*International Journal of Innovative Research and Review ISSN: 2347 – 4424 (Online)*
*An Online International Journal Available at http://www.cibtech.org/jirr.htm*
*2015 Vol. 3 (2) April-June, pp.45-56/Rajendiran et al.*
***Research Article***

# *IN VITRO* PROPAGATION OF *BENINCASA HISPIDA* (THUNB.) COGN. EXPLANTS AFTER ENHANCED UV-B EXPOSURE

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## ABSTRACT
The *in vitro* regeneration was tried with seeds, nodal stem explants and leaf explants (third 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 $m^{-2}$ $d^{-1}$) ash gourd (*Benincasa hispida* (Thunb.) Cogn.) to study their viability for germplasm storage. UV-B reduced the growth parameters in ash gourd on 15 and 30 DAS of growth. UV-B irradiated leaves of ash gourd exhibited different types of malformations. Unstressed ash gourd seeds failed to respond *in vitro* germination. UV-B exposed wet and dry ash gourd seeds also failed to germinate under *in vitro* culture. Callus induction occurred both in control and UV-B irradiated stem explants. Axillary bud proliferation occurred only in control stem explants. Callus induction occurred both in control and UV-B stressed ash gourd leaf explants. Both the stem and leaf explants of ash gourd are found to be tolerant to supplementary UV-B irradiation and hence suitable for germplasm conservation.

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

## INTRODUCTION
Ozone depletion occurs due to direct effect by ozone depleting substances (ODS) and by the indirect effect of accumulation of green house gases around the Earth released by factories, industries and automobiles. The hotness that normally would escape the troposphere and enter the stratosphere was held in this region, turning the stratosphere cooler. The cooler stratosphere in turn supports depletion of ozone, increasing UV-B influx into the Earth's surface thereby affecting the plants, animals and human. 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. Hence to select the best varieties of crops that are suitable for surviving in elevated UV-B environment and to conserve their germplasm, screening methods have to be developed. The present work is to assess the extent to which ash gourd can tolerate supplementary UV-B irradiation and to identify its germplasm for conservation and regeneration through tissue culture method.

## MATERIALS AND METHODS
Ash gourd (*Benincasa hispida* (Thunb.) Cogn.) was chosen for the study. Viable seeds of ash gourd were procured from Madagadipet Seeds Depot, 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-$B_{BE}$) 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-$B_{BE}$ 10 kJ $m^{-2}$ $d^{-1}$. The responses of ash 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 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-$B_{BE}$) 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).

**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 |

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.

***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.

## RESULT AND DISCUSSION

***In situ Studies***

There were less number of leaves (28.57 to 32.07 %) on 15 and 30 DAS under UV-B stress, but the plants of ash gourd under normal condition had more leaves (Table 1 to 2; Plate 1). Supplementary UV-B radiation decreased the total leaf area throughout the growth period, the maximum being 62.13 % on 15 DAS. The LAI was reduced by UV-B exposure by 25.89 % on 15 DAS, the maximum being 99.12 % over control on 30 DAS. An average reduction of 26.19 % and 14.47 % were observed on 15 and 30 DAS, respectively. UV-B stress decreased the fresh weight of leaves by 65.78 %, which showed recovery on 30 DAS with the reduction being only 38.42 %. The dry weight of foliage decreased by 43.27 % on 15 DAS and by 53.31 % 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 and inhibitions are part of general UV-B effects.

After continuous exposure to UV-B radiation, the leaves of ash gourd exhibited different types of malformations (Plate 2). The leaves became pale which later appeared waxy and shiny. The yellowing intensified and became discretely chlorotic. Browning developed in patches indicating necrosis of the underlying tissues during later stages. Older leaves which have received UV-B over a long time period of time exhibited necrotic lesions.

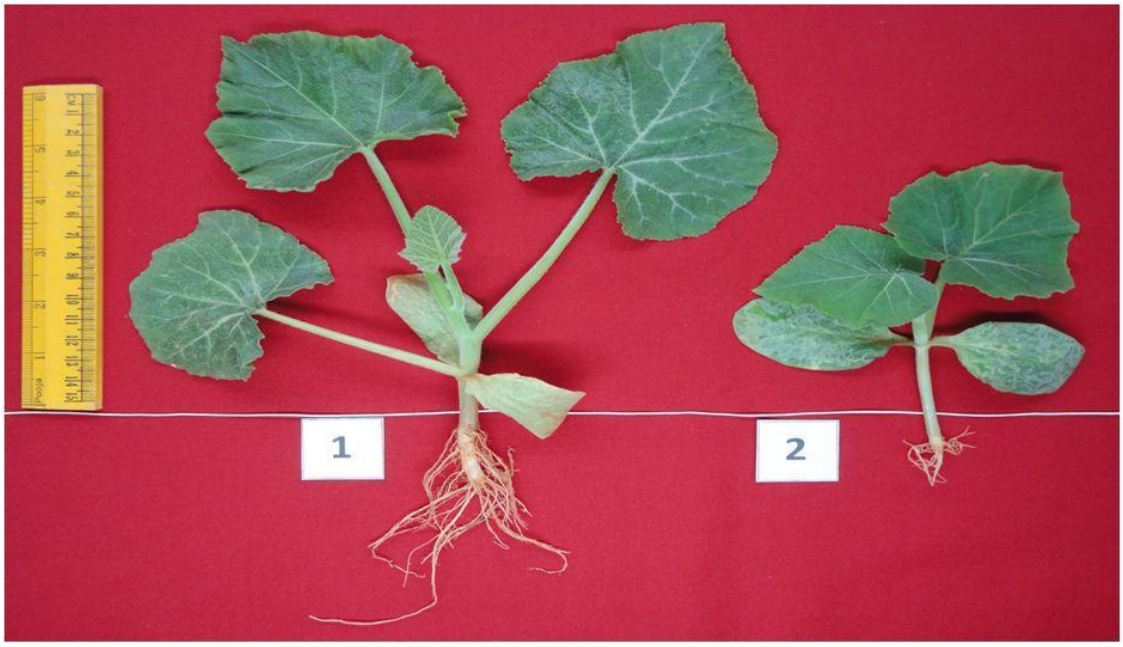


**Figure 1: On 15 DAS**


**Figure 2: On 30 DAS**

**Plate 1: The control and supplementary UV-B stressed plants of *Benincasa hispida* (Thunb.) Cogn. (1: Control, 2: UV-B)**

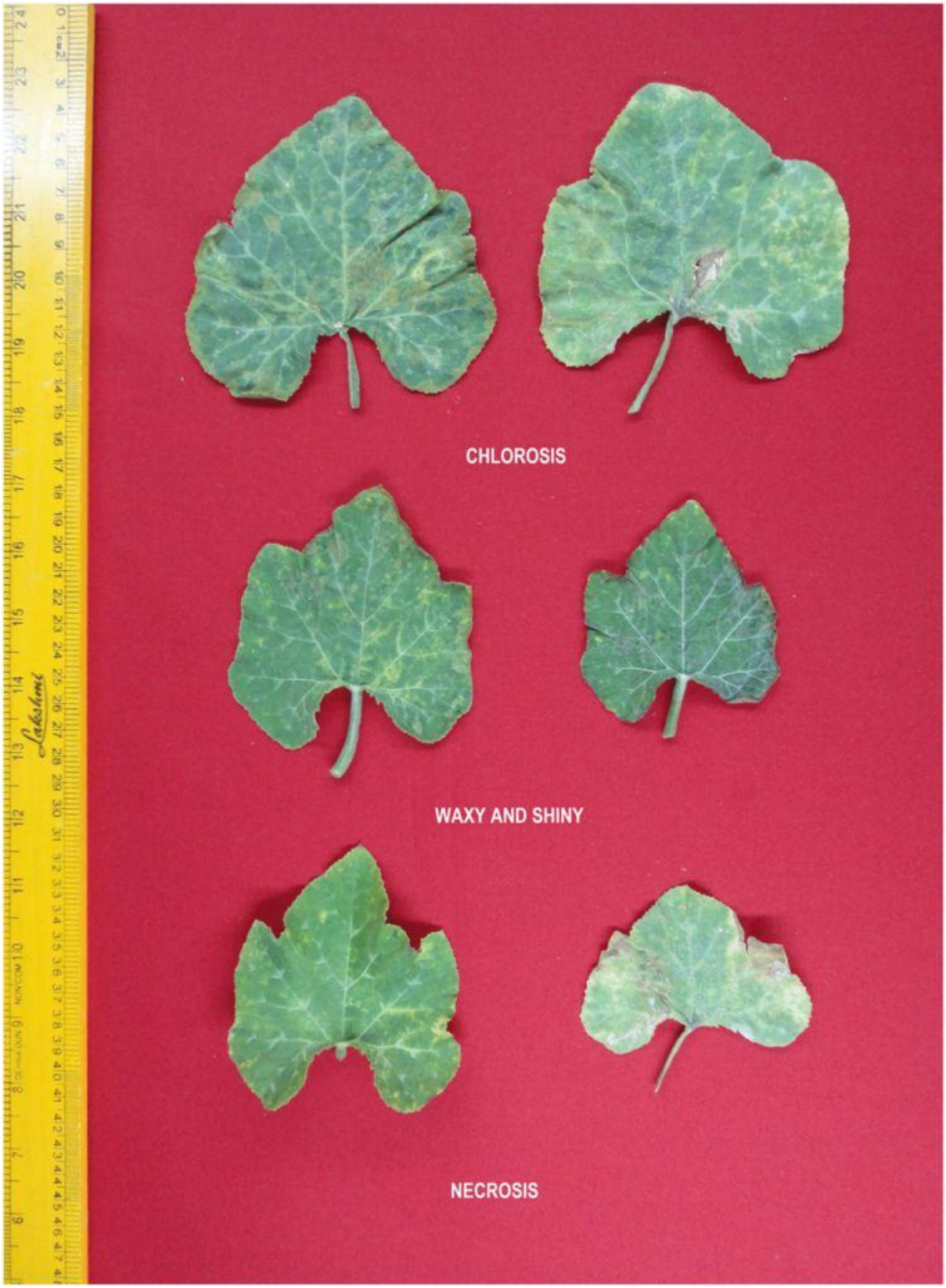


**Plate 2: Types of foliar injury caused by elevated UV-B radiation in *Benincasa hispida* (Thunb.) Cogn. on 30 DAS**

**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 *Benincasa hispida* (Thunb.) Cogn. in control and UV-B irradiated dry and soaked seeds. (DAI - Days after inoculation)**

**7 DAI: Stem explants Figure 1: Control**

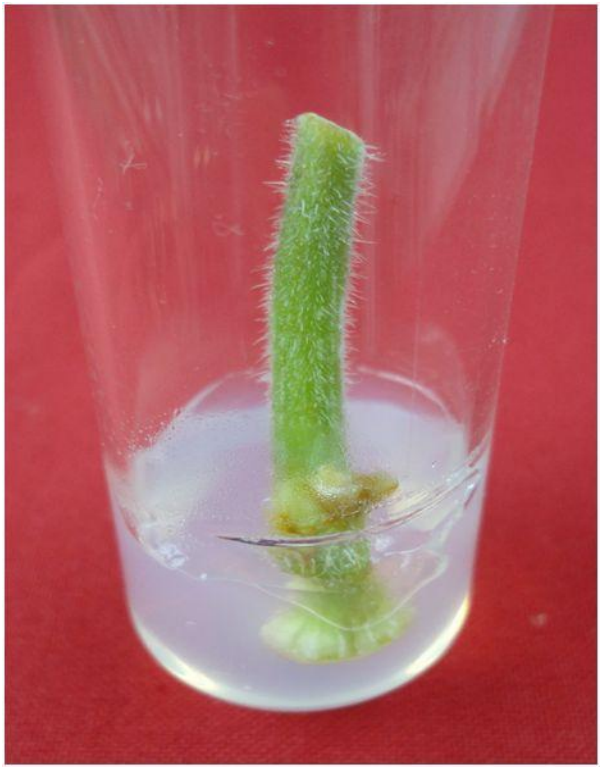

**Figure 2: UV- B**

**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 *Benincasa hispida* (Thunb.) Cogn. plants. (DAI - Days after inoculation)**

**Table 1: Changes in foliage of 15 DAS *Benincasa hispida* (Thunb.) Cogn. 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 | 5.3 | 238.32 | 0.306 | 0.042 | 2.642 | 1.972 |
| UV-B | 3.6 | 117.13 | 0.226 | 0.031 | 1.246 | 1.118 |

**Table 2: Changes in foliage of 30 DAS *Benincasa hispida* (Thunb.) Cogn. in control and UV-B irradiated plants– *In stiu***

| 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 | 7.6 | 378.79 | 31.552 | 0.080 | 18.165 | 5.209 |
| UV-B | 5.00 | 175.39 | 2.277 | 0.056 | 7.080 | 2.432 |

**Table 3: Changes in growth parameters of 15 DAS *Benincasa hispida* (Thunb.) Cogn. 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 | 23.06 | 10.83 | 12.23 | 2.77 | 1.833 | 5.445 | 8.27 | 0.657 | 2.710 | 4.434 | 0.06 |
| UV-B | 10.13 | 5.03 | 5.43 | 1.84 | 0.922 | 2.356 | 3.251 | 0.361 | 1.461 | 1.822 | 0.03 |

**Table 4: Changes in growth parameters of 30 DAS *Benincasa hispida* (Thunb.) Cogn. 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 | 165.36 | 141.66 | 17.7 | 4.37 | 4.121 | 10.285 | 20.406 | 1.801 | 4.673 | 8.808 | 0.08 |
| UV-B | 62.53 | 55.33 | 7.2 | 2.08 | 1.394 | 3.813 | 5.207 | 0.644 | 1.968 | 2.613 | 0.04 |

More abnormalities and injuries 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; Kokilavani and Rajendiran, 2015b).

Root length reduced by 53.42 % on 15 DAS and by 62.53 % till 30 DAS (Table 3 to 4; Plate 1) in response to UV-B radiation. Shoot length of UV-B stressed plants decreased by 48.73 % within 15 DAS and continued so till 30 DAS with 8.35 % reduction. The S / R ratio was decreased by UV-B stress by 30.75 % on 15 DAS and by 29.39 % below control 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 32.54 % on 15 DAS, the maximum reduction being 34.25 % on 30 DAS. A general decrease of 56.72 % in shoot fresh weight of UV-B treated plants was observed 15 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 by 51.87 % on 15 DAS with maximum reduction of 54.35 % on 30 DAS. A gradual reduction in the root biomass content starting from 50.09 % on 15 DAS and reaching 81.43 % on

30 DAS was caused by UV-B treatment. UV-B exposure suppressed dry weight of shoot by 46.08 % on 15 DAS, reaching a maximum of 67.22 % on 30 DAS over control. Plant dry weight increased with age but after UV-B stress, it fell below control by 46.92 % on 15 DAS and 72.42 % on 30 DAS.

Such inhibition of growth indicated by reductions in root and shoot length and biomass content due to UV-B stress are characteristic of UV-B stressed plants as in *Vigna unguiculata* reported by Kulandaivelu *et al.,* (1989), in *Phaseolus vulgaris* by Mark and Tevini (1997), in *Vigna mungo* by Rajendiran and Ramanujam (2000a) and in *Vigna radiata* by Rajendiran and Ramanujam (2003) and in ten varieties of cowpea by 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 reduction by 38.19 % compared to control on 15 DAS and by 31.88 % on 30 DAS (Table 3 to 4). Severe inhibitions of RGR by UV-B were observed in mungbean by Jain *et al.,* (1999) and in ten varieties of cowpea (Kokilavani and Rajendiran, 2014o).

***In vitro Studies***

Unstressed ash gourd seeds of failed to respond *in vitro* germination. The UV-B stressed dry and wet seeds responded same as the control seeds as they did not germinate under *in vitro* condition (Plate 3). 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 observed both in control stem explants as well as in stem explants harvested from *in situ* supplementary UV-B irradiated plants. However, axillary bud proliferation occurred only in control stem explants of ash gourd, while UV-B stressed stem explants failed to promote axillary bud proliferation. The induction of callus was delayed by one or two days in explants harvested from *in situ* UV-B irradiated plants compared with those of controls (Plate 4, Figure 1 to 2). Rajendiran *et al.,* (2014c) with *in vitro* regeneration of stem explants harvested from *in situ* grown UV-B stressed ten varieties of cowpea have reported comparable results.

Callus induction was observed both in control leaf explants as well as in leaf explants harvested from *in situ* supplementary UV-B irradiated crops. The induction of callus was delayed by one or two days in explants harvested from *in situ* UV-B irradiated crop compared with control (Plate 4, Figure 3 to 4). Such experiments were made by Rajendiran *et al.,* (2014d) with the leaf explants harvested from *in situ* grown UV-B stressed ten varieties of cowpea.

From the collected data *viz.,* growth under *in situ* condition and the responses of seeds and explants under *in vitro* culture, both the stem and leaf explants of ash gourd are recommended for germplasm conservation and regeneration in future elevated UV-B climate.

**ACKNOWLEDGEMENT**

The authors thank Prof. Dr. Thamizharasi Tamizhmani, Director, KMCPGS, Puducherry for providing research facilities.

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