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Research Article

IN VITRO PROPAGATION OF ULTRAVIOLET-B STRESSED MOMORDICA CHARANTIA L.

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ABSTRACT

In vitro regeneration carried out in bitter gourd (*Momordica charantia* L.) after *in situ* UV-B irradiation was unique of its own, as no work was reported earlier in the *in vitro* regeneration of UV-B exposed plant materials. Regeneration was tried with seeds, nodal stem explants and leaf explants (third node 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}) bitter gourd to study their viability for germplasm storage. UV-B reduced the growth parameters in bitter gourd on 15 DAS and 30 DAS of growth. UV-B exposed leaves of bitter gourd exhibited various kinds of abnormalities. Unstressed bitter gourd seeds responded *in vitro* germination. UV-B exposed bitter gourd seeds both in dry and wet treatments failed to germinate under *in vitro* culture. Callus induction occurred both in control and UV-B irradiated bitter gourd stem explants. Only control stem explants proliferated axillary bud. Callus induction occurred both in control and UV-B stressed bitter gourd leaf explants. Out of all the explants of bitter gourd taken for screening, both the stem and leaf explants are tolerant to elevated UV-B exposure and so best suited for germplasm conservation and regeneration.

Keywords: *Ultraviolet-B, Bitter Gourd, in Situ Growth, Seeds, Leaf Explants, Stem Explants, in Vitro Regeneration*

INTRODUCTION

Human activities have dumped ozone depleting substances (ODS) and green house gases around the earth favouring ozone depletion. The heat that normally would escape the troposphere and enter the stratosphere was obstructed, making the stratosphere cooler. Cooler temperatures in this layer increases ozone depletion and in turn the UV-B radiation increases, affecting the ecosystems. Enhanced 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 Sudha and Rajendiran, 2013a; Sudha and Rajendiran, 2013b; Sudha 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 need to be developed to select the best varieties of crops that can withstand elevated UV-B environment and to conserve their germplasm. On this line, the present work is an attempt for the first time to evaluate the sensitivity of bitter gourd to supplementary UV-B irradiation and to identify the germplasm of the crop for conservation and regeneration through tissue culture method.

MATERIALS AND METHODS

Bitter gourd (*Momordica charantia* L.) was chosen for the study. Viable seeds of bitter gourd were procured from Madagadipet Seeds Depot, Pondicherry. The seeds were selected for uniform color, 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}, and 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 dictate 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-B_{BE}) of 12.2 kJ m^{-2} d^{-1} equivalents to simulated 20 % ozone depletion at Pondicherry (12º2' N, India). The control plants, grown under natural solar radiation, received UV-B_{BE} 10 kJ m^{-2} d^{-1}. The responses of bitter gourd 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 dictate 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-B_{BE}) of 12.2 kJ m^{-2} d^{-1}. The control seeds were exposed to sunlight for same duration receiving UV-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% Baiting (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 (Technical 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 cytokines (6-benzyl amino purine – BAP and Kinetin ranging from 0.1 to 5.0 mgl^{-1}) and axons (IAA - Insole 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

Bitter gourd plants had fewer leaves (43.22 %) under UV-B stress on 15 DAS which showed recovery (4.42 %) on 30 DAS (Table 1 to 2; Plate 1). But plants under normal ambience had more number of leaves. Supplementary UV-B irradiation reduced the total leaf area throughout the growth period, the maximum being 73.43 % on 15 DAS followed by 43.32 % on 30 DAS in bitter gourd. The LAI was reduced by UV-B exposure by 12.87 % on 15 DAS and to a larger extent of 25.29 % below control on 30 DAS in bitter gourd. The SLW in UV-B irradiated reduced with age. An average decrease of 26.27 and 30.74 % were observed on 15 and 30 DAS respectively. UV-B stress decreased the fresh weight of leaves by 47.90 % on 15 DAS, with the maximum reduction being on 30 DAS by 81.93 %. The dry weight of foliage decreased by 13.56 to 78.83 % in all stages of 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). Changes in the leaf area and dry mass indicated that cell elongations as well as cell contents were affected by UV-B rays (Britz and Adamse, 1994).

Leaves of bitter gourd exhibited various kinds of abnormalities on continuous exposure to UV-B (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. The leaves exhibited bronzing and became dry and brittle. UV-B induced many abnormalities in black gram (Kokilavani and Rajendiran, 2013), cucumber (Kokilavani and Rajendiran, 2014b) and various varieties of cowpea (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).

UV-B exposure reduced root length significantly by 26.26 % on 15 DAS and continued to suppress it by 54.31 % till 30 DAS (Table 3 to 4; Plate 1). Shoot length of UV-B stressed plants decreased by 10.12 % within 15 DAS and continued so till 30 DAS with 21.13 % reduction. The S / R ratio was decreased by UV-B stress by 99.99 % on 15 DAS and 11.12 % on 30 DAS.

Fresh weight of roots increased with age in all treatments. But the biomass accumulation in root was inhibited after UV-B treatment by 44.71 % on 15 DAS which continued showing a reduction of 34.81 % on 30 DAS compared with control. A general decrease of 43.38 % on 15 DAS in shoot fresh weight of UV-B treated plants proved the maximum sensitivity shown by bitter gourd. The same trend was maintained till 30 DAS (44.52 %) of growth. The trends observed in root and shoot biomass pattern were reflected at the whole plant level too with inhibitions at UV-B by 17.17 % and 17.74 % on 15 and 30 DAS respectively.

A continuous reduction in the root biomass content starting severely from 48.77 % on 15 DAS and continuing with 37.37 % on 30 DAS was caused by UV-B treatment. UV-B exposure suppressed dry weight of shoot by 9.79 % on 15 DAS, reaching a maximum of 59.63 % on 30 DAS over control. Plant dry weight increased with age, but after UV-B stress it fell below control by 14.72 % on 15 DAS and 52.46 % on 30 DAS.

Inhibition of growth indicated by reductions in root and shoot length and biomass content due to UV-B stress were obvious 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 which showed a reduction in RGR by 17.59 % on 15 DAS and 18.17 % on 30 DAS (Table 3 to 4). Such inhibitions of RGR by UV-B were observed by Jain *et al.,* (1999) in mungbean and in ten varieties of cowpea (Kokilavani and Rajendiran, 2014o).

In Vitro Studies

The seeds of unstressed bitter gourd responded *in vitro* germination as they germinated profusely. The UV-B stressed dry and wet seeds failed to respond under *in vitro* condition (Table 5, Plate 3). Rajendiran *et al.,* (2014a, 2014b) after attempting *in vitro* regeneration of UV-B stressed seeds in ten varieties of cowpea have reported similar results.

Proliferation of axillary buds alone occurred in stem explants of *in situ* grown control plants of bitter gourd taken for study. Callus induction was observed in both control stem explants as well as in stem explants harvested from *in situ* supplementary UV-B irradiated crops (Plate 4, Figure 1 to 2). The induction of callus was delayed by one or two days in explants harvested from *in situ* UV-B irradiated bitter gourd plants compared with those of controls. Same trend was reported by Rajendiran *et al.*, (2014c) with *in vitro* regeneration of stem explants harvested from *in situ* grown UV-B stressed ten varieties of cowpea.

Figure 1: On 15 DAS

Figure 2: On 30 DAS

Plate 1: The control and supplementary UV-B stressed plants of *Momordica charantia* L. (1: Control, 2: UV-B)

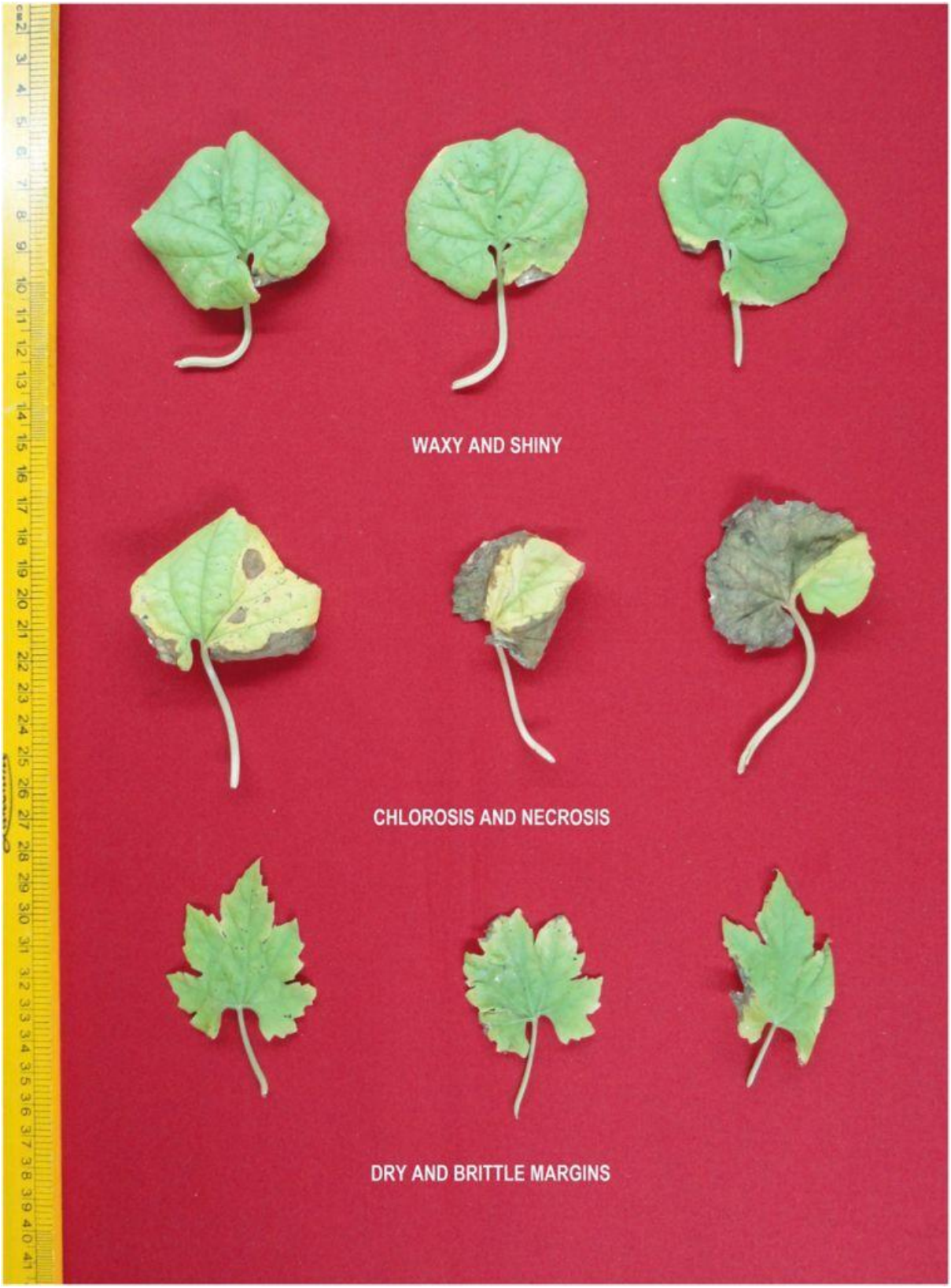

Plate 2: Types of foliar injury caused by elevated UV-B radiation in *Momordica charantia* L. on 30 DAS

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 *Momordica charantia* L. 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 *Momordica charantia* L. plants. (DAI - Days after inoculation)

Table 1: Changes in foliage of 15 DAS Momordica charantia L. 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	12.33	496.81	11.24	0.080	1.425	0.804
UV-B	7.00	131.96	9.79	0.059	0.742	0.695

Table 2: Changes in foliage of 30 DAS *Momordica charantia* L. 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	22.6	1407.37	12.89	0.099	12.347	3.408
UV-B	21.6	797.71	9.63	0.039	2.230	0.721

Table 3: Changes in growth parameters of 15 DAS *Momordica charantia* L. 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	38.93	10.66	28.26	0.18	0.463	2.433	2.896	0.474	0.805	1.279	0.03
UV-B	32.26	7.86	25.4	0.17	0.250	1.377	2.398	0.242	0.726	1.091	0.02

Table 4: Changes in growth parameters of 30 DAS *Momordica charantia* L. 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	93.00	27.8	65.2	0.25	0.507	2.506	3.013	0.980	2.055	3.036	0.45
UV-B	61.76	12.7	49.06	0.22	0.330	1.390	2.479	0.614	0.829	1.443	0.36

Table 5: Changes in growth parameters of 15 DAI *Momordica charantia* L. in control and UV-B irradiated dry seeds

*– In vitro.*reatment	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	8	3.4	0.23	1.130	0.669	1.799	0.063	0.049	0.112	0.01
UV-B	-	-	-	-	-	-	-	-	-	-	-

Callus induction was observed in bitter gourd 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). The induction of callus was delayed by one or two days in explants harvested from *in situ* UV-B irradiated crop varieties compared with those of controls. Rajendiran *et al.,* (2014d) had comparable results with *in vitro* regeneration of leaf explants harvested from *in situ* grown UV-B stressed ten varieties of cowpea.
From the data obtained in 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 bitter gourd taken for screening, both the stem and leaf explants are best suited for germplasm conservation and regeneration.

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