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RESEARCH ARTICLE

IN VITRO SEED GERMINATION AND GROWTH OF THREE VARIETIES OF GREEN GRAM AFTER ULTRAVIOLET-B IRRADIATION

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ABSTRACT

In vitro seed germination and growth was carried out with control and ultraviolet-B irradiated seeds (UV-B = 2 hours daily with 1 hour recovery time @ 12.2 kJ m^{-2} d^{-1}; ambient = 10 kJ m^{-2} d^{-1}) of three selected varieties *viz.* CO-8, NVL-585 and VAMBAN-2 of green gram (*Vigna radiata* (L.) Wilczek.) to evaluate the seed viability on culture media for germplasm conservation. Unstressed and UV-B stressed CO-8, NVL-585 and VAMBAN-2 both in dry and wet conditions responded *in vitro* germination. UV-B irradiation suppressed height of seedlings at both dry and wet conditions in all varieties of green gram compared with respective controls. However, root length of UV-B stressed VAMBAN-2 performed 7.14 % better than control. Shoot length of NVL-585 responded well recording little reduction (4 % and 15.83 %) after UV-B exposure to dry and wet seeds. UV-B stressed NVL-585 dry and wet seeds accumulated less plant biomass (14.57 % and 10.52 %) than their controls. VAMBAN-2 dry and wet UV-B exposure produced equal number of leaves compared to controls.

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INTRODUCTION

In the stratosphere, in the region between 15 to 50 kilometres above the Earth's surface, ozone plays a vital role by absorbing harmful ultraviolet radiation from the sunlight. Stratospheric ozone is threatened by the human-made ozone depleting substances (ODS) that have been released into the atmosphere.

Elevated ultraviolet-B (UV-B) radiation (280-320 nm) is a harmful abiotic stress to the biosphere (Caldwell *et al*. 1998) as it destroys leaf epidermis of plants (Kokilavani and Rajendiran 2013, Kokilavani and Rajendiran 2014a, Kokilavani and Rajendiran 2014b, Kokilavani and Rajendiran 2014c, Kokilavani and Rajendiran 2014d, Kokilavani and Rajendiran 2014f, Kokilavani and Rajendiran 2014g, Kokilavani and Rajendiran 2014h, Kokilavani and Rajendiran 2014j, Kokilavani and Rajendiran 2014k, Kokilavani and Rajendiran 2014l, Kokilavani and Rajendiran 2014m, Kokilavani and Rajendiran 2014n, Kokilavani and Rajendiran 2015a and Kokilavani and Rajendiran 2015b), causes stomatal abnormalities in the epidermis of cotyledons in germinating seedlings (Rajendiran *et al.* 2015b and Rajendiran *et al.* 2015c), suppresses the vital photosynthetic process (Kulandaivelu *et al.* 1989, Sullivan *et al*. 1994 and Rajendiran 2001), causes reduction in plant growth and development (Rajendiran and Ramanujam 2000, Rajendiran and Ramanujam 2003, Rajendiran and Ramanujam 2004, Kokilavani and Rajendiran 2014o and Rajendiran *et al.* 2015j), reduces fruit yield (Mark and Tevini 1997, Rajendiran and Ramanujam 2004, Kokilavani and Rajendiran 2014e and Rajendiran *et al.* 2015j) and reduces nodulation and symbiotic nitrogen fixation in legumes (Rajendiran and Ramanujam 2006, Sudaroli Sudha and Rajendiran 2013a, Sudaroli Sudha and Rajendiran 2013b, Kokilavani and Rajendiran 2014i, Sudaroli Sudha and Rajendiran 2014a, Sudaroli Sudha and Rajendiran 2014b, Sudaroli Sudha and Rajendiran 2014c, Arulmozhi and Rajendiran 2014a, Arulmozhi and Rajendiran 2014b, Arulmozhi and Rajendiran 2014c, Vijayalakshmi and Rajendiran 2014a, Vijayalakshmi and Rajendiran 2014b and Vijayalakshmi and Rajendiran 2014c).

In this context, an experiment was conducted to identify the variety of green gram that can withstand supplementary UV-B radiation and to test the seeds through *in vitro* culture methods for the purpose of germplasm conservation and regeneration.

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MATERIALS AND METHODS

In vitro UV-B radiation

Green gram (*Vigna radiata* (L.) Wilczek.), the nitrogen fixing grain legume belonging to family Fabaceae was chosen for the study. Viable seeds of the three varieties of green gram *viz.* CO-8, NVL-585 and VAMBAN-2 were procured from Saravana Farms, Villupuram, Tamil Nadu and from local farmers in Pondicherry, India.

The seeds were selected for uniform colour, size and weight and used in the experiments. Ultraviolet-B (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).

Experimental design

Seeds were divided into two lots - one for growing under normal ambience (control) and another for receiving Ultraviolet-B (UV-B). Each lot was again subdivided into two groups, where, one received treatment in dry condition (dry seeds) while the other received treatment in wet condition (wet seeds) after soaking in water over night.

In vitro culture of seeds

Seeds after appropriate aseptic treatment were used for *in vitro* culture. Seeds 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). The final wash was given with aqueous sterilized solution of (0.1%) ascorbic acid. The surface sterilized seeds were dipped in 90% ethanol for a short period (40 seconds).

The seeds were inoculated horizontally 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 were recorded after 15 DAI (days after inoculation). 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 (pounds per square inch) 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 at 121˚C for appropriate period of time.

Measurement of plant growth

Ten plants from each treatment were carefully uprooted on 15 DAI and their axial growth (roots and shoot length and plant height) and fresh biomass were measured. They were then dried in an oven at 80º C for 48 h and weighed again for dry mass measurements. Alongside, morphological and developmental abnormalities if any, caused by UV-B radiation were also recorded. Assessment of growth of three varieties of green gram on 15 DAI were recorded and calculated using standard methods. The leaf area (the leaflets from all the nodes) was determined at various stages using Area meter (Analytical Development Corporation, UK, model AM100). The total leaf area per plant was obtained by summing up the area of the leaves from all the nodes of the plant. Leaf area index (LAI) (Williams 1946), specific leaf weight (SLW) (Pearce *et al.* 1968) and shoot / root ratio (Racey *et al.* 1983) were calculated using the following formulae.

$$LAI = \frac{\text{Leaf area of the plants } (cm^2)}{\text{Ground area occupied } (cm^2)}$$

$$SLW = \frac{\text{Leaf dry weight (g)}}{\text{Leaf area } (m^2)}$$

$$\text{S/R ratio} = \frac{\text{Shoot weight (g)}}{\text{Root weight (g)}}$$

Photography

The anatomical features were viewed through Nikon Labomed microscope under incident and translucent light and photographed using Sony digital camera fitted with Olympus adaptor. The culture tubes with seeds and seedlings were photographed in daylight using a Sony digital camera fitted with appropriate close-up accessories.

Dendrogram

At least three replicates were maintained for all treatments and control. The experiments were repeated to confirm the trends. The result of single linkage clustering (Maskay 1998) was displayed graphically in the form of a diagram called dendrogram (Everstt 1985). The similarity indices between the three varieties of green gram under study were calculated using the formula given by Bhat and Kudesia (2011).

$$\text{Similarity index} = \frac{\text{Total number of similar characters}}{\text{Total number of characters studied}} \times 100$$

Based on the similarity indices between the three varieties of green gram, dendrograms were drawn to derive the interrelationship between them and presented in Tables 5, 6 and Plates 5, 6.

RESULT AND DISCUSSION

Standardisation of culture media for seed germination

For the standardisation of culture media, seeds harvested from green gram variety *viz.*, NVL-585 grown under control condition were used (Plate 1). The seeds were inoculated on MS medium for culture initiation containing different concentration and combination of cytokinins (6-benzyl amino purine - BAP = 2.0 mgL^{-1} and Kinetin = 0.1, 0.25 and 0.5 mgL^{-1}) and auxins (IAA - Indole acetic acid = 1.0 mgL^{-1}). The combination of cytokinins (6-benzyl amino purine - BAP = 2.0 mgL^{-1} and Kinetin = 0.25 mgL^{-1}) and auxins (IAA - Indole acetic acid = 1.0 mgL^{-1}) was found to be best suited for initiating germination in seeds (Plate 1).

In vitro germination of seeds and growth of seedlings

The seeds of control and UV-B treated CO-8, NVL-585 and VAMBAN-2 varieties of green gram both in dry and wet conditions responded to *in vitro* germination (Table 1 to 2; Plate 2 to 4). The control and UV-B stressed dry and wet seeds of all the three varieties responded well to *in vitro* germination. UV-B exposure reduced root length significantly by 8.09 % to 21.43 % on 15 DAI in all the three varieties, with VAMBAN-2 performing poorly (7.14 %) in dry UV-B treatment than the control (Table 1). UV-B exposed dry and wet seeds of all varieties of green gram recorded decreased shoot growth by 9.54 % to 24.76 % compared to their respective controls (Table 1 to 2; Plate 2 to 4).

Overall, the height of the seedlings was suppressed by UV-B irradiation at both dry and wet conditions in all the varieties of green gram compared with their respective controls (Plate 2 to 4). The S / R ratio was decreased under UV-B stress on 15 DAI by 1.56 % to 6.71 % both in dry and wet seed exposures in NVL-585 and VAMBAN-2. However, UV-B stressed CO-8 dry and wet seeds showed enhanced S/R ratio by 11.11 % to 26.76 % over control. Biomass accumulation in root was inhibited by UV-B irradiation by 13.93 % to 92.63 % in both dry and wet seed treatments, the maximum being in VAMBAN-2 dry seed UV-B exposure.

A general decrease of 2 % to 41.33 % in shoot fresh weight after UV-B exposure to dry and wet seeds was observed, the maximum reduction in shoot biomass being in UV-B irradiated VAMBAN-2 dry seeds. Plant fresh biomass accumulation was inhibited in UV-B irradiated dry seeds and wet seeds of all the three varieties of green gram by 6.13 % to 67.74 % below control. Reduction in the root biomass content from 2 % to a maximum of 78.26 % was caused by UV-B treatment in both dry and wet seed treatments over control (Plate 1 to 2). UV-B exposure suppressed dry weight of shoot by 4 % to 28 % on 15 DAI over control in dry and seed treatments. Plant dry weight increased with age in control and in all treatments. But after UV-B stress, it fell below control by 6.8 % to 46.53 % on 15 DAI both after dry and wet seed exposures in all the three varieties of green gram (Table 1 to 2).

Table 1 Changes in growth parameters of three varieties of 15 DAI *Vigna radiata* (L.) Wilczek in control and UV-B irradiated dry seeds – *In vitro*.

Varieties	Treatment	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)
CO-8	Control	14	18.9	1.35	0.366	0.677	1.043	0.039	0.041	0.080
	UV-B	11	16.5	1.50	0.241	0.445	0.686	0.021	0.039	0.060
NVL-585	Control	13.6	17.5	1.28	0.512	0.579	1.091	0.053	0.049	0.102
	UV-B	12.5	15.8	1.26	0.376	0.556	0.932	0.052	0.043	0.095
VAMBAN-2	Control	7	18	2.57	0.434	0.406	0.840	0.049	0.051	0.100
	UV-B	7.5	17	2.42	0.032	0.239	0.271	0.023	0.049	0.072

Table 2 Changes in growth parameters of three varieties of 15 DAI *Vigna radiata* (L.) Wilczek in control and UV-B irradiated wet seeds – *In vitro.*

Varieties	Treatment	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)
CO-8	Control	14	19.9	1.42	0.366	0.677	1.043	0.039	0.057	0.096
	UV-B	10	18	1.87	0.315	0.664	0.979	0.031	0.041	0.072
NVL-585	Control	18	21	1.16	0.604	0.556	1.160	0.052	0.043	0.095
	UV-B	11.7	15.8	1.24	0.512	0.526	1.038	0.043	0.046	0.089
VAMBAN-2	Control	5	11.2	2.24	0.434	0.239	0.840	0.049	0.052	0.101
	UV-B	4.3	9	2.09	0.010	0.153	0.163	0.005	0.049	0.054

Table 3 Changes in foliage of three varieties of 15 DAI *Vigna radiata* (L.) Wilczek in control and UV-B irradiated dry seeds – *In vitro.*

Varieties	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)
CO-8	Control	5	13.2	1.050	0.001	0.071	0.018
	UV-B	5	1.81	0.288	0.002	0.028	0.005
NVL-585	Control	3	13.86	0.360	0.001	0.012	0.016
	UV-B	2	0.66	0.052	0.003	0.006	0.002
VAMBAN-2	Control	2	1.98	0.157	0.005	0.021	0.010
	UV-B	2	1.32	0.105	0.002	0.009	0.003

Fig. 1 K = 0.1 mgL^{-1}

Fig. 2 K = 0.25 mgL^{-1}

Fig. 3 K = 0.5 mgL^{-1}

Plate 1 Standardisation of Kinetin (K) concentration in culture media for *in vitro* seed germination using *Vigna radiata* (L.) Wilczek var. NVL-585 control samples. (7 DAI - Days after inoculation)

1 DAI **Fig. 1** Control

Fig. 2 UV-B on dry seed

Fig. 3 UV-B on soaked seed

7 DAI **Fig. 4** Control

Fig. 5 UV-B on dry seed

Fig. 6 UV-B on soaked seed

15 DAI **Fig. 7** Control

Fig. 8 UV-B on dry seed

Fig. 9 UV-B on soaked seed

Plate 3 *In vitro* seed germination and growth of *Vigna radiata* (L.) Wilczek var. NVL-585 in control and UV-B irradiated dry and soaked seeds. (DAI - Days after inoculation)

1 DAI **Fig. 1** Control

Fig. 2 UV-B on dry seed

Fig. 3 UV-B on soaked seed

7 DAI **Fig. 4** Control

Fig. 5 UV-B on dry seed

Fig. 6 UV-B on soaked seed

15 DAI **Fig. 7** Control

Fig. 8 UV-B on dry seed

Fig. 9 UV-B on soaked seed

Plate 4 *In vitro* seed germination and growth of *Vigna radiata* (L.) Wilczek var. VAMBAN-2 in control and UV-B irradiated dry and soaked seeds. (DAI - Days after inoculation)

Table 4 Changes in foliage of tree varieties of 15 DAI *Vigna radiata* (L.) Wilczek in control and UV-B irradiated wet seeds– *In vitro.*

Varieties	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)
CO-8	Control	7	4.62	0.030	0.001	0.071	0.009
	UV-B	5	1.32	0.016	0.006	0.065	0.008
NVL-585	Control	2	2.64	0.210	0.002	0.024	0.006
	UV-B	1	0.66	0.105	0.004	0.012	0.003
VAMBAN-2	Control	2	2.24	0.178	0.004	0.021	0.010
	UV-B	2	1.32	0.105	0.001	0.007	0.002

These results are in accordance with the findings of Rajendiran *et al.* (2014c) who have reported the failure of seeds of three varieties of cowpea to proliferate callus after ultraviolet-B irradiation out of the ten varieties tried for *in vitro* regeneration.

The varied responses shown by the control explants *viz.*, leaf, stem and seeds to *in vitro* culture depend on the sensitivity of the plants to *in vitro* conditions, while the extend of UV-B induced damages of the cells are responsible for delay or failure of seed germination (Rajendiran *et al.* 2014a, Rajendiran *et al.* 2014b and Rajendiran *et al.* 2014c).

Foliage of seedlings

The plants under normal condition had better number of leaves but there were fewer leaves only under UV-B stressed dry and wet seeds (Table 3 to 4; Plate 2 to 4). However, UV-B stressed VAMBAN-2 under dry and wet conditions and CO-8 under dry condition had similar number of leaves compared with their controls. NVL-585 variety of green gram under dry and wet seed UV-B exposure recorded 33.33 % to 50 % less leaves than their controls. UV-B irradiation reduced the total leaf area severely below control both under dry and wet seed treatments of all the three varieties of green gram by 33.33 % to 95.23 %, the maximum being in NVL-585 variety. The LAI was reduced by UV-B exposure to a larger extent by 41 % to 85.55 % both under dry and wet seed treatments.

The SLW in UV-B irradiated seedlings under dry and wet conditions increased manifold in CO-8 and NVL-585, while a decrease (60 to 75 %) in SLW was recorded in VAMBAN-2 under both dry and wet seed UV-B exposure. UV-B stress decreased the fresh weight of leaves by 8.40 % to 60.56 % when compared to their respective controls under dry and wet seed exposures of all the varieties of green gram (Table 3 to 4). The trend observed in fresh weight continued with dry weight of foliage also. UV-B exposure decreased the dry weight of leaves by 11.11 % to 87.50 % below control under dry and wet seed exposure in CO-8, NVL-585 and VAMBAN-2 (Table 3 to 4). These results are in accordance with the reports of Rajendiran *et al.* (2015a) in *Amaranthus dubius* Mart. Ex. Thell., Rajendiran *et al.* (2014b) in ten varieties of cowpea, Rajendiran *et al.* (2015d) in *Macrotyloma uniflorum* (Lam.) Verdc., Rajendiran *et al.* (2015e) in *Momordica charantia* L., Rajendiran *et al.* (2015f) in *Spinacia oleracea* L., Rajendiran *et al.* (2015g) in *Trigonella foenum-graecum* (L.) Ser., Rajendiran *et al.* (2015h) in *Benincasa hispida* (Thunb.) Cogn. and Rajendiran *et al.* (2015i) in *Portulaca oleracea* L. foliage harvested from seedlings grown under *in situ* normal and UV-B exposured conditions.

Dendrogram

Growth parameters in dry seeds

The growth parameters studied in three varieties of green gram under *in vitro* culture, after exposure of dry seeds to UV-B radiation, showed variations in germination of seeds, plant height, number of leaves, total leaf area, fresh weight, dry weight, and relative growth rate on 15 DAI. The similarity index between CO-8 and NVL-585 was the least with a value of 41.66 %. But CO-8 and NVL-585 varieties of green gram remained as one group recording 47.22 and 48.6 % similarity values respectively with VAMBAN-2 which stayed alone in the cluster (Table 5; Plate 5).

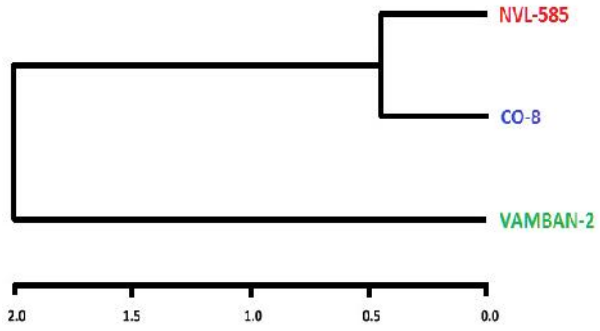

Plate 5 Dendrogram showing the interrelationship between the three varieties of *Vigna radiata* (L.) Wilczek in growth parameters in control and UV-B irradiated dry seeds - *In vitro.*

Table 5 The similarity indices in growth parameters of three varieties of *Vigna radiata* (L.) Wilczek in control and UV-B exposed dry seeds – *In vitro.*

Varieties	CO-8	NVL-585	VAMBAN-2
CO-8	100%	41.66%	47.22%
NVL-585	41.66%	100%	48.60%
VAMBAN-2	47.22%	48.60%	100%

Growth parameters in wet seeds

Three varieties of green gram under *in vitro* culture, after exposure of wet seeds to UV-B radiation, showed variations in germination of seeds, plant height, number of leaves, total leaf area, fresh weight, dry weight, and relative growth rate on 15 DAI. The least similarity index of 45.83 % brought together CO-8 and VAMBAN-2 varieties as one group. NVL-585 exhibited more affinity towards CO-8 (55.86 %) than VAMBAN-2 (54.16 %), but remained as a distant individual in the cluster (Table 6; Plate 6).

Table 6 The similarity indices in growth parameters of three varieties of *Vigna radiata* (L.) Wilczek in control and UV-B exposed wet seeds – *In vitro.*

Varieties	CO-8	NVL-585	VAMBAN-2
CO-8	100%	55.55%	45.83%
NVL-585	55.55%	100%	54.16%
VAMBAN-2	45.83	54.16%	100%

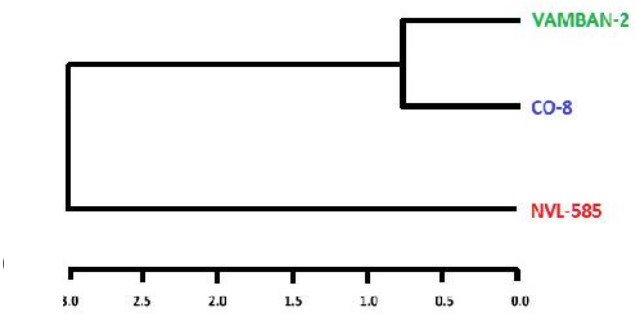

Plate 6 Dendrogram showing the interrelationship between the three varieties of *Vigna radiata* (L.) Wilczek in growth parameters in control and UV-B irradiated wet seeds - *In vitro.*

The present investigation recommends the seeds of CO-8, NVL-585 and VAMBAN-2 varieties of green gram for germplasm conservation as the seedlings of these varieties established well in culture media after UV-B stress. However, VAMBAN-2 variety of green gram was found to be less comfortable in culture medium, after wet UV-B irradiation as evident by the reduced growth of the seedlings.

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