International Journal of Food, Agriculture and Veterinary Sciences ISSN: 2277-209X (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jfav.htm
2014 Vol. 4 (3) September-December, pp. 190-199/ Rajendiran et al.
Research Article

IN VITRO CALLUS PROLIFERATION FROM STEM EXPLANTS OF TEN VARIETIES OF COWPEA AFTER *IN SITU* ULTRAVIOLET-B EXPOSURE

***Rajendiran K., Kokilavani V. and Murugananthan P.**
Department of Botany, K.M. Centre for Post Graduate Studies, Pondicherry – 605 008
**Author for Correspondence*

ABSTRACT

The *in vitro* callus proliferation carried out with ten varieties *viz.* CW-122, COVU-1, COFC-8, CO-1, COVU-2, KM-1, CO-6, VAMBAN, CO-3 and PUDUVAI of cowpea, *Vigna unguiculata* (L.) Walp. 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 samples. Callus induction was tried with stem explants (nodal region from 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}) ten varieties of cowpea to study their viability for germplam storage. Callus induction occurred in COFC-8, CO-6 and VAMBAN both in control and UV-B irradiated stem explants. However, only control stem explants from CO-1 and CO-3 proliferated callus. UV-B irradiated crop varieties delayed callus induction, reduced callus biomass and produced smaller and more parenchyma cells. The callus of *in situ* UV-B stressed CO-6 variety of cowpea accumulated 91.69 % fresh biomass more than that of control. The present study proves that nodal stem explants from COFC-8, CO-6 and VAMBAN varieties of cowpea are the best choice for germplasm conservation for cultivation in UV-B elevated environment, as they responded to *in vitro* culture by proliferating callus profusely.

Keywords: *Ultraviolet-B, Cowpea, Ten Varieties, Stem Explant, Callus Proliferation*

INTRODUCTION

Ozone depletion threatens to continue due to increases in ozone depleting substances (ODS) as well as thickness of green house gases around the earth released by human activities. The heat that normally would escape the troposphere and enter the stratosphere no longer does so, making the stratosphere cooler. Far below normal temperatures in this layer increases ozone depletion. As a result, the UV-B radiation will increase, affecting the ecosystems. Elevated ultraviolet-B (UV-B) radiation (280-320 nm) is a dangerous atmospheric stress (Caldwell *et al*., 1983) as it affects foliar epidermis (Bornman and Vogelmann, 1991; Rajendiran and Ramanujam, 2000; 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; Arulmozhi and Rajendiran, 2014; Vijayalakshmi and Rajendiran, 2014) in sensitive plants. Hence 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 germplasms. On this line, the present work is an attempt for the first time to find out the best variety of cowpea that can tolerate supplementary UV-B irradiation and to identify the germplasm of the crop for conservation and regeneration through tissue culture method.

MATERIALS AND METHODS

Cowpea (*Vigna unguiculata* (L) Walp.), the nitrogen fixing grain legume was chosen for the study. Viable seeds of the ten varieties of cowpea *viz.* CW-122, COVU-1, COFC-8, CO-1, COVU-2, KM-1, CO-6,

VAMBAN, CO-3 were procured from Saravana Farms, Villupuram, Tamil Nadu and PUDUVAI 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}. Nodal shoot segments (stem explants) from third node from top of canopy were harvested from 30 DAS crops that received supplementary UV-B irradiation and sunlight in the *in situ* condition.

Nodal shoot segments (stem explants) after appropriate aseptic treatment were used for *in vitro* culture. Nodal shoot segments 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 stem 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 were recorded after 15 DAI (days after inoculation) and callus proliferation after 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.

Chemical Composition of MS Medium **(Murashige and Skoog, 1962)**

Constituents	Quantity (mg l^{-})
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

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 stem 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 term dendrogram is used in numerical taxonomy for any graphical drawing giving a tree-like description of a taxonomic system. The similarity indices between the ten varieties of cowpea 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 ten varieties of cowpea, dendrograms were draw to derive the interrelationship between them and presented in tables and plates.

RESULTS AND DISCUSSION

Proliferation of callus occurred in stem explants of only in five out of ten varieties of cowpea taken for study (Table 1; Plate 1). Callus induction was observed in COFC-8, CO-6 and VAMBAN both in control stem explants as well as in stem explants harvested from *in situ* supplementary UV-B irradiated crops (Table 1; Plate 1, 2). However, only control stem explants from CO-1 and CO-3 proliferated callus (Table 1; Plate 1, 2). 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 (Table 1).

Figure 1: CW-122 C **UV-B**

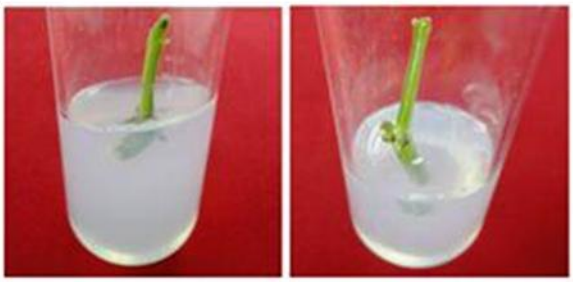

Figure 2: COVU-1 C **UV-B**

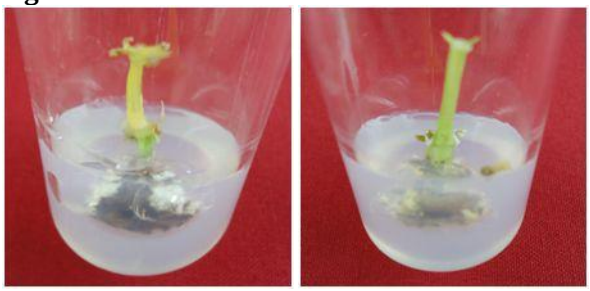

Figure 3: COFC-8 C **UV-B**

Figure 4: CO-1 C **UV-B**

Figure 5: COVU-2 C **UV-B**

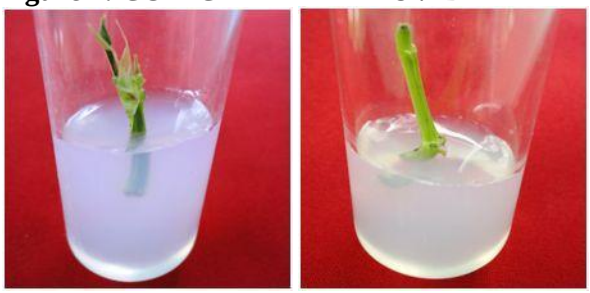

Figure 6: KM-1 C **UV-B**

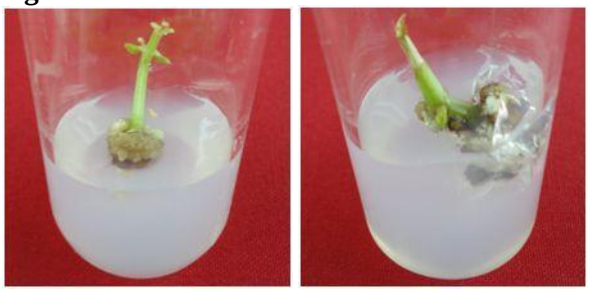

Figure 7: CO-6 C **UV-B**

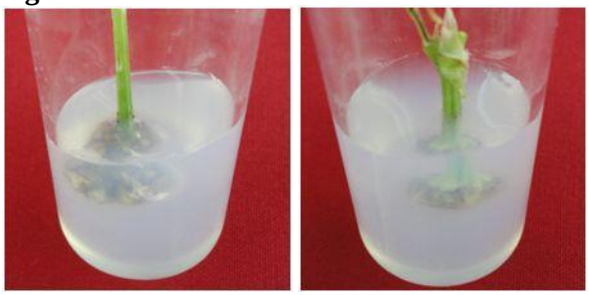

Figure 8: VAMBAN C **UV-B**

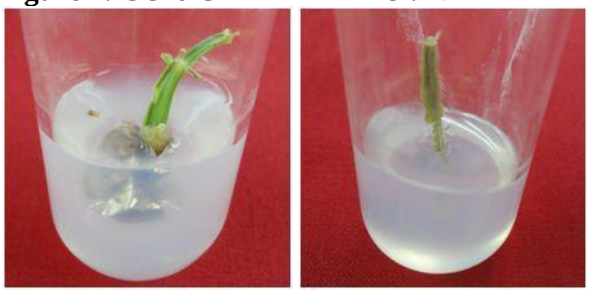

Figure 9: CO-3 C **UV-B**

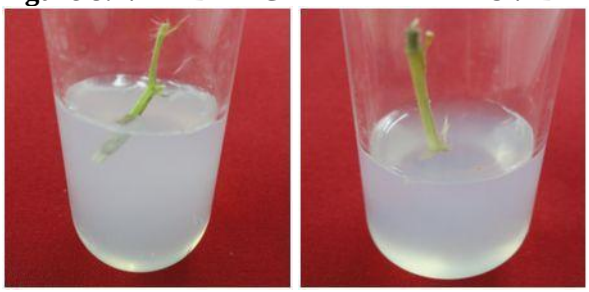

Figure 10: PUDUVAI C **UV-B**

Plate 1: *In vitro* callus proliferation from stem explants in 5 out of 10 varieties of *Vigna unguiculata* (L.) Walp. of control (C) and Ultraviolet-B (UV-B) irradiated plants

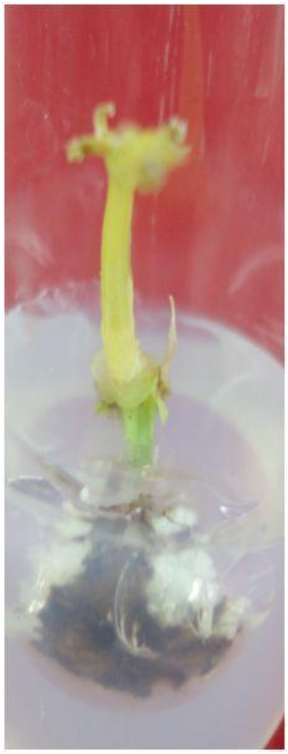

Figure 1: COFC-8 C

Figure 2: COFC-8 UV-B

Figure 3: CO-6 C

Figure 4: CO-6 UV-B

Figure 5: VAMBAN C

Figure 6: VAMBAN UV-B

Figure 7: CO-1 C

Figure 8: CO-3 C

Plate 2: A closer view of callus formed in 5 varieties of *Vigna unguiculata* (L.) Walp. from stem explants of control (C) and Ultraviolet-B (UV-B) irradiated plants.

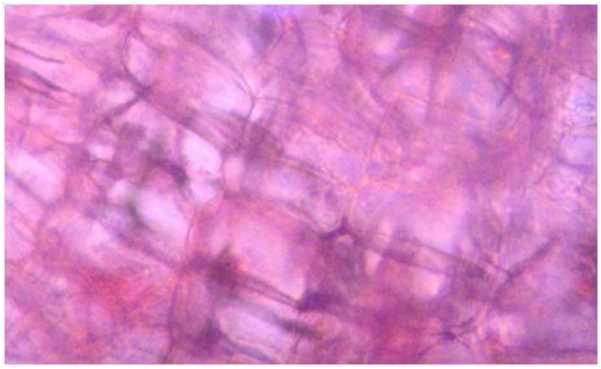

Figure 1: COFC-8 Control

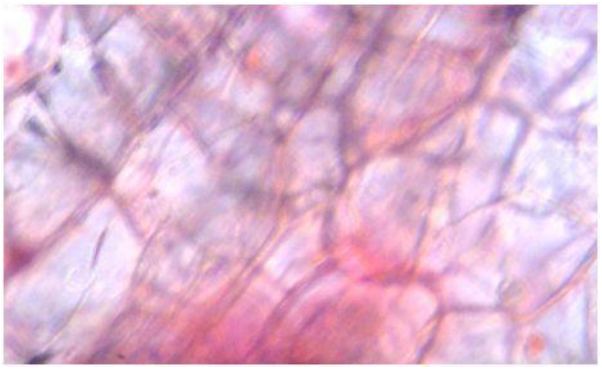

Figure 2: COFC-8 UV-B

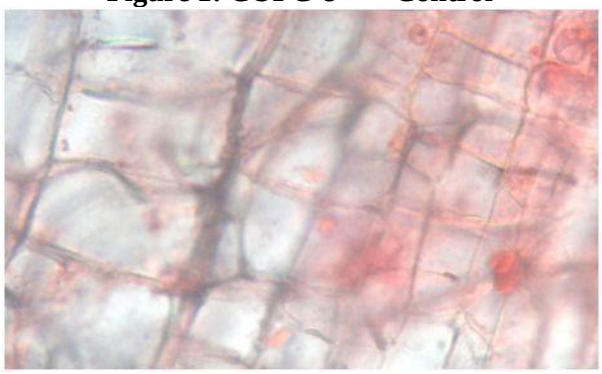

Figure 3: CO-1 Control

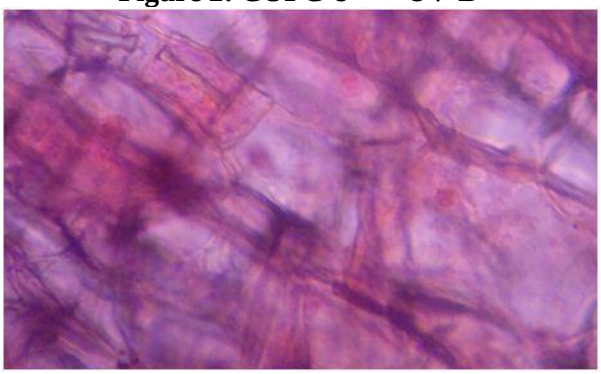

Figure 4: CO-3Control

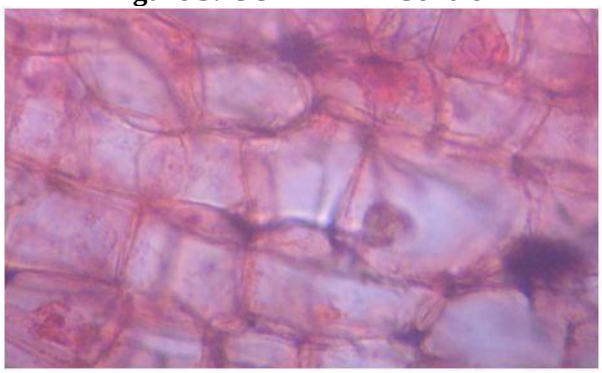

Figure 5: CO-6 Control

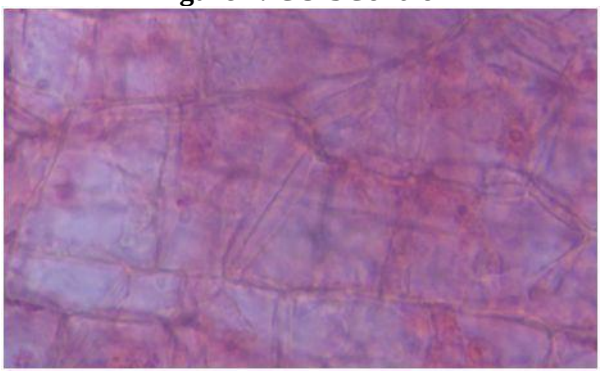

Figure 6: CO-6 UV-B

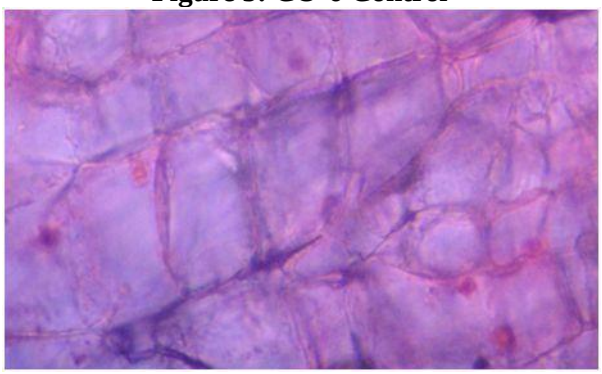

Figure 7: VAMBAN Control

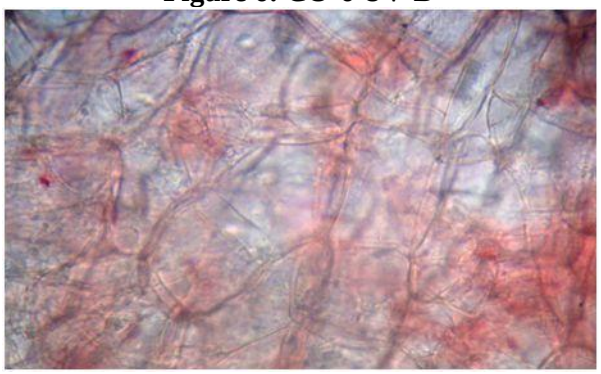

Figure 8: VAMBAN UV-B

Plate 3: Cross section of callus formed in 5 varieties of *Vigna unguiculata* (L.) Walp. from stem explants of control and UV-B irradiated plants. (All figs 400x)

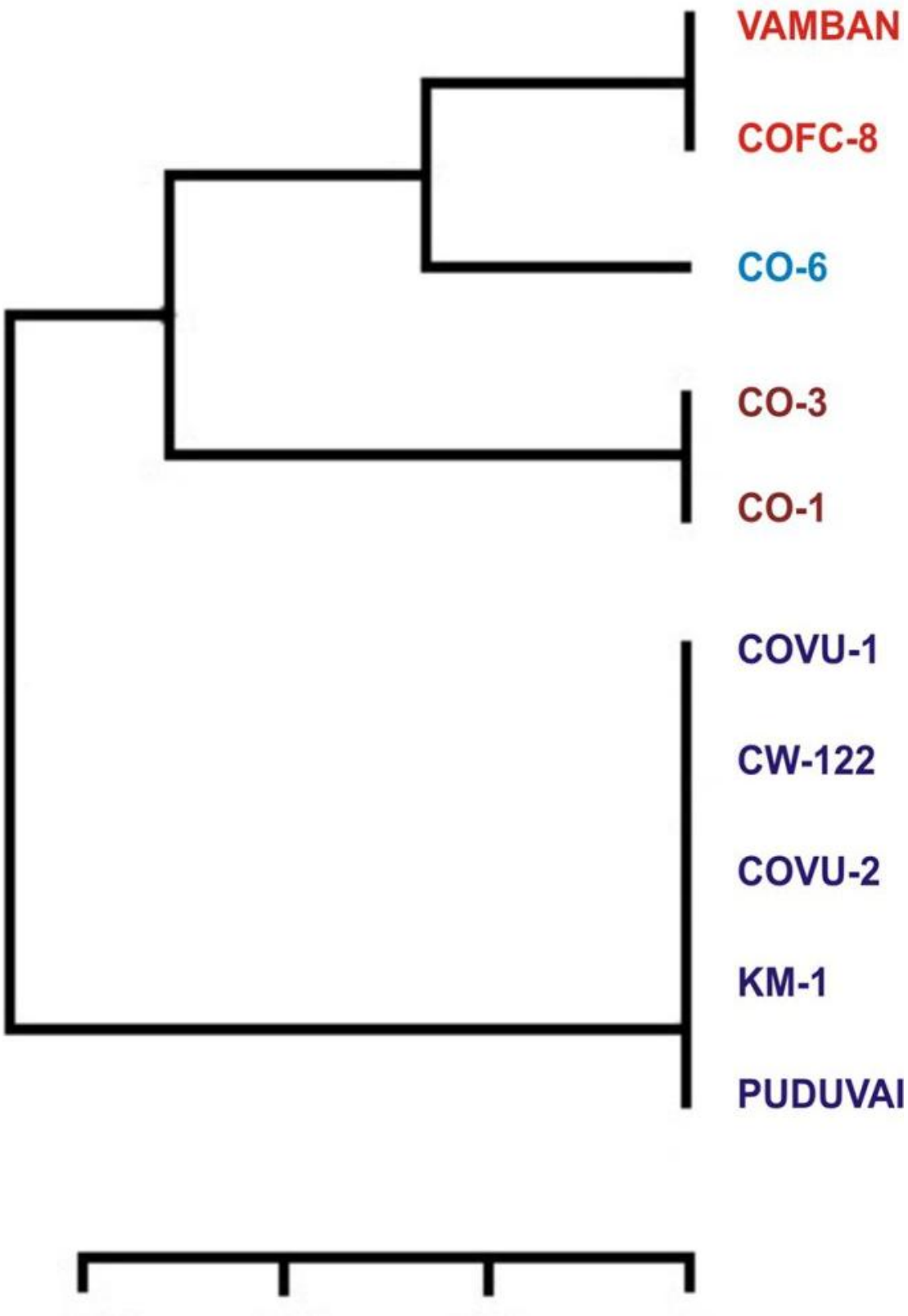

Plate 4: Dendrogram showing the interrelationship between the ten varieties of *Vigna unguiculata* (L.) Walp. in callus proliferation from stem explants of supplementary UV-B irradiated plants

Table 1: Characteristics of callus proliferation in stem explants of ten varieties of 30 DAI *Vigna unguiculata* (L.) Walp. from control and supplementary UV-B exposed conditions

Varieties	Treatment	Time taken for initiation (d)	Fresh weight (g)	Dry weight (g)	Parenchyma cell Frequency (µm)	Parenchyma cell size (µm) Length	Breadth
CW-122	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-
COVU-1	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-
COFC-8	Control	22	0.754	0.163	290.42 ± 0.73	161.52 ± 1.85	126.37 ± 1.21
	UV-B	24	0.691	0.115	316.83 ± 1.45	88.34 ± 0.46	61.63 ± 0.62
CO-1	Control	26	1.464	0.588	620.43 ± 1.22	125.86 ± 0.11	79.14 ± 0.56
	UV-B	-	-	-	-	-	-
COVU-2	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-
KM-1	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-
CO-6	Control	24	0.603	0.093	448.85 ± 2.04	154.26 ± 0.95	140.81 ± 0.53
	UV-B	25	0.915	0.194	594.06 ± 0.93	74.64 ± 1.22	70.72 ± 0.32
VAMBAN	Control	22	1.482	0.722	369.45 ± 0.74	167.45 ± 0.27	132.91 ± 0.48
	UV-B	25	0.761	0.108	475.22 ± 1.66	67.63 ± 1.44	53.42 ± 0.97
CO-3	Control	24	0.986	0.142	488.41 ± 0.27	149.61 ± 0.93	141.35 ± 1.28
	UV-B	-	-	-	-	-	-
PUDUVAI	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-

Table 2: The similarity indices in callus proliferation from stem explants of ten varieties of *Vigna unguiculata* (L.) Walp. after supplementary UV-B exposure

Varieties	CW-122	COVU-1	COFC-8	CO-1	COVU-2	KM-1	CO-6	VAMBAN	CO-3	PUDUVAI
CW-122	-	-	-	-	-	-	-	-	-	-
COVU-1	-	-	-	-	-	-	-	-	-	-
COFC-8	-	-	100%	33.3%	-	-	66.6%	100%	33.3 %	-
CO-1	-	-	33.3%	100%	-	-	50%	33.3%	100 %	-
COVU-2	-	-	-	-	-	-	-	-	-	-
KM-1	-	-	-	-	-	-	-	-	-	-
CO-6	-	-	66.6%	50%	-	-	100%	66.6%	50%	-
VAMBAN	-	-	100%	33.3%	-	-	66.6%	100%	33.3 %	-
CO-3	-	-	33.3%	100%	-	-	50%	33.3%	100 %	-
PUDUVAI	-	-	-	-	-	-	-	-	-	-

A reduction in the fresh biomass of callus by 8.30 % in COFC-8 and by 48.64 % in VAMBAN was caused by *in situ* supplementary UV-B treatment. However, the callus of *in situ* UV-B stressed CO-6 variety of cowpea accumulated 91.69 % fresh biomass more than that of control (Table 1). The inhibitory tendency of UV-B continued in dry weight of callus of COFC-8 and VAMBAN also. The callus of *in situ* UV-B irradiated CO-6 variety weighed more by 107.04 % above control on 30 DAI (Table 1).

The parenchyma cells appeared isodiametric with thin cell walls and were distributed evenly all through the callus in control samples. The parenchyma cells that have proliferated from the *in situ* UV-B irradiated calluses were comparatively smaller and more in number by 9.09 % in COFC-8, by 32.35 % in CO-6 and by 28.57 % in VAMBAN over their controls (Table 1; Plate 3). Over all, the size of the parenchyma cells were reduced by 45.33 to 59.80 % in all the calluses inducted from *in situ* UV-B exposed COFC-8, CO-6 and VAMBAN explants (Table 1; Plate 3).

The time taken for callus initiation, fresh and dry weight of callus, frequency and size of parenchyma cells in callus in stem explants of ten varieties of cowpea exhibited differences on 30 DAI after *in situ* supplementary UV-B radiation. The similarity index between five varieties *viz.*, COVU-1, CW-122, COVU-2, KM-1 and PUDUVAI was 100 % and they formed one group showing 100 % similarities as the stem explants harvested from both control and UV-B stressed crops failed to produce callus (Table 2; Plate 4). VAMBAN together with COFC-8 leveled with CO-6 as the explants from both control and UV-B irradiated crops of three varieties responded well in callus proliferation. The other group consisting of CO-3 and CO-1 with 100 % similarity between them proliferated callus only in control explants and so related with VAMBAN, COFC-8 and CO-6 (Table 2; Plate 4). The nodal stem explants from COFC-8, CO-6 and VAMBAN varieties of cowpea responded quickly to *in vitro* callus proliferation proving that they are the best explants for germplasm conservation for cultivating in UV-B elevated environment in future.

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

REFERENCES
Arulmozhi D and Rajendiran K (2014). Effect of supplementary ultraviolet-B radiation on nodulation and nitrogen metabolism in *Lablab purpureus* L. var. Goldy. *International Journal of Advanced Biological Research* **4**(3) 343-346.
Bhat TM and Kudesia R (2011). Evaluation of Genetic Diversity in Five Different Species of Family Solanaceae using Cytological Characters and Protein Profiling. *Genetic Engineering and Biotechnology Journal* 1- 8.
Bornman JF and Vogelmann TC (1991). Effect of UV-B radiation on leaf optical properties measured with fibre optics. *Journal of Experimental Botany* **42** 647 - 554.
Caldwell MM, Gold WG, Harris G and Ashurst CW (1983). A modulated lamp systam for solar UV-B (280-320 nm supplementation studies in the field. *Photochemistry and Photobiology* **37** 479 - 485.
Everstt B (1985). *Clustering Analysis* (John Wiley and Sons, New York).
Kokilavani V and Rajendiran K (2013). Ultraviolet-B induced changes in the leaf epidermal and anatomical characteristics of *Vigna mungo* L. var. KM-2. *International Journal of Science and Nature* **5**(1) 126-130.
Kokilavani V and Rajendiran K (2014a). Ultraviolet-B induced changes in the leaf architecture of *Cucumis sativus* L. var. CO 1. *International Journal of Geology, Earth and Environmental Sciences* **4**(2) 208-215.
Kokilavani V and Rajendiran K (2014b). Alterations in leaf architecture of *Ocimum sanctum* L. under elevated ultraviolet-B stress. *Global Journal of Bio-Science and Biotechnology* **3**(4) 374-378.
Maskay N (1998). Single linkage clustering. In: *Encyclopedia of Biostatistics*, edited by Armintage P and Cotton T (Wiley, New York) **5** 4121- 4122.
Murashige T and Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum***15** 473 - 497.
Rajendiran K and Ramanujam MP (2000). Efficacy of triadimefon treatment in ameliorating the UV-B stress in green gram. - In: *National Symposium on Environmental Crisis and Security in the New Millennium*, edited by Khan M (National Environmental Science Academy, New Delhi) 41-42.
Rajendiran K and Ramanujam MP (2003). Alleviation of ultraviolet-B radiation-induced growth inhibition of green gram by triadimefon. *Biologia Plantarum* **46** 621-624.
Rajendiran K and Ramanujam MP (2004). Improvement of biomass partitioning, flowering and yield by triadimefon in UV-B stressed *Vigna radiata* (L.) Wilczek. *Biologia Plantarum* **48** 145-148.
Rajendiran K and Ramanujam MP (2006). Interactive effects of UV-B irradiation and triadimefon on nodulation and nitrogen metabolism in *Vigna radiata* plants. *Biologia Plantarum* **50**(4) 709-712.
Sudaroli Sudha J and Rajendiran K (2013a). Effect of elevated UV-B irradiation on the nodulation and nitrogen metabolism in *Sesbania grandiflora* (L.) Pers. *International Journal of Science and Nature* **4**(4) 664 - 667.
Sudaroli Sudha J and Rajendiran K (2013b). Effect of elevated UV-B irradiation on the nodulation and nitrogen metabolism in *Vigna unguiculata* (L.) Walp. c.v. BCP-25. *International Journal of Food, Agriculture and Veterinary Sciences* **3**(3) 77 - 81.
Vijayalakshmi R and Rajendiran K (2014). Impact of ultraviolet-B radiation on nodulation and nitrogen metabolism in *Phaseolus vulgaris* L. cv. Prevail. *International Journal of Advanced Biological Research* **4**(3) 339 - 342.