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Research Article

IN VITRO CALLUS PROLIFERATION FROM LEAF EXPLANTS OF GREEN GRAM AFTER *IN SITU* UV-B EXPOSURE

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Abstract

Callus induction was tried with leaf explants (third leaf from top of canopy) harvested from *in situ* control and supplementary UV-B irradiated (UV-B = 2 hours daily @ 12.2 kJ m^{-2} d^{-1}; ambient = 10 kJ m^{-2} d^{-1}) three varieties of green gram *viz.* CO-8, NVL-585 and VAMBAN-2 to study their viability for germplam conservation. Callus induction occurred both in control and UV-B stressed NVL-585 leaf explants. VAMBAN-2 both in unstressed and UV-B stressed conditions did not initiate callus. Only control leaf explants from CO-8 proliferated callus. Callus of UV-B irradiated NVL-585 weighed less (51.28 %) than control. Parenchyma cells were smaller in callus inducted from *in situ* UV-B exposed NVL-585 leaf explants. The leaf explants from UV-B stressed NVL-585 varieties of green gram responded to *in vitro* callus proliferation making them fit for germplasm conservation for cultivating in UV-B elevated environment.

Key words: Callus proliferation; Green gram; Leaf explants; Three varieties; Ultraviolet-B.

Introduction

Ozone depletion is facilitated by increased stratospheric particles as provided by stratospheric clouds in the polar regions and globally by volcanic eruptions. A drop in the level of ozone allows ultraviolet-B (UV-B) radiation (280-320 nm) into the earth surface creating a dangerous atmospheric stress to wild plants and domesticated crops (Caldwell *et al*., 1998) as it destroys the leaf epidermis (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; Kokilavani and Rajendiran, 2015b) even when plants in reflex pump epicuticular wax to leaf surface rapidly (Gowsalya and Rajendiran, 2015; Thiruvarasan and Rajendiran, 2015; Vidya and Rajendiran 2015) causes stomatal aberrations in cotyledonary epidermis of germinating seedlings (Rajendiran *et al.*, 2015c; Rajendiran *et al.,* 2015d) suppresses photosynthesis (Kulandaivelu *et al.,* 1989; Sullivan *et al.,* 1994; Rajendiran, 2001) causes abnormalities in morphology thereby reducing plant growth (Rajendiran and Ramanujam, 2003; Rajendiran and Ramanujam, 2004; Kokilavani and Rajendiran, 2014o; Rajendiran *et al.*, 2015b; Rajendiran *et al.*, 2015k; Rajendiran *et al.*, 2015n; Rajendiran *et al.,* 2015p; Rajendiran *et al.,* 2015q), reduces yield (Mark and Tevini, 1997; Rajendiran and Ramanujam, 2004; Kokilavani and Rajendiran, 2014e; Rajendiran *et al.,* 2015m; Rajendiran *et al.,* 2015p; Rajendiran *et al.,* 2015q) and disturbs nodulation and symbiotic nitrogen fixation (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; Vijayalakshmi, Rajendiran 2014c; Rajendiran *et al.,* 2015l; Rajendiran *et al.,* 2015o) in many sensitive crops. Hence, *in vitro* screening needs to be carried out for selecting the germplasm of UV-B tolerant crop varieties prior to their conservation and regeneration. This study deals with identifying the varieties of green gram that can survive under supplementary UV-B irradiation and the possibilities of utilizing the leaf explants for germplasm preservation.

Materials and Methods

In situ UV-B radiation

Green gram (*Vigna radiata* (L.) Wilczek.), the nitrogen fixing grain legume 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. 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 5 DAS (days after seed germination). 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}. Leaf explants (third leaf from top of canopy) were harvested from 30 DAS crops that received supplementary UV-B irradiation and sunlight in the *in situ* condition.

In vitro culture with leaf explants

Leaf explants after appropriate aseptic treatment were used for *in vitro* culture. Leaf discs 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 leaf discs 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 mg L^{-1}) and auxins (IAA - Indole acetic acid ranging from 0.1 to 1.0 mg L^{-1}) were incorporated in the medium for inducing callus. 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 callus proliferation were recorded after 30 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 (mgL^{-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
	$MnSO_4.4H_2$	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.

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 leaf explants and callus 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, dendrogram was drawn to derive the interrelationship between them and presented in Table 2 and Plate 5.

Results and Discussion

Standardisation of culture medium for leaf explant

For the standardisation of culture media leaf explants from green gram variety *viz.*, NVL-585 grown under control condition were used (Plate 1). The explants 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 callus in leaf explants (Plate 1) and used for leaf explants harvested from all varieties of green gram (Plate 2).

In vitro callus induction

In leaf explants proliferation of callus occurred only in two green gram varieties out of the three varieties taken for study (Table 1; Plate 2, 3). Callus induction was observed in control leaf explants of CO-8 and both in control leaf explants as well as in leaf explants harvested from *in situ* supplementary UV-B irradiated NVL-585 crops. However, all leaf explants from VAMBAN-2 failed to induct callus. The induction of callus was delayed by one day in explants harvested from *in situ* UV-B irradiated NVL-585 compared with the control (Table 1). However, in CO-8 callus was inducted one day earlier to NVL-585 control. The callus of *in situ* UV-B stressed NVL-585 variety of green gram weighed less by 51.28 % compared to control. The control CO-8 had better callus as evident by an accumulation in the fresh biomass by 10 % over the control callus of NVL-585. The trend observed in fresh weight continued in dry weight of callus also. The callus of *in situ* UV-B irradiated NVL-585 variety weighed less by 78.42 % below control on 30 DAI, while CO-8 control recorded the maximum (Table 1). The parenchyma cells of calluses proliferated from leaf explants were isodiametric with thin cell walls and were distributed evenly all through the callus in control samples (Plate 4, Fig. 1, 2). The parenchyma cells that have proliferated from the *in situ* UV-B irradiated callus were smaller by 29.67 % and more in number by 17 % in NVL-585 than their control (Plate 4, Fig. 2, 3). This is in accordance with the findings of Rajendiran *et al.* (2014a) who have reported the failure of leaf explants of some of the varieties of cowpea to proliferate callus after ultraviolet-B irradiation under *in vitro* culture. Rajendiran *et al.* (2014b) and Rajendiran *et al.* (2014c) opine that the response shown by stem explants and seeds to *in vitro* culture differ based on the sensitivity of the crops to *in vitro* culture conditions. Rajendiran *et al.* (2015a) in *Amaranthus dubius* Mart. Ex. Thell., Rajendiran *et al.* (2015e) in *Macrotyloma uniflorum* (Lam.) Verdc., Rajendiran *et al.* (2015f) in *Momordica charantia* L., Rajendiran *et al.* (2015g) in *Spinacia oleracea* L., Rajendiran *et al.* (2015h) in *Trigonella foenum-graecum* (L.) Ser., Rajendiran *et al.* (2015i) in *Benincasa hispida* (Thunb.) Cogn. and Rajendiran *et al.* (2015j) in *Portulaca oleracea* L. have also recorded varied results with leaf explants of *in situ* UV-B stressed plants under *in vitro* culture. In addition, *in vitro* germination of F_1 seeds harvested from *in situ* UV-B stressed black gram (Thiruvarasan and Rajendiran 2015), cowpea (Gowsalya and Rajendiran 2015) and green gram varieties (Vidya and Rajendiran 2015) also showed differences in viability.

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* regeneration from leaf explants using *Vigna radiata* (L.) Wilczek var. NVL-585 control samples. (7 DAI - Days after inoculation)

Table 1: Characteristics of callus proliferation in leaf explants of three varieties of 30 DAI *Vigna radiata* (L.) Wilczek from control and supplementary UV-B exposed conditions - *In vitro*.

Varieties	**Treatment**	**Time taken for initiation (d)**	**Fresh weight (g)**	**Dry weight (g)**	**Parenchyma cell Frequency (µm)**	**Parenchyma cell size (µm)**	
						Length	**Breadth**
CO-8	Control	23	0.896	0.157	479. ±0.45	147.57±0.83	142.54±1.76
	UV-B	-	-	-	-	-	-
NVL-585	Control	24	1.564	0.576	389.47±1.43	124.66±0.17	78.54±0.76
	UV-B	25	0.762	0.124	323.77±1.45	87.67±0.45	65.67±0.76
VAMBAN-2	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-

Table 2: The similarity indices in callus proliferation from leaf explants of three varieties of *Vigna radiata* (L.) Wilczek after supplementary UV-B exposure - *In vitro*.

Varieties	**CO-8**	**NVL-585**	**VAMBAN-2**
CO-8	100%	42.86%	14.29%
NVL-585	42.86%	100%	-
VAMBAN-2	14.29%	-	100%

Fig. 1: CO-8 Control UV-B

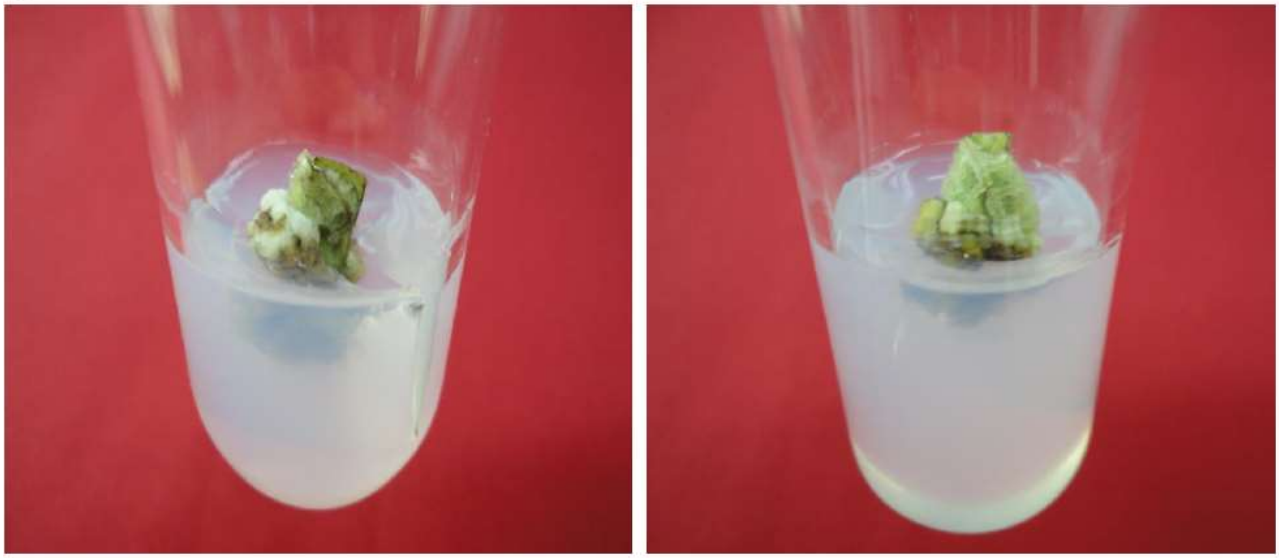

Fig. 2: NVL-585 Control UV-B

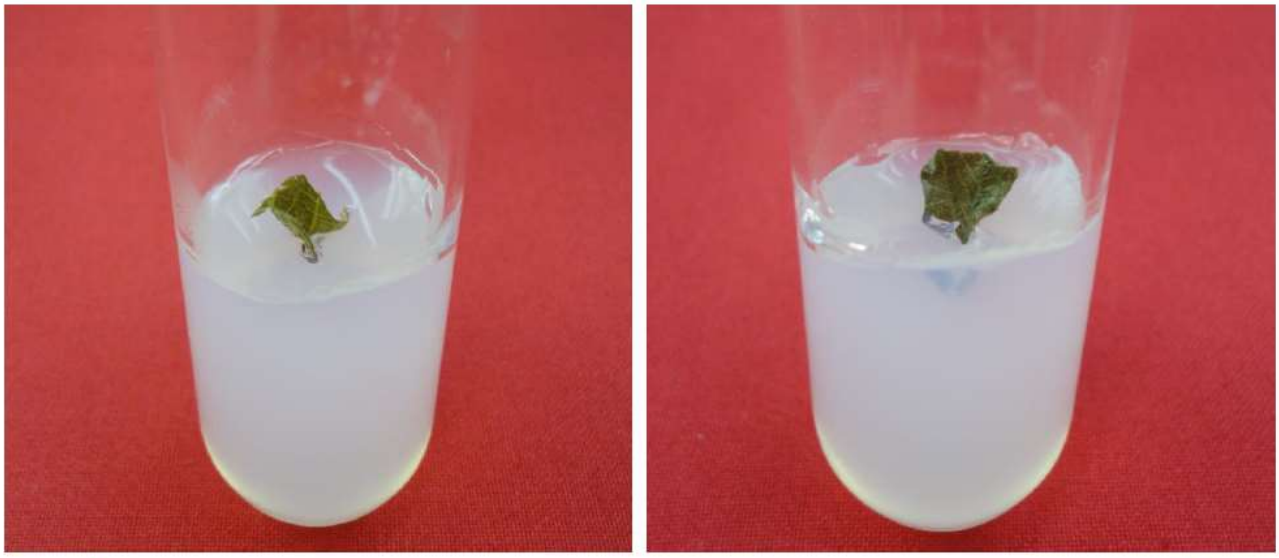

Fig. 3: VAMBAN-2 Control UV-B

Plate 2: Comparison of *in vitro* callus proliferation from leaf explants of three varieties of *Vigna radiata* (L.) Wilczek on 30 DAI (Days after inoculation).

Fig. 1: CO-8 - Control

Fig. 2: NVL-585 - Control

Fig. 3: NVL-585 - UV-B

Plate 3: A closer view of calluses formed in two out of three varieties of *Vigna radiata* (L.) Wilczek from leaf explants of control and UV-B irradiated plants.

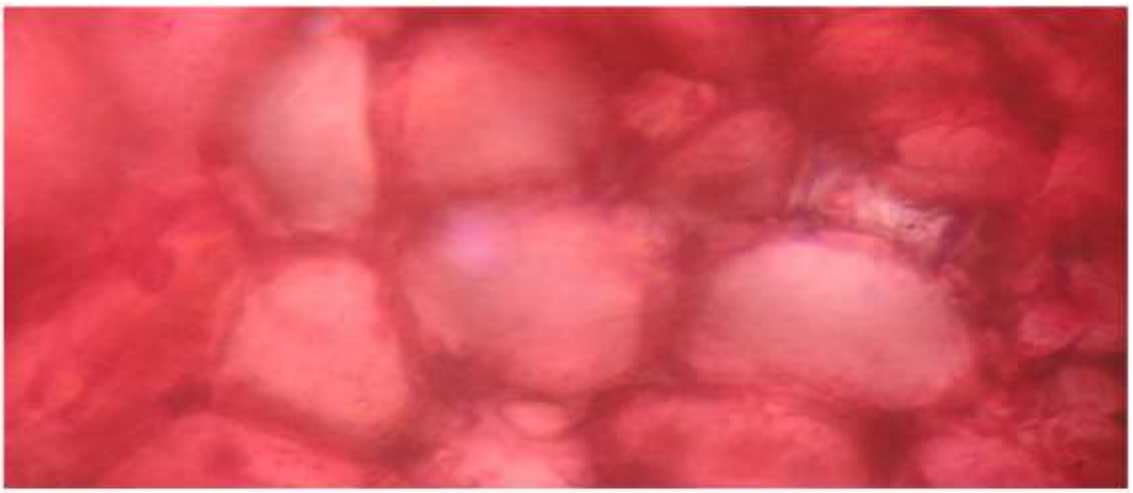

Fig. 1: CO-8 - Control

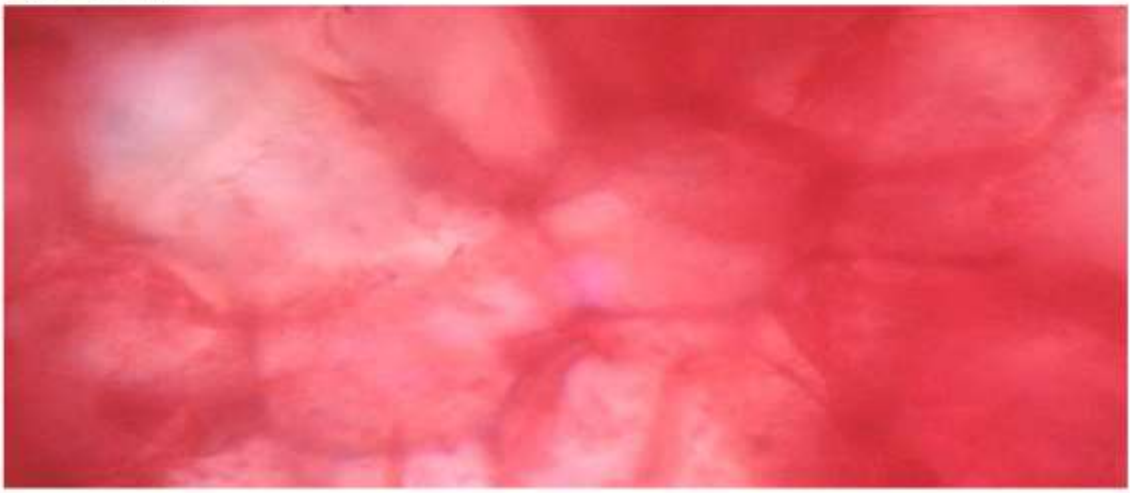

Fig. 2: NVL-585 - Control

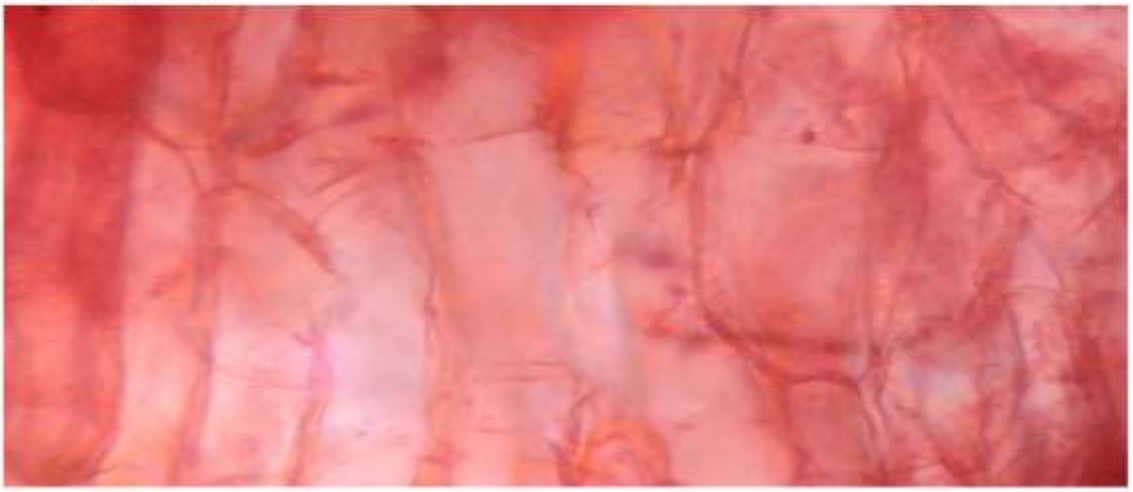

Fig. 3: NVL-585 - UV-B

Plate 4: Cross section of calluses formed in two out of three varieties of *Vigna radiata* (L.) Wilczek from leaf explants of control and UV-B irradiated plants. (All figs. 400x)

Dendrogram

The variation shown by leaf explants of three varieties of green gram after *in situ* supplementary UV-B radiation in the parameters *viz.*, time taken for callus initiation, fresh and dry weight of callus, frequency and size of parenchyma cells in callus was reflected in the dendrogram. The two varieties *viz.*, CO-8 and NVL-585 had 42.86 % similarity and they formed one group as their leaf explants harvested from either control or UV-B stressed crops or both proliferated callus. VAMBAN-2 remained alone in the cluster showing very little similarity (14.29 %) with other two varieties, as both control and UV-B stressed explants failed to induct callus (Table 2; Plate 5).

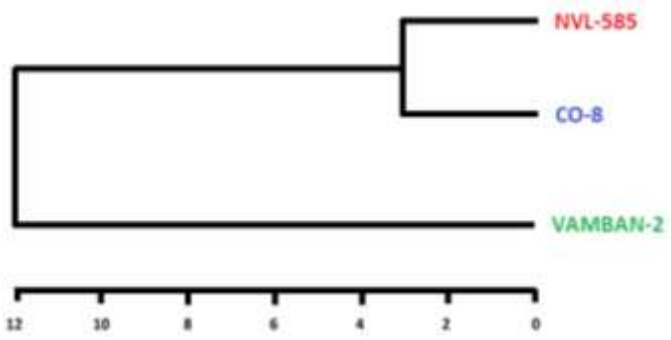

Plate 5: Dendrogram showing the interrelationship between the three varieties of *Vigna radiata* (L.) Wilczek in callus proliferation from leaf discs of control and supplementary UV-B irradiated plants - *In vitro*.

Conclusion

From the present experiment it is evident that the leaves of NVL-585 variety of green gram are the appropriate explants for germplasm conservation for surviving in UV-B elevated environment, as they are the only plant material to respond to *in vitro* culture after UV-B irradiation.

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