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RESEARCH ARTICLE

IN VITRO CALLUS PROLIFERATION FROM LEAF EXPLANTS OF THREE VARIETIES OF COWPEA AFTER IN SITU UV-B RADIATION

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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}) GOWMATHI, FOLA and NS-634 varieties of cowpea to study their efficiency for germplam conservation. Callus induction occurred both in control and UV-B stressed GOWMATHI leaf explants. Both control and UV-B stressed FOLA leaf explants failed to initiate callus. Induction of callus occurred in UV-B stressed NS-634 leaf explants alone, while no callus was formed by the control. As only the leaf explants from GOWMATHI and NS-634 varieties of cowpea responded to *in vitro* callus proliferation, they are the best suited for germplasm conservation for cultivating in UV-B elevated habitat.

INTRODUCTION

Stratospheric ozone layer that guards the Earth from harmful ultraviolet radiation, was destroyed directly by ozone depleting substances (ODS) and indirectly by green house gases formed by human activity. These two processes have added a siphon downstream, removing ozone faster than natural ozone creation reactions can make. Ultraviolet-B (UV-B) radiation (280-320 nm) is a dangerous atmospheric stress to plants (Caldwell *et al.* 1998) which destroys foliar 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 and Kokilavani and Rajendiran 2015b), causes stomatal anomalies in cotyledonary epidermis (Rajendiran *et al.* 2015b and Rajendiran *et al.* 2015c), inhibits photosynthesis by disturbing photosystem II (Kulandaivelu *et al.* 1989, Sullivan *et al.* 1994 and Rajendiran 2001), reduces growth of plants (Rajendiran and Ramanujam 2000, Rajendiran and Ramanujam 2003, Rajendiran and Ramanujam 2004, Kokilavani and Rajendiran 2014o and Rajendiran *et al.* 2015j), decreases yield (Mark and Tevini 1997, Rajendiran and Ramanujam 2004, Kokilavani and Rajendiran 2014e and Rajendiran *et al.* 2015j) and suppresses nodulation and nitrogen metabolism (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 sensitive plants. In this context, *in vitro* screening of leaf explants from cowpea varieties was carried out to select the suitable variety that can survive UV-B stress and to conserve their germplasm.

MATERIALS AND METHODS

In situ UV-B radiation

Cowpea (*Vigna unguiculata* (L.) Walp.) belonging to the family Fabaceae which is a nitrogen fixing grain legume was

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chosen for the study. Viable seeds of the three varieties of cowpea *viz.* GOWMATHI, FOLA and NS-634 (Namdhari Seeds) 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. 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 discs from trifoliate leaves (third 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 of leaf explants

Leaf explants after appropriate aseptic treatment were used for *in vitro* culture. Leaf 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). 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 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 (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.

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 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 three varieties of cowpea, dendrograms were drawn to derive the interrelationship between them and presented in Table 2 and Plate 5.

RESULT AND DISCUSSION

Standardisation of culture medium for leaf explants

For the standardisation of culture media, leaf explants from cowpea variety *viz.*, GOWMATHI grown under control condition were used. 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).

In vitro callus and axillary bud proliferation

In leaf explants, proliferation of callus occurred only in two cowpea varieties out of the three varieties taken for the experiment (Table 1; Plate 2). Callus induction was observed in GOWMATHI both in control and UV-B exposed leaf explants. However, in NS-634 only leaf explants harvested from *in situ* supplementary UV-B irradiated crop inducted callus. FOLA leaf explants failed to induct callus both from control and UV-B irradiated conditions.

Rajendiran *et al.* (2014a) have reported the failure of leaf explants of few varieties of cowpea to proliferate callus after ultraviolet-B irradiation out of the selected ten varieties tried for *in vitro* regeneration. The induction of callus was delayed by one day in explants harvested from *in situ* UV-B irradiated crop varieties compared with those of controls in GOWMATHI (Table 1). The calluses from the controls of GOWMATHI and UV-B irradiated FOLA were inducted on the same day. A reduction in the fresh biomass by 3 % in GOWMATHI was recorded in the *in situ* supplementary UV-B treated leaf explants compared to its control callus (Table 1; Plate 3).

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

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

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

Plate 1 Standardisation of Kinetin (K) concentration in culture media for *in vitro* regeneration from leaf explants using *Vigna unguiculata* (L.) Walp. var. GOWMATHI control samples. (7 DAI - Days after inoculation)

However, the callus of UV-B exposed NS-634 variety of cowpea weighed on par with the control of GOWMATHI. 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). The parenchyma cells that have proliferated from the *in situ* UV-B irradiated calluses were smaller (62 %) and more in number (28.73 %) in GOWMATHI, with UV-B exposed NS-634 callus proliferating smaller cells (21.40 %) compared with GOWMATHI controls (Table 1; Plate 4).

Table 1 Characteristics of callus proliferation in leaf explants of three varieties of 30 DAI *Vigna unguiculata* (L.) Walp. from con 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
GOWMATHI	Control	25	0.468	0.176	652.83± 1.26	175.36± 0.34	187.25± 1.33
	UV-B	26	0.454	0.169	465.26± 0.57	65.46± 0.67	75.65± 0.45
FOLA	Control	-	-	-	-	-	-
	UV-B	-	-	-	-	-	-
NS-634	Control	-	-	-	-	-	-
	UV-B	25	0.452	0.159	564.83 ± 2.21	485.62 ± 1.05	64.78 ± 0.57

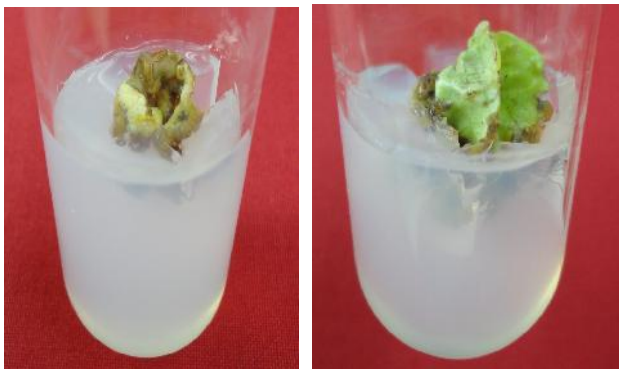

Fig. 1 Gowmathi control UV-B

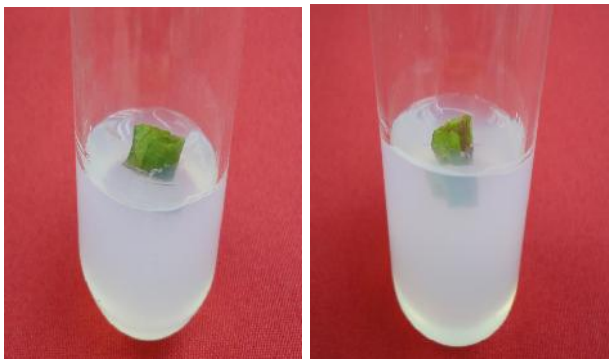

Fig. 2 FOLA Control UV-B

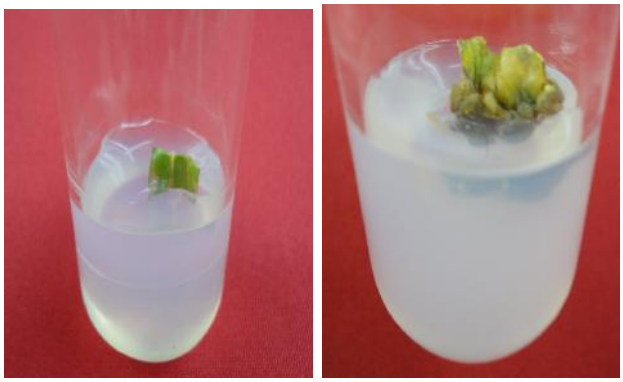

Fig. 3 NS-634 Control UV-B

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

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

Varieties	GOWMATHI	FOLA	NS-634
GOWMATHI	100%	-	25%
FOLA	-	100%	12.5%
NS-634	25%	12.5%	100%

The size of the parenchyma cells were reduced by 60 % in all the calluses inducted from *in situ* UV-B exposed GOWMATHI and NS-634 explants (Table 1; Plate 4). Rajendiran *et al.* (2014a), Rajendiran *et al.* (2014b) and Rajendiran *et al.* (2014c) opined that seed, leaf and stem explants exhibited varied responses to *in vitro* culture either due to the sensitivity of crop varieties to *in vitro* conditions or by the influence of severity of UV-B induced tissue damage in the explants. Rajendiran *et al.* (2015a) in *Amaranthus dubius* Mart. Ex. Thell., 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. have reported similar results with leaf explants harvested from plants after UV-B exposure.

Fig. 1 Gowmathi – Control

Fig. 2 Gowmathi - UV-B

Fig. 3 NS-634 - UV-B

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

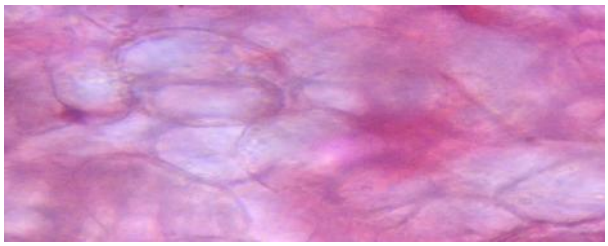

Fig. 1 Gowmathi – control

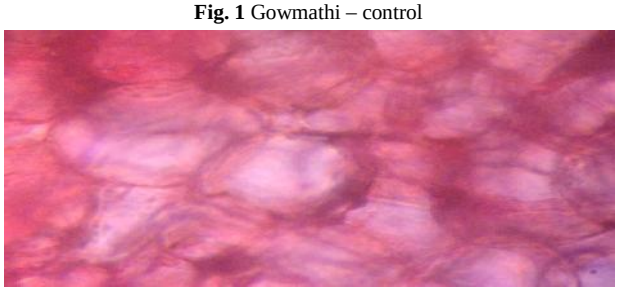

Fig. 2 Gowmathi - UV-B

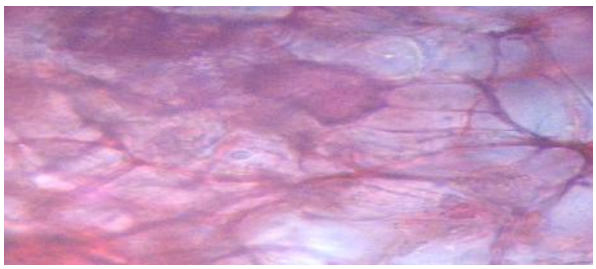

Fig. 3 NS-634 - UV-B

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

Dendrogram

The two varieties *viz.*, FOLA and GOWMATHI had no similarity and they formed one group as the leaf explants harvested from both control and UV-B stressed crops of FOLA failed to proliferate callus. Even though UV-B stressed explants of NS-634 inductedcallus, due to the variation in time taken for callus initiation, fresh and dry weight of callus, frequency and size of parenchyma cells in callus in leaf explants from other varieties, it showed only 25 % and 12.5 % similarities with GOWMATHI and FOLA respectively, to remain alone in the cluster (Table 2; Plate 5).

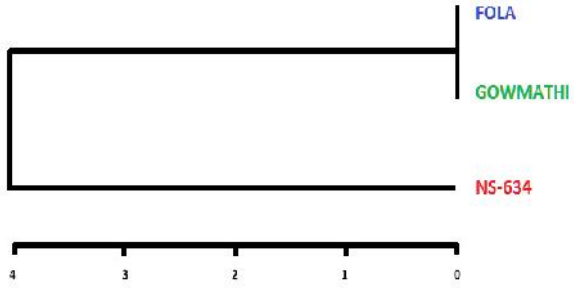

Plate 5 Dendrogram showing the interrelationship between the three varieties of *Vigna unguiculata* (L.) Walp. in callus proliferation from leaf discs of control and supplementary UV-B irradiated plants - *In vitro.*

CONCLUSION

From the present study it is evident that the leaves of GOWMATHI and NS-634 varieties of cowpea are the explants suitable for germplasm conservation for propagation in UV-B elevated environment, as they proliferated callus under *in vitro* condition despite UV-B stress. However, NS-634 variety of cowpea was found to be more comfortable under UV-B irradiation as it required UV-B exposure for *in vitro* germination.

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