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Research Article

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

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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}) ten varieties of cowpea *viz.* CW-122, COVU-1, COFC-8, CO-1, COVU-2, KM-1, CO-6, VAMBAN, CO-3 and PUDUVAI to study their viability for germplam conservation. Callus induction was recorded in COFC-8, CO-1, CO-6 and VAMBAN both from control and UV-B leaf explants harvested from *in situ* condition. But only control leaf explants from CO-3 proliferated callus. UV-B delayed callus induction, depressed biomass accumulation and proliferated smaller and numerous parenchyma cells. However, accumulation of fresh biomass by 0.82 % in VAMBAN and by 6.53 % in COFC-8 over control was recorded in the *in situ* supplementary UV-B treated callus. Both the stem and leaf explants harvested from normal and elevated UV-B irradiated *in situ* grown varieties of COFC-8, VAMBAN, CO-6 and only leaf explants of CO-1 inducted callus. However callus induction occurred in both control stem and leaf explants of CO-3 and only in control stem explants of CO-1. As only the leaf explants from COFC-8, CO-1, CO-6 and VAMBAN varieties of cowpea responded to *in vitro* callus proliferation, they are the best explants for germplasm conservation for cultivating in UV-B elevated environment in future.

Keywords: *Ultraviolet-B, Cowpea, Ten Varieties, Callus Proliferation, Leaf Explants*

INTRODUCTION
The ozone layer in the stratosphere protects life on earth from exposure to dangerous levels of ultraviolet light. It does so by filtering out harmful ultraviolet radiation from the sun. When ozone-degrading chemicals are emitted, they mix with the atmosphere and eventually rise to the stratosphere. Chlorine and bromine catalyzes the destruction of ozone. This destruction is occurring at a more rapid rate than ozone can be created through natural processes. The degradation of the ozone layer leads to higher levels of ultraviolet radiation reaching Earth's surface. This in turn can lead to a greater incidence of skin cancer, cataracts, and impaired immune systems, and is expected also to reduce crop yields, diminish the productivity of the oceans, and possibly to contribute to the decline of amphibious populations that is occurring around the world. Ultraviolet-B (UV-B) radiation (280-320 nm) is a dangerous atmospheric stress (Caldwell *et al.*, 1983) as it was found to affect foliar epidermis (Bornman and Vogelmann, 1991; Rajendiran and Ramanujam, 2000; Kokilavani and Rajendiran, 2013; Kokilavani and Rajendiran, 2014a; Kokilavani and Rajendiran, 2014b), suppress photosynthesis (Rajendiran and Ramanujam, 2003; Rajendiran and Ramanujam, 2004) and inhibit 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 all sensitive crops. In this context, *in vitro* screening methods have to be developed to select suitable crop varieties that can survive in elevated UV-B radiation and also to conserve their germplasms. The present study was carried out to find the varieties of cowpea that can tolerate supplementary UV-B exposure as well as to identify the germplasm of the crop for conservation and regeneration through *in vitro* culture.

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}. 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.

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 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.

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

Constituents	Quantity (mgl^{-})
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

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 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).

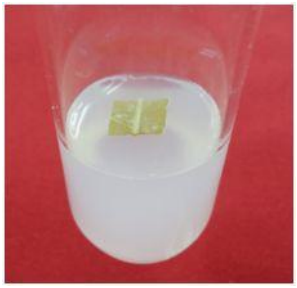
$$\text{Similarity index} = \frac{\text{Total number of similar characters}}{\text{Total number of characters studied}} \times 100$$

Based on the similarity indices between the ten varieties of cowpea, dendrograms were draw.

RESULTS AND DISCUSSION

In leaf explants proliferation of callus occurred only in five cowpea varieties out of the ten varieties taken for study. Callus induction was observed in COFC-8, CO-1, CO-6 and VAMBAN both in control leaf explants as well as in leaf explants harvested from *in situ* supplementary UV-B irradiated crops (Table 1; Plate 1, 2). However, only control leaf explants from 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. However, in CO-1 callus was inducted on the same day as that of the control (Table 1).


Figure 1: CW-122 C


UV-B

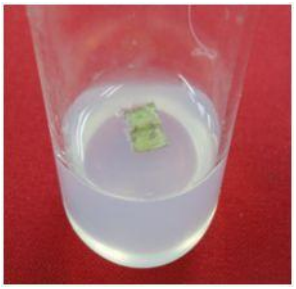


Figure 2: COVU-1 C


UV-B


Figure 3: COFC-8 C


UV-B


Figure 4: CO-1 C

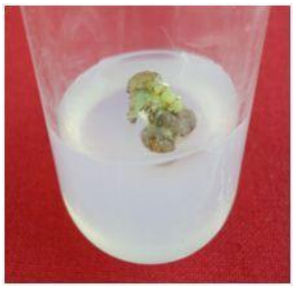

UV-B


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 leaf 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 Control

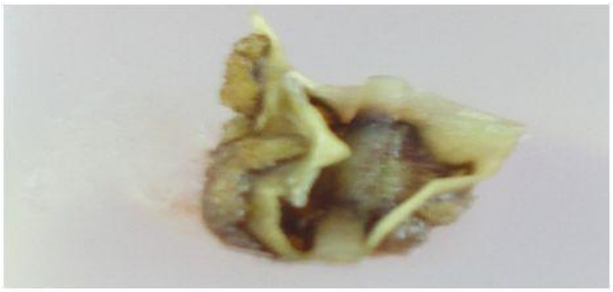


Figure 2: COFC-8 UV-B

Figure 3: CO-1 Control

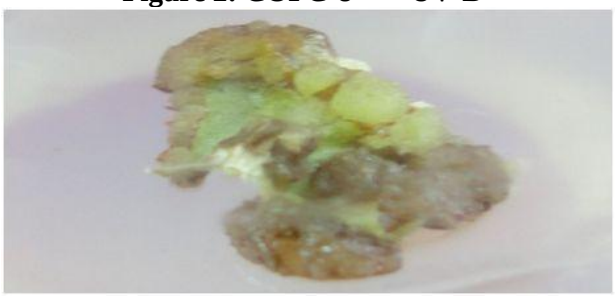


Figure 4: CO-1UV-B

Figure 5: CO-6 Control

Figure 6: CO-6 UV-B

Figure 7: VAMBAN Control

Figure 8: VAMBAN UV-B

Figure 9: CO-3Control

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

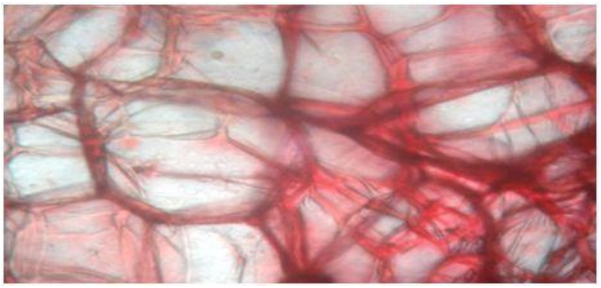


Figure 1: COFC-8 Control

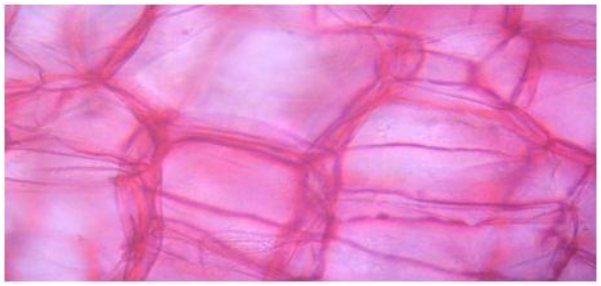


Figure 2: COFC-8 UV-B

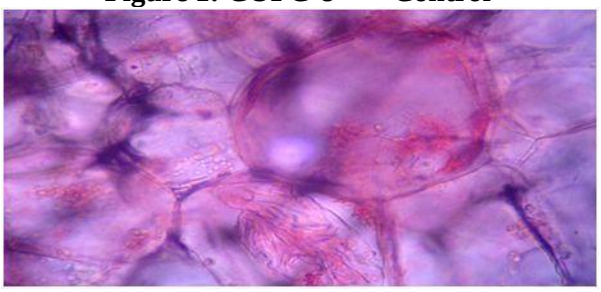


Figure 3: CO-1 Control

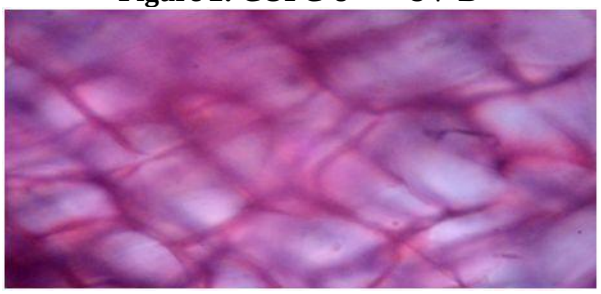


Figure 4: CO-1UV-B

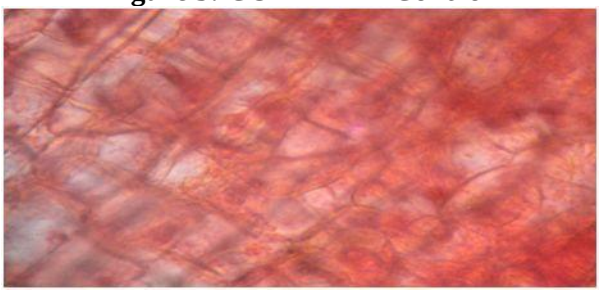


Figure 5: CO-6 Control

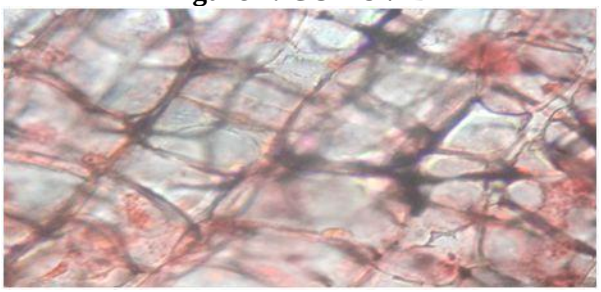


Figure 6: CO-6 UV-B

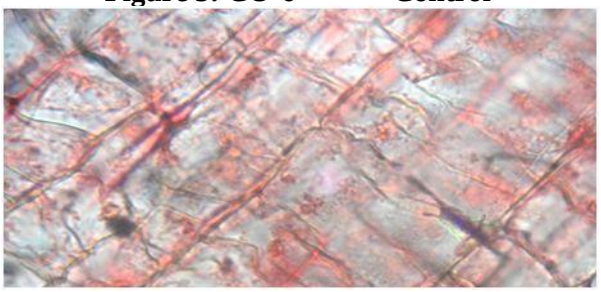


Figure 7: VAMBAN Control

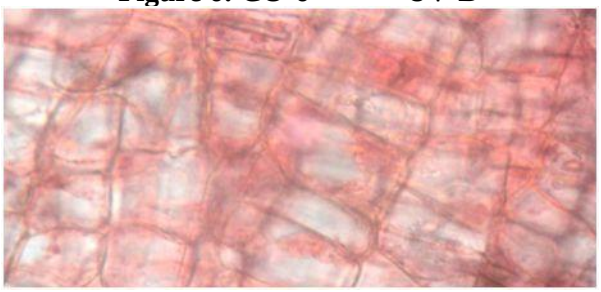


Figure 8: VAMBAN UV-B

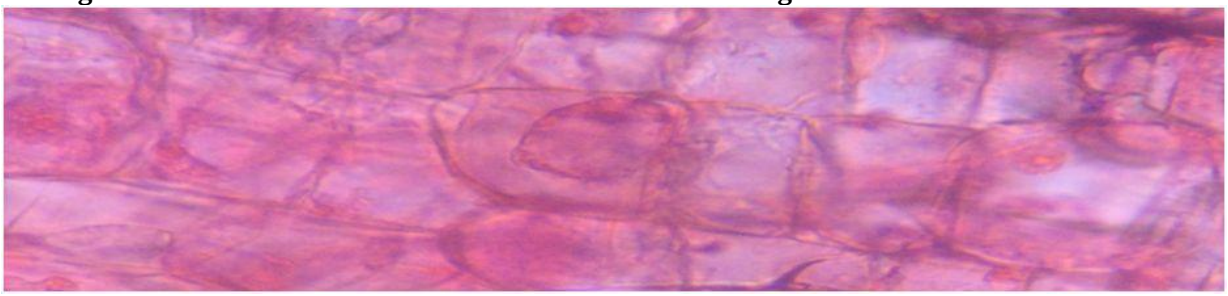


Figure 9: CO-3Control

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

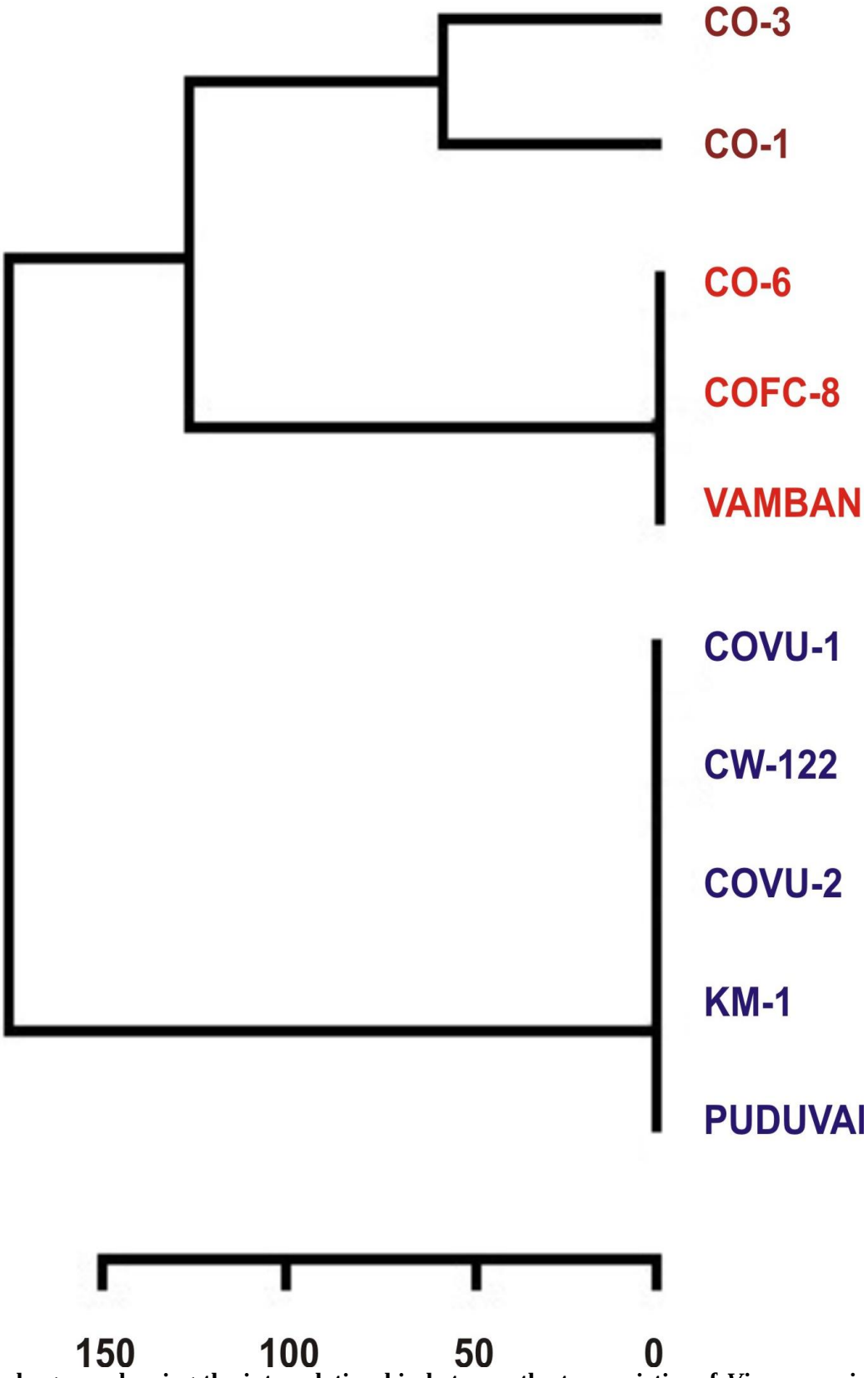


Plate 4: Dendrogram showing the interrelationship between the ten varieties of *Vigna unguiculata* (L.) Walp. in callus proliferation from leaf discs of supplementary UV-B irradiated plants

Table 1: Characteristics of callus proliferation in leaf explants of ten varieties of 30 DAI *Vigna unguiculata* (L.) Walp. 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
CW-122	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-
COVU-1	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-
COFC-8	Control	24	0.442	0.170	382.81 ± 1.24	178.33 ± 0.41	189.23 ± 1.39
	UV-B	26	0.470	0.191	475.25 ± 0.58	66.41 ± 0.96	74.85 ± 0.27
CO-1	Control	22	0.528	0.092	514.23 ± 3.60	218.83 ± 0.64	246.44 ± 0.32
	UV-B	22	0.515	0.086	561.22 ± 2.11	83.61 ± 1.58	88.13 ± 1.46
COVU-2	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-
KM-1	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-
CO-6	Control	24	0.753	0.213	633.66 ± 0.47	132.73 ± 1.91	176.36 ± 1.21
	UV-B	26	0.445	0.064	686.45 ± 0.29	60.12 ± 2.54	52.85 ± 1.40
VAMBAN	Control	25	0.567	0.072	475.24 ± 1.77	158.44 ± 0.14	171.66 ± 0.36
	UV-B	26	0.572	0.069	580.85 ± 2.46	50.62 ± 1.17	66.13 ± 0.52
CO-3	Control	24	0.465	0.057	396.06 ± 0.48	136.43 ± 1.91	118.85 ± 2.37
	UV-B	-	-	-	-	-	-
PUDUVAI	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-

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

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%	50%	-	-	100%	100%	33.3 %	-
CO-1	-	-	50%	100%	-	-	50%	50%	66.6 %	-
COVU-2	-	-	-	-	-	-	-	-	-	-
KM-1	-	-	-	-	-	-	-	-	-	-
CO-6	-	-	100%	50%	-	-	100%	100%	33.3 %	-
VAMBAN	-	-	100%	50%	-	-	100%	100%	33.3 %	-
CO-3	-	-	33.3%	66.6%	-	-	33.3%	33.3%	100 %	-
PUDUVAI	-	-	-	-	-	-	-	-	-	-

An accumulation in the fresh biomass by 0.82 % in VAMBAN and by 6.53 % in COFC-8 was recorded in the *in situ* supplementary UV-B treated callus. However, the callus of *in situ* UV-B stressed CO-1 and CO-6 varieties of cowpea weighed less by 2.44 % and 40.89 % compared to control (Table 1). The trend observed in fresh weight continued in dry weight of callus also. The callus of *in situ* UV-B irradiated COFC-8 variety weighed more by 12.38 % above control on 30 DAI, while CO-1, CO-6 and VAMBAN recorded a reduction by 3.87 to 69.97 % (Table 1).

The parenchyma cells of calluses proliferated from leaf explants were similar to that of stem explants in being 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 smaller and more in number by 5.25 % in CO-1, by 8.33 % in CO-6, by 22.22 % in VAMBAN and by 24.13 % in COFC-8 over their controls (Table 1; Plate 3). The size of the parenchyma cells were reduced by 54.7 to 68.05 % in all the calluses inducted from *in situ* UV-B exposed COFC-8, CO-1, 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 of leaf explants from ten varieties of cowpea after *in situ* supplementary UV-B radiation showed variations when analyzed through dendrogram. The five varieties *viz.*, COVU-1, CW-122, COVU-2, KM-1 and PUDUVAI had 100 % similarity and they formed one group as the stem explants harvested from both control and UV-B stressed crops failed to proliferate callus. CO-6, COFC-8 and VAMBAN grouped together as the explants from both control and UV-B irradiated crops of three varieties initiated callus (Table 2; Plate 4). The other group consisted of CO-3 and CO-1 with 100 % similarity between them. Even though both the control and UV-B stressed explants of CO-1 proliferated callus, due to similarities in other parameters CO-3 equalled CO-1 as one group.

From the present study it is evident that leaf explants from COFC-8, CO-1, CO-6 and VAMBAN varieties of cowpea are the fittest for germplasm conservation for cultivating in UV-B elevated environment, as they proliferated callus under *in vitro* condition.

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