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Research Article

IN VITRO PROPAGATION OF EXPLANTS FROM *TRIGONELLA FOENUM-GRAECUM* (L.) SER. AFTER SUPPLEMENTARY UV-B IRRADIATION

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ABSTRACT
In fenugreek (*Trigonella foenum-graecum* (L.) Ser.) after *in vitro* regeneration was carried out with seeds, stem and leaf explants (third node from top of canopy) harvested on 30 days after seed germination from *in situ* control and supplementary ultraviolet-B irradiated (UV-B = 2 hours daily @ 12.2 kJ m^{-2} d^{-1}; ambient = 10 kJ m^{-2} d^{-1}) plants to study their viability for germplasm storage. UV-B reduced the growth parameters in fenugreek on 15 and 30 DAS of growth. On continuous exposure to UV-B radiation the leaves of fenugreek exhibited various kinds of injuries. Unstressed fenugreek seeds responded to *in vitro* germination. UV-B exposed dry seeds failed to germinate under *in vitro* culture. UV-B stressed wet seeds germinated under *in vitro* condition. UV-B irradiation suppressed height of seedlings under wet seed treatment compared with control. Stem explants proliferated axillary bud in both control and UV-B irradiated conditions. Control and UV-B stressed stem explants failed to induct callus. Callus induction did not occur both in control and UV-B stressed fenugreek leaf explants. Both the seeds and stem explants of fenugreek are best suited for germplasm conservation for cultivation in climate elevated UV-B.

Keywords: *Ultraviolet-B, Fenugreek, In Situ Growth, Seeds, Leaf Explants, Stem Explants, In Vitro Regeneration*

INTRODUCTION
Ozone depletion is one of the man-made calamities that occurs due to increases in ozone depleting substances (ODS) and thickness of green house gases around the earth released by human activities. The heat that escapes the troposphere and enters the stratosphere no longer does so, making the stratosphere cooler. Falling below normal temperatures in this layer increases ozone depletion. Due to this, the UV-B radiation will increase, affecting the natural and artificial ecosystems. Elevated ultraviolet-B (UV-B) radiation (280-320 nm) is a dangerous atmospheric stress (Caldwell *et al.*, 1983, Jordan 1997, Caldwell *et al.*, 1998) as it affects foliar epidermis (Bornman and Vogelmann, 1991; Rajendiran and Ramanujam, 2000a; Rajendiran and Ramanujam, 2000b; Rajendiran, 2001; Kokilavani and Rajendiran, 2013; Kokilavani and Rajendiran, 2014a; Kokilavani and Rajendiran, 2014b), suppresses photosynthesis (Rajendiran and Ramanujam, 2003; Rajendiran and Ramanujam, 2004) and inhibits nodulation and nitrogen metabolism (Rajendiran and Ramanujam, 2006; Rajendiran and Ramanujam, 2003; Sudaroli and Rajendiran, 2013a; Sudaroli and Rajendiran, 2013b; Sudaroli and Rajendiran, 2014; Arulmozhi and Rajendiran, 2014a; Arulmozhi and Rajendiran, 2014b; Arulmozhi and Rajendiran, 2014c; Vijayalakshmi and Rajendiran, 2014a; Vijayalakshmi and Rajendiran, 2014b; Vijayalakshmi and Rajendiran, 2014c) in sensitive plants. Screening methods have to be developed to select the best varieties of crops that are suitable for surviving in elevated UV-B environment and to conserve their germplasm. Hence an attempt to analyse the tolerance of fenugreek to supplementary UV-B irradiation and to identify its germplasm for conservation and regeneration through *in vitro* technique was conducted.

MATERIALS AND METHODS
Fenugreek (*Trigonella foenum-graecum* (L.) Ser.) was chosen for the study. Viable seeds of fenugreek were procured from local farmers in Pondicherry. The seeds were selected for uniform colour, size and weight and used in the experiments. The crops were grown in pot culture in the naturally lit greenhouse (day temperature maximum 38 ± 2 ºC, night temperature minimum 18 ± 2 ºC, relative humidity 60 ± 5 %,

maximum irradiance (PAR) 1400 µmol m^{-2} s^{-1}, photoperiod 12 to 14 h). Supplementary UV-B radiation was provided in UV garden by three UV-B lamps (*Philips TL20W/12 Sunlamps*, The Netherlands), which were suspended horizontally and wrapped with cellulose diacetate filters (0.076 mm) to filter UV-C radiation (< 280 nm). UV-B exposure was given for 2 h daily from 10:00 to 11:00 and 15:00 to 16:00 starting from the 5th day after sowing. Plants received a biologically effective UV-B dose ($UV\text{-}B_{BE}$) of 12.2 kJ m^{-2} d^{-1} equivalent to a simulated 20 % ozone depletion at Pondicherry (12º2' N, India). The control plants, grown under natural solar radiation, received $UV\text{-}B_{BE}$ 10 kJ m^{-2} d^{-1}. The responses of fenugreek in control and supplementary UV-B irradiation under *in situ* condition were assessed in terms of growth on 15 and 30 DAS.

Supplementary UV-B radiation was provided by one UV-B lamp (Philips TL 20W/12 Sunlamps, The Netherlands) which was suspended horizontally over the seeds. UV-B dose was maintained by adjusting the distance (30 cm) between seeds and the lamp. The lamp was wrapped with cellulose diacetate filters (0.076 mm) to filter UV-C radiation (< 290 nm). The filters were changed periodically to maintain uniform optical properties. UV-B exposure to seeds was given only once for two hours duration with one hour recovery time in between. Seeds received a biologically effective UV-B dose ($UV\text{-}B_{BE}$) of 12.2 kJ m^{-2} d^{-1}. The control seeds were exposed to sunlight for same duration receiving $UV\text{-}B_{BE}$ 10 kJ $m^{-2}d^{-1}$ with one hour recovery time in between (Caldwell, 1971).

Seeds, nodal shoot segments (stem explants) and leaf discs (leaf explants) after appropriate aseptic treatment were used for *in vitro* culture. The explants were thoroughly washed with water containing 0.1% Bavistin (a systemic fungicide BASF, India Ltd., Bombay) for 4-5 minutes. They were surface sterilized with 0.1% $HgCl_2$ for 4-5 minutes and washed 6 to 8 times with autoclaved water under Laminar Air Flow Cabinet (Technico Systems, Chennai) and inoculated aseptically onto culture medium. The final wash was given with aqueous sterilized solution of (0.1%) ascorbic acid. The surface sterilized explants were dipped in 90% ethanol for a short period (40 seconds).

The seeds, stem and leaf explants were inoculated vertically on MS medium for culture initiation. Different concentration and combination of cytokinins (6-benzyl amino purine – BAP and Kinetin ranging from 0.1 to 5.0 mgl^{-1}) and auxins (IAA - Indole acetic acid ranging from 0.1 to 1.0 mgl^{-1}) were incorporated in the medium for inducing bud breaking. These cultures were incubated at 28 ± 2 ˚C in the dark for 2-3 days. Subsequently these were kept under diffused light (22 µ mol m^{-2} s^{-1} SFP- spectral flux photon) for 8 to 10 days. The light was provided by fluorescent tubes and incandescent bulbs. Temperature was maintained by window air conditioners. Positive air pressure was maintained in the culture rooms, in order to regulate temperature and to maintain aseptic conditions.

The cultures were regularly monitored and the growth parameters and callus proliferation were recorded after 15 DAI (days after inoculation) and 30 DAI. The experiments were carried out with three replicates per treatment.

The plant tissue culture media generally comprise of inorganic salts, organic compounds, vitamins, gelling agents like agar-agar. All the components were dissolved in distilled water except growth regulators. Auxins were dissolved in 0.5N NaOH or ethanol and cytokinins were dissolved in dilute 0.1N HCl or NaOH. For the present study MS basal medium (Murashige and Skoog 1962) was used as nutrient medium.

MS basal medium was used either as such or with certain modification in their composition. Sucrose and sugar cubes were added as a source of carbohydrate. The pH of the media was adjusted to 5.8±2 with 0.5N NaOH or 0.1N HCl before autoclaving. The medium was poured in the culture vessels. Finally the medium was steam sterilized by autoclaving at 15 psi pressure at 121˚C for 15 minutes.

Chemical composition of MS medium (Murashige and Skoog, 1962)

Constituents	Quantity (mg L^{-1})
Macronutrients	
NH_4NO_3	1650
KNO_3	1900

	$CaCL_2.2H_2O$	440
	$MgSO_4.7H_2O$	370
	KH_2PO_4	170
	Na.EDTA	37.23
	$FeSO_4.7H_2O$	27.95
Micronutrients		
	KI	0.83
	H_3BO_3	6.20
	$MnSO_4.4H_2O$	22.30
	$ZnSO_4.7H_2O$	8.60
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4,5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
	Meso-Inositol	100
	Glycine	2.0
	Thiamine. HCl	0.1
	Nicotinic acid	0.5
	Pyridoxine. HCl	0.5
	Sucrose (%w/v)	3 %
	pH	5.8

Preparation of MS medium

Approximately 90 % of the required volume of the deionized-distilled water was measured in a container of double the size of the required volume. Dehydrated medium was added into the water and stirred to dissolve the medium completely. The solution was gently heated to bring the powder into solution. Desired heat stable supplements were added to the medium solution. Deionized-distilled water was added to the medium solution to obtain the final required volume. The pH was adjusted to required level with NaOH or HCl. The medium was finally dispensed into culture vessels. The medium was sterilized by autoclaving at 15 psi (pounds per square inch) at 121˚C for appropriate period of time.

Photography

Plants grown under *in situ* condition and *in vitro* cultures tubes were photographed in daylight using a Sony digital camera fitted with appropriate close-up accessories.

RESULTS AND DISCUSSION

In Situ Studies

Supplementary UV-B irradiation reduced the number of leaves by (20 to 33.33 %) in fenugreek (Table 1 to 2; Plate 1). Total leaf area and leaf area index followed the same trend. Specific leaf weight in UV-B irradiated decreased with age. The values were equal to control on 15 DAS and decreased by 31.84 % on 30 DAS. UV-B stress decreased the fresh weight of leaves by 26.51 % to 31.84 %, with the maximum reduction being on 30 DAS. The dry weight of foliage decreased by 33.43 % and 46.53 % on 15 DAS and 30 DAS respectively in UV-B exposed plants (Table 1 to 2; Plate 1). Reductions in leaf area and mass were observed in the field-grown sweetgum plants exposed to elevated UV-B radiation (Sullivan *et al.*, 1994) and *in situ* pot-grown ten varieties of cowpea (Kokilavani and Rajendiran, 2014a, 2014b, 2014c, 2014d, 2014e, 2014f, 2014g, 2014h, 2014i, 2014j, 2014k, 2014l, 2014m, 2014n, 2014o). According to Britz and Adamse (1994) changes in the leaf area and dry mass indicated that cell elongations as well as cell contents were affected. Britz and Adamse (1994) also opine that inhibitions are part of general UV-B effects.

In response to prolonged exposure to UV-B radiation, the leaves of fenugreek exhibited different types of malformations (Plate 2). The leaves became generally pale which became waxy and shiny. The yellowing intensified and became discretely chlorotic. Browning developed in patches indicating necrosis of the

underlying tissues during later stages. Necrotic lesions appeared in older leaves which have received UV-B over a long time and the leaf margins turned curly, dry and brittle. Similar abnormalities were reported in black gram (Kokilavani and Rajendiran, 2013), cucumber (Kokilavani and Rajendiran, 2014b) and several varieties of cowpea grown under *in situ* UV-B exposure (Kokilavani and Rajendiran, 2014a; Kokilavani and Rajendiran, 2014b; Kokilavani and Rajendiran, 2014c; Kokilavani and Rajendiran, 2014e; Kokilavani and Rajendiran, 2014f; Kokilavani and Rajendiran, 2014g; Kokilavani and Rajendiran, 2014j; Kokilavani and Rajendiran, 2014k; Kokilavani and Rajendiran, 2014l; Kokilavani and Rajendiran, 2014m; Kokilavani and Rajendiran, 2014n; Kokilavani and Rajendiran, 2014o; Kokilavani and Rajendiran, 2015a and Kokilavani and Rajendiran, 2015b).

Growth of all the varieties of cowpea was progressively inhibited by the UV-B radiation. UV-B exposure reduced root length significantly by 1.01% to 15.58 % on all stages of growth till 30 DAS. Shoot length of UV-B stressed plants decreased by 6.67 % within 15 DAS and continued so till 30 DAS with 27.93 % reduction (Table 3 to 4; Plate 1). The S / R ratio was decreased by UV-B stress by 26.24 % on 15 DAS and continued to go below control by 38.75 % on 30 DAS. Fresh weight of roots increased with age in all treatments. But the biomass accumulation in root was inhibited by UV-B treatment by 4.33 % on 15 DAS, the maximum reduction being on 30 DAS by 9.25 %. A general decrease of 36.12 % in shoot fresh weight of UV-B treated plants was observed on 30 DAS. The same trend was maintained till 30 DAS of growth. The trends observed in root and shoot biomass pattern were reflected at the whole plant level too with inhibitions at UV-B, maximum reduction of 51.02 % on 15 DAS and only little suppression on 30 DAS (4.35 %). A reduction in the root biomass content by 30.76 % was observed on 15 DAS which showed recovery as the root dry weight showed only 4.73 % reduction on 30 DAS after UV-B treatment. UV-B exposure suppressed dry weight of shoot by 17.78 % on 15 DAS, reaching a maximum of 31.54 % on 30 DAS compared with control. Plant dry weight increased with age, but after UV-B stress, it fell below control by 31.71 to 46.53 % the maximum being on 30 DAS.

Inhibition of growth indicated by reductions in root and shoot length and biomass content due to UV-B stress were apparent at all stages. Such inhibitions are characteristic of UV-B stressed legumes as in *Vigna unguiculata (*Kulandaivelu *et al.*, 1989), *Phaseolus vulgaris* (Mark and Tevini, 1997), *Vigna mungo* (Rajendiran and Ramanujam, 2000a) and *Vigna radiata* (Rajendiran and Ramanujam, 2003) and ten varieties of cowpea (Kokilavani and Rajendiran, 2014o). The stunting of UV-B stressed plants is attributed to destruction of endogenous IAA whose photo-oxidative products may be inhibitory (Kulandaivelu *et al.*, 1989; Tevini and Teramura, 1989) as indicated by a decrease in IAA content concomitant with a corresponding increase in IAA oxidase activity in rice leaves (Huang *et al*., 1997).

The relative growth rate (RGR) was lowered in all UV-B irradiated plants showing a reduction by 99.60 % from control on 15 DAS. RGR was reduced on 30 DAS after UV-B exposure as it reached 48.97 % below control (Table 3 to 4). Similar inhibitions of RGR by UV-B were observed in mungbean by Jain *et al.*, (1999) and in ten varieties of cowpea by Kokilavani and Rajendiran (2014o).

In Vitro Studies

The *in vitro* study carried out with fenugreek after *in situ* and *in vitro* UV-B irradiations was unique of its own as no work was reported earlier in the *in vitro* regeneration of UV-B exposed plant samples. The seeds of unstressed fenugreek responded *in vitro* germination. The UV-B stressed dry seeds did not respond. However UV-B stressed wet seeds germinated under *in vitro* condition. UV-B stressed wet seeds responded 50 % less compared to control under *in vitro* germination on 15 DAI. Unstressed plants showed profuse root growth on 15 DAI (Table 5; Plate 3). UV-B exposure in wet seeds reduced shoot length by 21.42 % on 15 DAI, while unstressed seeds showed profuse shoot growth on 15 DAI. Overall, the height of the seedling was suppressed by UV-B irradiation in dry treatment compared with control. The S / R ratio was increased by UV-B stress on 15 DAI by 20.39 % over control after wet seed exposure. Biomass accumulation in root was inhibited by UV-B irradiation by 22.58 % in wet seed treatments compared to control. A general decrease of 6.80 % in shoot fresh weight after UV-B exposure to wet seeds was observed on 15 DAI. The trends observed in root / shoot biomass pattern were reflected at the whole seedling level too with inhibitions (13.11 %) at UV-B irradiated dry seeds.

Table 1: Changes in foliage of 15 DAS *Trigonella foenum-graecum* (L.) Ser. in control and UV-B irradiated plants– *In situ*

Treatment	Number of leaves	Total leaf area (cm^2)	Leaf area index	Specific leaf weight (g^{-2})	Fresh weight of foliage (g)	Dry weight of foliage (g)
Control	3	12.29	0.031	0.094	0.308	0.283
UV-B	2	6.16	0.028	0.094	0.226	0.188

Table 2: Changes in foliage of 30 DAS *Trigonella foenum-graecum* (L.) Ser. in control and UV-B irradiated plants – *In situ*

Treatment	Number of leaves	Total leaf area (cm^2)	Leaf area index	Specific leaf weight (g^{-2})	Fresh weight of foliage (g)	Dry weight of foliage (g)
Control	5	36.74	0.037	0.015	0.808	0.052
UV-B	4	20.88	0.029	0.010	0.551	0.027

Table 3: Changes in growth parameters of 15 DAS *Trigonella foenum-graecum* (L.) Ser. in control and UV-B irradiated plants – *In situ*

Treatment	Plant height (cm)	Root length (cm)	Shoot length (cm)	Shoot / root ratio	Root fresh wt. (g)	Shoot fresh wt. (g)	Plant fresh wt. (g)	Root dry wt. (g)	Shoot dry wt. (g)	Plant dry wt. (g)	Relative growth Rate
Control	8.7	1.54	7.16	2.57	0.143	0.367	0.784	0.006	0.029	0.086	-7.01
UV-B	6.46	1.30	5.16	1.57	0.149	0.235	0.385	0.004	0.024	0.059	-0.02

Table 4: Changes in growth parameters of 30 DAS *Trigonella foenum-graecum* (L.) Ser. in control and UV-B irradiated plants – *In situ*

Treatment	Plant height (cm)	Root length (cm)	Shoot length (cm)	Shoot / root ratio	Root fresh wt. (g)	Shoot fresh wt. (g)	Plant fresh wt. (g)	Root dry wt. (g)	Shoot dry wt. (g)	Plant dry wt. (g)	Relative growth Rate
Control	20.37	6.87	13.5	1.37	0.379	0.521	1.709	0.124	0.331	0.587	0.23
UV-B	19.7	6.80	12.6	1.01	0.344	0.349	1.243	0.118	0.201	0.347	0.12

Table 5: Changes in growth parameters of 15 DAI *Trigonella foenum-graecum* (L.) Ser. in control and UV-B irradiated soaked seeds – *In vitro*

Treatment	Plant height (cm)	Root length (cm)	Shoot length (cm)	Shoot / root ratio	Root fresh wt. (g)	Shoot fresh wt. (g)	Plant fresh wt. (g)	Root dry wt. (g)	Shoot dry wt. (g)	Plant dry wt. (g)	Relative growth Rate
Control	11	4	7	1.48	0.160	0.238	0.398	0.148	0.153	0.302	- 0.02
UV-B	7.5	2	5.5	1.78	0.124	0.222	0.346	0.111	0.153	0.264	-0.03

Figure 1: On 15 DAS

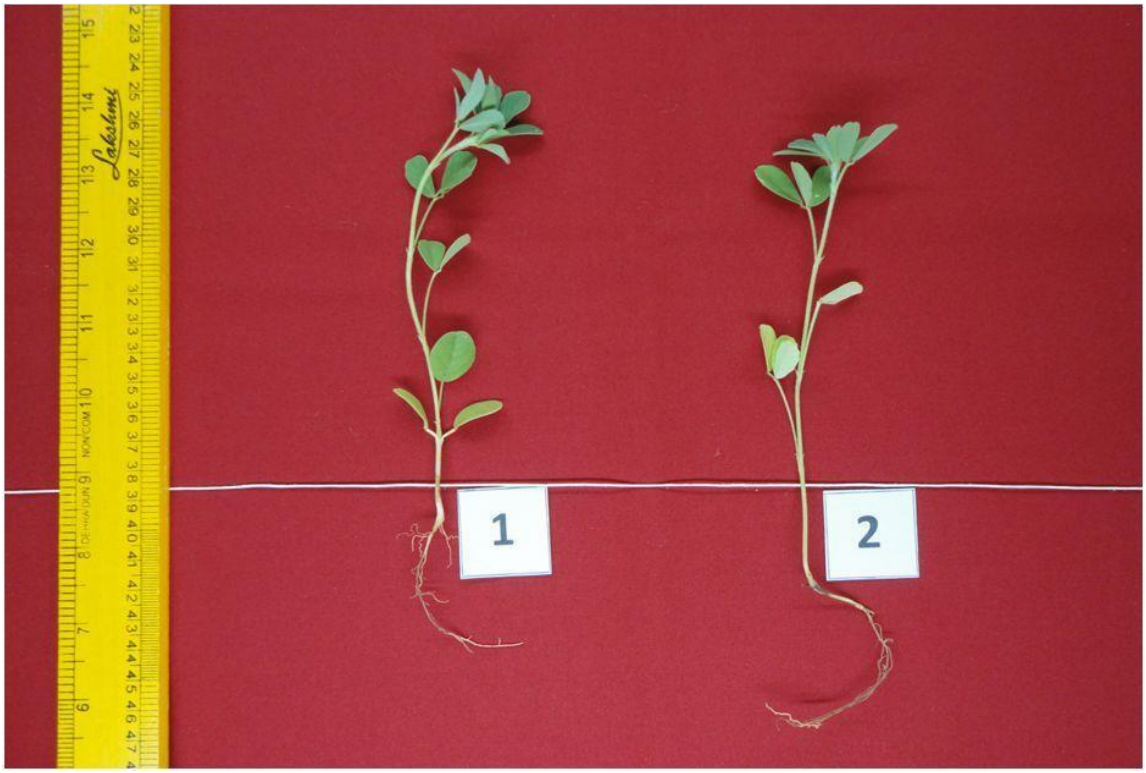

Figure 2: On 30 DAS

Plate 1: The control and supplementary UV-B stressed plants of *Trigonella foenum-graecum* (L.) Ser. (1: Control, 2: UV-B)

Plate 2: Types of foliar injury caused by elevated UV-B radiation in *Trigonella foenum-graecum* (L.) Ser. on 30 DAS

1 DAI Figure 1: Control

Figure 2: UV-B on dry seed

Figure 3: UV-B on soaked seed

7 DAI Figure 4: Control

Figure 5: UV-B on dry seed

Figure 6: UV-B on soaked seed

Plate 3: *In vitro* seed germination and growth of *Trigonella foenum-graecum* (L.) Ser. in control and UV-B irradiated dry and soaked seeds. (DAI - Days after inoculation)

7 DAI: Stem explants Figure 1: Control

Figure 2: UV- B

7 DAI: Leaf explants Figure 3: Control

Figure 4: UV- B

Plate 4: *In vitro* callus proliferation from stem and leaf explants of control and UV-B irradiated *Trigonella foenum-graecum* (L.) Ser. plants. (DAI - Days after inoculation)

Reduction in the root biomass content by 25.40 % on 15 DAS was caused by UV-B treatment in wet seed treatments. Suppression of dry weight of shoot after UV-B exposure was only 0.13 % below control on 15 DAI in wet seed treatment. Plant dry weight increased with age in control and in all treatments. But after UV-B stress, it fell below control by 12.43 % on 15 DAI after wet seed exposure. The relative growth rate (RGR) of seedlings was increased by 15 % in UV-B irradiated wet seeds on 15 DAI. The plants under normal condition had better number of leaves but there were fewer leaves only under UV-B stressed wet seeds. Similar results were reported by Rajendiran *et al.,* (2014a, 2014b) after experimenting with the *in vitro* regeneration of UV-B stressed seeds in ten varieties of cowpea.

Callus induction was not observed in fenugreek both in control stem explants as well as in stem explants harvested from *in situ* supplementary UV-B irradiated crops. However, axillary buds proliferated in control stem explants as well as from *in situ* UV-B stressed stem explants (Plate 4, Figure 1 to 2)). Similar experiments were carried out by Rajendiran *et al.,* (2014c) with *in vitro* regeneration of stem explants harvested from *in situ* grown UV-B stressed ten varieties of cowpea.

In leaf explants, proliferation of callus did not occur both in control leaf explants as well as in leaf explants harvested from *in situ* supplementary UV-B irradiated crops (Plate 4, Figure 3 to 4). Similar attempts were made by Rajendiran *et al.,* (2014d) with *in vitro* regeneration of leaf explants harvested from *in situ* grown UV-B stressed ten varieties of cowpea.

Taking into consideration of all the parameters studied *viz.,* growth under *in situ* condition and the responses of seeds and explants under *in vitro* culture, the present study recommended that out of the explants of fenugreek taken for screening, both the seeds and stem explants are considered to be best suited for germplasm conservation and regeneration.

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