International Journal Of

Recent Scientific Research

ISSN: 0976-3031

Volume: 7(1) January -2016

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THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 1, pp. 8205-8211, January, 2016

International Journal of Recent Scientific Research

RESEARCH ARTICLE

RESPONSE OF STEM EXPLANTS OF *IN SITU* UV-B IRRADIATED BLACK GRAM VARIETIES TO *IN VITRO* CULTURE

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ARTICLE INFO

Article History:

Received 15th September, 2015
Received in revised form 21st November, 2015
Accepted 06th December, 2015
Published online 28st January, 2016

Key words:

Black gram, callus proliferation, stem explant, three varieties, ultraviolet-B.

ABSTRACT

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}) three varieties of black gram *viz.* VAMBAN-3, NIRMAL-7 and T-9 to assess their viability for germplasm storage. Callus induction failed to occur in control and UV-B exposed VAMBAN-3, NIRMAL-7 and T-9 stem explants. However, stem explants from unstressed NIRMAL-7 proliferated axillary bud. The present study proves that nodal stem explants from the three varieties of black gram taken for the experiment are not suitable for germplasm conservation for cultivation in UV-B elevated environment.

INTRODUCTION

Biotechnology influences germplasm conservation by providing alternatives in some cases to conserving whole organism, assisting with the exchange of germplasm, applying the techniques of molecular biology to the problems of managing and using germplasm and by the increased demand for germplasm and conservation services by the biotechnologists themselves. As *in vitro* cell and tissues are providing new approaches to multiply germplasm resources and open opportunities for long-term cryopreservation, these techniques can be utilized to screen the explants of crop plants for regeneration and survival in changing climatic conditions in future.

Ozone depletion is one among the reasons for climate change formed due to increases in ozone depleting substances (ODS) as well as thickness of green house gases around the earth released by human activities. Ozone depletion allows enormous ultraviolet-B (UV-B) radiation (280-320 nm) which is a dangerous atmospheric stress to plants (Caldwell *et al*. 1998) as it affects 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 and Kokilavani and Rajendiran 2015b), causes abnormalities in cotyledonary epidermis (Rajendiran *et al.* 2015b and Rajendiran *et al.* 2015c) suppresses photosynthesis (Kulandaivelu *et al.* 1989, Sullivan *et al.* 1994 and Rajendiran 2001), retards growth (Rajendiran and Ramanujam 2000, Rajendiran and Ramanujam 2003, Rajendiran and Ramanujam 2004, Kokilavani and Rajendiran 2014o and Rajendiran *et al.* 2015j), reduces harvest (Mark and Tevini 1997, Rajendiran and Ramanujam 2004, Kokilavani and Rajendiran 2014e and Rajendiran *et al.* 2015j) and disturbs 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. The present work was carried out to assess the ability of stem explants of black gram varieties

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grown under *in situ* supplementary UV-B irradiation to regenerate under *in vitro* condition.

MATERIALS AND METHODS

In situ UV-B radiation

Black gram (*Vigna mungo* (L.) Hepper) the nitrogen fixing grain legume of Fabaceae was chosen for the study. Viable seeds of the three varieties of black gram *viz.* VAMBAN-3, NIRMAL-7 and T-9 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}. 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.

In vitro culture of stem explants

Nodal shoot segments (stem explants) after appropriate aseptic treatment were used for *in vitro* culture. Stem 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.

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 (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 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 similarity indices between the three varieties of black 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 black gram, dendrograms were draw to derive the interrelationship between them and presented in Table 1 and Plate 4.

RESULTS AND DISCUSSION

For the standardisation of culture media stem explants from NIRMAL-7 variety of black gram 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 axillary bud and callus proliferation in stem explants (Plate 1) and used for inoculations of stem explants of all black gram varieties (Plate 2).

Proliferation of callus failed to occur in stem explants of all the three varieties of black gram *viz.*, VAMBAN-3, NIRMAL-7 and T-9 both in control stem explants as well as in stem explants harvested from *in situ* supplementary UV-B irradiated crops (Plate 3). However axillary bud initiation occurred from the stem explants of NIRMAL-7 excised from black gram crops grown under *in situ* control condition (Plate 3, Fig. 2). All the explants from *in situ* grown VAMBAN-3 and T-9 both under control and UV-B failed to initiate axillary buds (Plate 3, Fig. 1, 3).

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

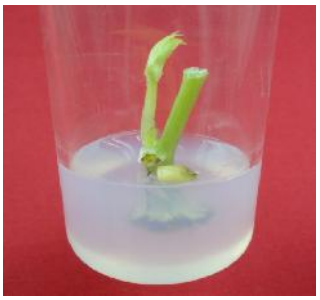

Fig. 2 K = 0.25 mgL^{-1}

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

Plate 1 Standardisation of Kinetin (K) concentration in culture media for *in vitro* regeneration from stem explants using *Vigna mungo* (L.) Hepper var. NIRMAL-7 control samples on 7 DAI (Days after inoculation).

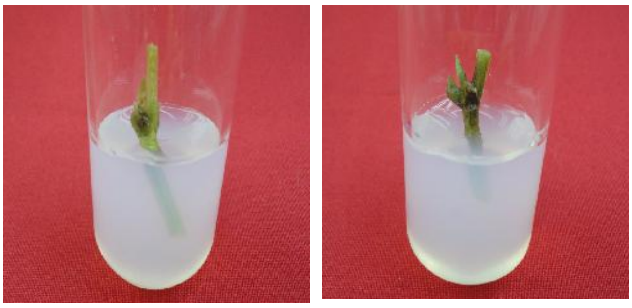

Fig. 1 VAMBAN-3 **Control** **UV-B**

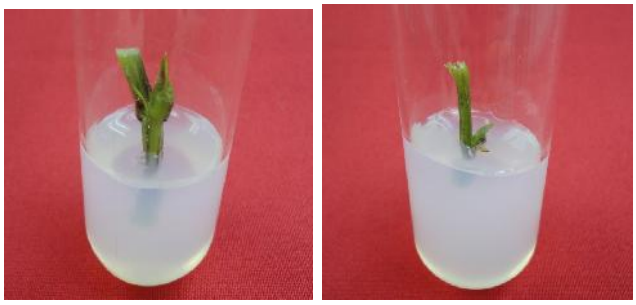

Fig. 2 NIRMAL-7 **Control** **UV-B**

Fig. 3 T-9 **Control** **UV-B**

Plate 2 Innoculum for i*n vitro* regeneration of three varieties of *Vigna mungo* (L.) Hepper from stem explants of control and ultraviolet-B (UV-B) irradiated plants.

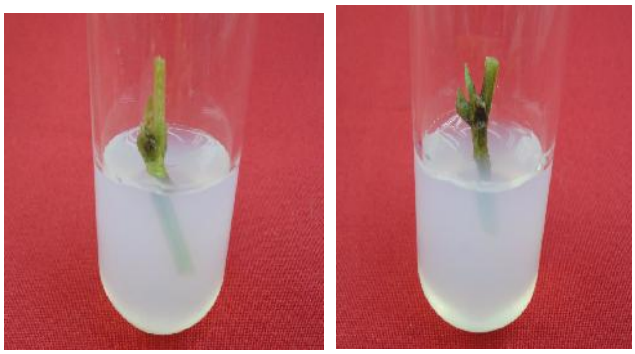

Fig. 1 VAMBAN-3 **Control** **UV-B**

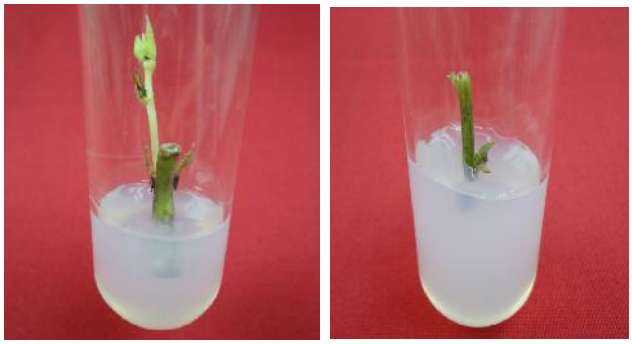

Fig. 2 NIRMAL-7 **Control** **UV-B**

Fig. 3: T-9 **Control** **UV-B**

Plate 3 Comparison of *in vitro* callus / axillary bud proliferation from stem explants of three varieties of *Vigna mungo* (L.) Hepper on 30 DAI (Days after inoculation).

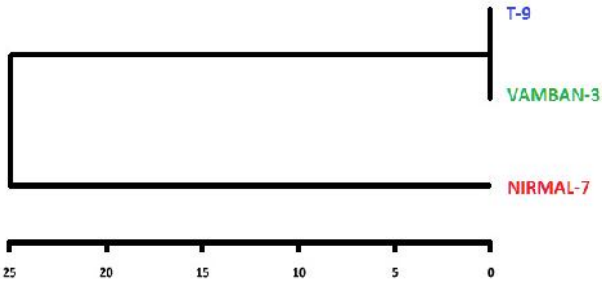

Plate 4 Dendrogram showing the interrelationship between the three varieties of *vigna mungo* (L.) Hepper in callus/axillary bud proliferation from stem explants of control and supplementary uv-birradiated plants-in vitro

This is in accordance with the findings of Rajendiran *et al.* (2014b) who have reported the failure of stem explants of three varieties of cowpea to proliferate callus after ultraviolet-B irradiation out of the ten varieties tried for *in vitro* regeneration. Rajendiran *et al.* (2014a) and Rajendiran *et al.* (2014c) opined that the varied responses shown by the seeds, leaf and stem explants to *in vitro* culture depend on the sensitivity of the plants to *in vitro* conditions.

Diverse results were also reported by 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. stem explants harvested from plants after exposure to UV-B.

The similarity index between VAMBAN-3 and T-9 varieties of black gram was 100 % and they formed one group as their stem explants harvested from both control and UV-B stressed crops failed to induct callus and axillary buds. NIRMAL-7 remained alone in the cluster showing only 25 % affinity with the group, as its control stem explants initiated axillary bud (Table 1; Plate 4).

Table 1 The similarity indices in callus / axillary bud proliferation from stem explants of three varieties of *Vigna mungo* (L.) Hepper after supplementary UV-B exposure – *In vitro*.

Varieties	VAMBAN-3	NIRMAL-7	T-9
VAMBAN-3	100%	25%	100%
NIRMAL-7	25%	100%	25%
T-9	100%	25%	100%

CONCLUSION

The nodal stem explants from VAMBAN-3, NIRMAL-7 and T-9 varieties of black gram did not respond to *in vitro* callus proliferation proving that they are not the suitable plant materials for germplasm storage for cultivation in UV-B elevated environment. For arriving at a conclusion on the selection of explants for germplasm conservation and regeneration in black gram varieties, further research on the screening of seed and leaf explants for *in vitro* regeneration from UV-B irradiated crops is needed.

Acknowledgement

The authors thank Prof. Dr. Thamizharasi Tamizhmani, Director, KMCPGS, Pondicherry, India for providing research facilities and Dr. M. P. Ramanujam for his support and encouragement.

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How to cite this article:

Rajendiran K., Thiruvarasan K. and Vijayalakshmi R., Response of Stem Explants of *In Situ* UV-B Irradiated Black Gram Varieties To *In Vitro* Culture. *Int J Recent Sci Res.* 7(1), pp. 8205-8211.

TSSN 0976-3031