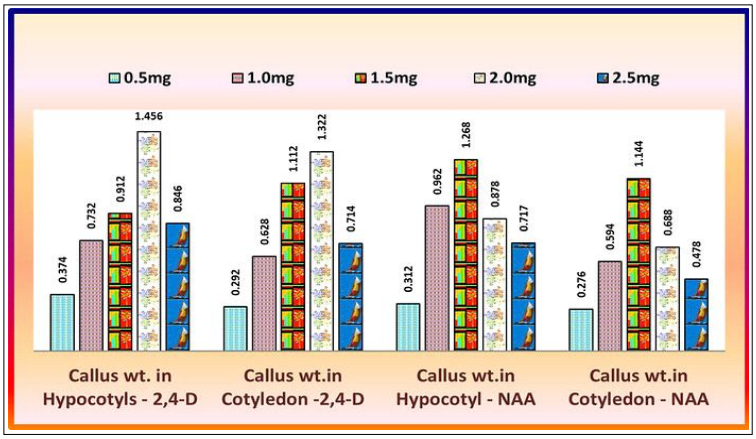

Chart 1C: Influence of various levels of 2,4-D and NAA on callus wt. in hypocotyls and cotyledon.

Table 2: Effect of various concentrations of BAP (with same concentration of Adenine sulphate: 1.0 mg/l) on shoot regeneration from hypocotyl and cotyledonary nodal segments of fenugreek cultured on MS medium.

Explant	BAP (mg/l)	Adenine sulphate (mg/l)	Frequency (%) of shoot regeneration	Number of shoots per gm of callus	Shoot length (cm)
Hypocotyl	0.5	1.0	39.11 (39.80)	1.659 (2.80)	1.41
	1.0	1.0	49.38 (57.60)	2.142 (4.60)	1.63
	1.5	1.0	56.55 (69.60)	3.065 (9 .40)	1.89
	2.0	1.0	58.85 (73.20)	2.604 (6.80)	1.81
	2.5	1.0	50.31 (59.20)	1.839 (3.40)	1.51
	S.Em.		0.77	0.027	0.01
	C.D. at 5%		2.26	0.215	0.04
	C.V.%		3.37	7.218	1.65
Cotyledon	0.5	1.0	35.54 (33.80)	1.478 (3.40)	1.37
	1.0	1.0	45.46 (50.80)	2.041 (4.60)	1.58
	1.5	1.0	54.10 (50.80)	2.861 (8.20)	1.68
	2.0	1.0	57.69 (71.40)	2.321 (5.60)	1.61
	2.5	1.0	48.56 (56.20)	1.776 (3.20)	1.45
	S.Em.		0.72	0.032	0.01
	C.D. at 5%		2.12	0.235	0.03
	C.V.%		3.34	8.488	1.44

Nos. of Repetition: 5. * Frequency (%) of shoot regeneration: Values in parenthesis are original mean values whereas outside values are arc sine transformed values. Number of shoots per gm of callus: Values in parenthesis are original mean values whereas outside values are square root transformed values.

The shoot length of the shoots obtained from hypocotyl segment, ranged from 1.41 to 1.89 cm and from cotyledon, it was ranged from 1.37 to 1.68 cm. Shoot length increased with increase in BAP up to the level of 1.5 mg/l in both types of the explants but it was decreased after the increasing the levels from 2.0 mg/l BAP and above. Similarly, as above, the shoot regeneration frequency reported more in 2.0 mg/l BAP, while the shoot length was achieved higher in 1.5 mg/l BAP for both explants.

Root formation

The results of root induction obtained in regenerated plantlets showed in Table 3, Fig 6 and Chart 3. Morphogenetic root response ranged from 9.80 to 74.00% between the IAA concentration of 0.1 to 0.4 mg/l. After IAA concentration of 0.4 mg/l, morphogenetic response of root was not observed even up to the 0.6 mg/l. Maximum morphogenetic root response was observed on White's medium supplemented with 0.2 mg/l IAA (74.00%) with thick

Table 3: Morphogenetic response of root in white's rooting medium fortified with different concentration of IAA.

IAA (mg/l)	Morphogenetic response (%)	No. of roots/plantlet	Root morphology
0.1	39.81 (41.00)	1.51 (1.8)	Thick and medium
0.2	59.37 (74.00)	1.75 (2.6)	Thick and long
0.3	33.44 (30.40)	1.30 (1.2)	Thick and short
0.4	18.21 (9.80)	1.22 (1.0)	Thin and short
0.5	0.00 (0.0)	0.70 (0.0)	-
0.6	0.00 (0.0)	0.70 (0.0)	-
S.Em.	0.79	0.06	-
C.D. at 5%	2.36	0.18	-
C.V.%	4.67	9.53	-

Nos. of repetition: 5.

Morphogenetic response (%): Values in parenthesis are original mean values whereas outside values are arc sine transformed values.

* No. of roots/plantlet: Values in parenthesis are original mean values whereas outside values are $\sqrt{X + 0.5}$ transformed values.

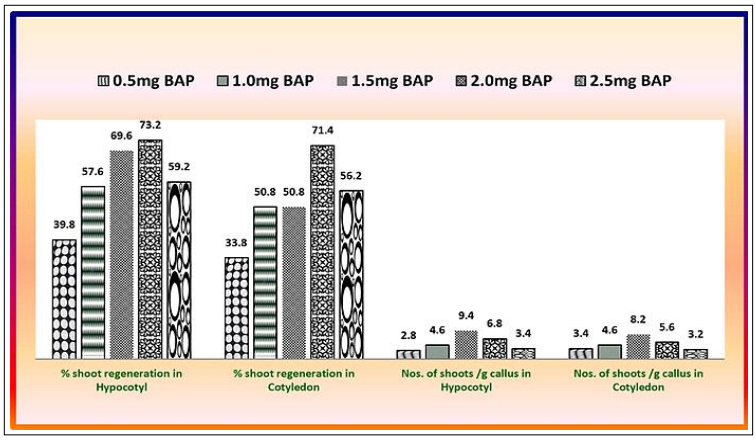

Chart 2: Effect of various concentrations of BAP on shoot regeneration from hypocotyls and cotyledon.

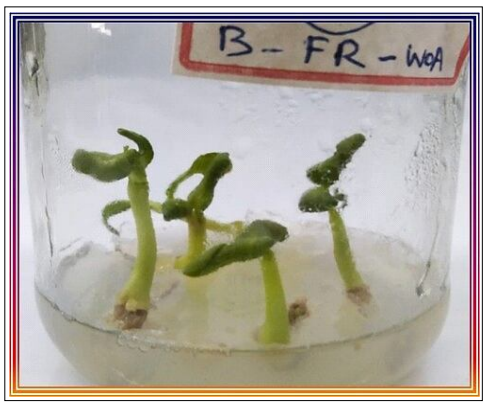

Fig 5: Development of somaclones from callus culture.



Fig 6: Root formation from fenugreek shoot.

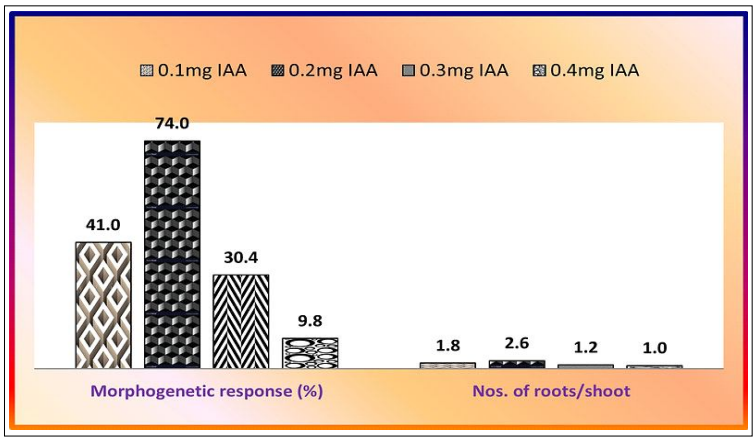

Chart 3: Morphogenetic response of root in white's rooting medium fortified with different concentration of IAA.

Table 4: Progression of powdery mildew in regenerated somaclones compared to the parent GM-2 (Data of somaclones superior to parental line during screening have been included).

Line/somaclone code	Disease intensity* (%)	Disease reaction	Line/somaclone code	Disease intensity (%)	Disease reaction
GM-2 (P*)	39	MS			
GMS-9	37	MS	GMS-29	24	MR
GMS-145	35	MS	GMS-128	24	MR
GMS-91	35	MS	GMS-89	21	MR
GMS-3	33	MS	GMS-45	21	MR
GMS-105	33	MS	GMS-121	20	MR
GMS-56	31	MS	GMS-143	20	MR
GMS-93	31	MS	GMS-17	19	MR
GMS-78	30	MS	GMS-66	17	MR
GMS-151	30	MS	GMS-133	17	MR
GMS-99	30	MS	GMS-85	9	R
GMS-158	28	MS	GMS-110	8	R
GMS-12	28	MS	GMS-139	8	R
GMS-139	28	MS	GMS-6	7	R
GMS-61	25	MR	GMS-31	6	R

P*- Parent line (GM-2); GMS- Gujarat Methi Somaclone; MS-Moderately susceptible; MR- Moderately resistance; R- Resistance.
*Disease intensity- Mean value.

and long roots, whereas minimum was noticed in 0.4 mg/l IAA (9.80%) with thin and short roots. These results were confirmed with Burdak *et al.* (2017). It is true that addition of auxins in the culture medium produced roots in crop species like carrot (Pant and Manandhar, 2007) and brinjal (Narayanaswami, 1994).

Number of roots per plantlet was obtained highest in the treatment White's medium with 0.2 mg/l IAA (2.6) and it was significantly superior over all the treatments whereas lowest was reported in 0.4 mg/l. IAA (1.0). The concentration above 0.4 mg/l IAA, could not showed root induction up to the 0.6 mg/l IAA. Same kind of response was observed by Burdak *et al.* (2017).

Downy mildew resistance

The somaclones regenerated were evaluated in comparison with parent variety GM-2, under screen house condition for powdery mildew resistance. After inoculation, 20 somaclones of 160 (11.12%) produced less severe symptoms than parental material, while eight somaclones (5%), showed the highest levels of resistance, under controlled conditions (Table 4). Though complete resistance has not been observed, yet five somaclones showed resistance reaction and least disease intensity, can be further evaluated to incorporate in the powdery mildew resistance breeding program in fenugreek or to improve the resistance level of parental line which showed moderate susceptible reaction in our experimental design. The effectiveness of somaclones in disease resistance have also been established by various researchers in different crops, including chickpea (Parkash *et al.,* 1994), barley (Li *et al.,* 2001), peanut (Yusnita *et al.,* 2005), garden pea (Sharma *et al.*, 2010) and sugarcane (Kona *et al.,* 2019).

CONCLUSION

It can be concluded that in *vitro* induction of somaclonal variation can be a viable and sustainable alternative in developing powdery mildew resistant fenugreek. Among all the treatments, 2.0 mg/l 2,4-D resulted in regeneration of maximum number of somaclones in GM-2 variety. The resistant somclones compared to parental line can be screened under field conditions for various agronomic feautures to develop powdery mildew resistant varieties with competitive yield in fenugreek.

Conflict of interest

All authors declare that they have no conflicts of interest.

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