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Research Article

SCREENING OF EXPLANTS OF ULTRAVIOLET-B EXPOSED *AMARANTHUS DUBIUS* MART. EX THELL. FOR *IN VITRO* PROPAGATION

***Rajendiran K., Ariswary D., Arulmozhi D. and Kokilavani V.**
Department of Botany, K.M. Centre for Post Graduate Studies, Pondicherry - 605 008, India
**Author for Correspondence*

ABSTRACT
The *in vitro* regeneration was carried out with spleen amaranth (*Amaranthus dubius* Mart. ex Thell.) 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. Regeneration was tried with seeds, shoot apical meristem, nodal stem explants (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}) spleen amaranth to study their viability for germplasm storage. UV-B reduced the growth parameters in spleen amaranth at all stages of growth. On prolonged exposure to UV-B the leaves exhibited various kinds of foliar injuries. Unstressed spleen amaranth seeds failed to respond *in vitro* germination. UV-B exposed dry and wet spleen amaranth seeds failed to germinate under *in vitro* culture. Callus induction did not occur in UV-B irradiated stem explants. Only control stem explants from proliferated calluses. Regeneration of axillary buds occurred both in control and UV-B exposed spleen amaranth stem explants. Callus induction did not occur both in control and UV-B stressed leaf explants. The present study proves that nodal stem explants from spleen amaranth are the best choice for germplasm conservation for cultivation in UV-B elevated environment.

Keywords: *Ultraviolet-B, Spleen Amaranth, In Situ Growth, Seeds, Leaf Explants, Stem Explants, In Vitro Regeneration*

INTRODUCTION
Ozone depleting substances (ODS) as well as thickness of green house gases around the earth released by human activities caused depletion in ozone layer. The heat that has to escape the troposphere and enter the stratosphere was held by this blanket of gases, making the stratosphere cooler, which in turn enhances ozone depletion. As a result, the UV-B radiation will reach Earth's surface, affecting the ecosystems. Elevated ultraviolet-B (UV-B) radiation (280-320 nm) is a dangerous atmospheric stress (Caldwell *et al.*, 1983; Jordan, 1997; Caldwell *et al.*, 1998) as it affects foliar epidermis (Bornman and Vogelmann, 1991; Rajendiran and Ramanujam, 2000a; Rajendiran and Ramanujam, 2000b; Rajendiran, 2001; 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; Sudaroli and Rajendiran, 2014; Arulmozhi and Rajendiran, 2014a; Arulmozhi and Rajendiran, 2014b; Arulmozhi and Rajendiran, 2014c; Vijayalakshmi and Rajendiran, 2014a; Vijayalakshmi and Rajendiran, 2014b; Vijayalakshmi and Rajendiran, 2014c) 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. This is an attempt for the first time to screen whether spleen amaranth can tolerate supplementary UV-B irradiation and to identify the germplasm of the crop for conservation and regeneration through biotechnology.

MATERIALS AND METHODS
Spleen amaranth (*Amaranthus dubius* Mart. ex Thell.) was chosen for the study. Viable seeds of spleen amaranth were procured from Madagadipet Seeds Depot, 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-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-B_{BE} 10 kJ m^{-2} d^{-1}. The responses of spleen amaranth in control and supplementary UV-B irradiation under *in situ* condition were assessed in terms of growth on 15 and 30 DAS.

Supplementary UV-B radiation was provided by one UV-B lamp (Philips TL 20W/12 Sunlamps, The Netherlands) which was suspended horizontally over the seeds. UV-B dose was maintained by adjusting the distance (30 cm) between seeds and the lamp. The lamp was wrapped with cellulose diacetate filters (0.076 mm) to filter UV-C radiation (< 290 nm). The filters were changed periodically to maintain uniform optical properties.

UV-B exposure to seeds was given only once for two hours duration with one hour recovery time in between. Seeds received a biologically effective UV-B dose (UV-B_{BE}) of 12.2 kJ m^{-2} d^{-1}. The control seeds were exposed to sunlight for same duration receiving UV-B_{BE} 10 kJ $m^{-2}d^{-1}$ with one hour recovery time in between (Caldwell, 1971).

Seeds, nodal shoot segments (stem explants) and leaf discs (leaf explants) after appropriate aseptic treatment were used for *in vitro* culture. The 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 and leaf 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.

Seed, stem and leaf 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 and callus proliferation were recorded after 15 DAI (days after inoculation) and 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 (pound per square inch) pressure at 121˚C for 15 minutes.

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

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

Plants grown under *in situ* condition and *in vitro* cultures tubes were photographed in daylight using a Sony digital camera fitted with appropriate close-up accessories.

RESULTS AND DISCUSSION

***In situ* studies**

Supplementary UV-B irradiation reduced the number of leaves (45.22 %) on 15 DAS and (47.41 %) on 30 DAS under UV-B stress but plants under normal ambience had more number of leaves (Table 1 to 2; Plate 1). UV-B irradiation reduced the total leaf area throughout the growth period, the maximum being 72.46 % on 30 DAS. The LAI was reduced by UV-B exposure to a larger extent, the maximum being 36.59 % over control on 15 DAS which showed signs of recovery (16.9 %) on 30 DAS. The SLW in UV-B irradiated plants increased with age. An average increase of 5.38 to 3.95 % was observed on 15 and 30 DAS, respectively. UV-B stress decreased the fresh weight of leaves by 38.72 % on 15 DAS, with the maximum reduction being 61.07 % on 30 DAS. The dry weight of foliage decreased by 31.23 % on 15 DAS in UV-B exposed plants which recovered (27.17 %) on 30 DAS (Table 1 to 2; Plate 1). Reductions in leaf area and mass were observed in the field-grown sweetgum plants exposed to elevated UV-B

radiation (Sullivan *et al.,* 1994) and *in situ* pot-grown ten varieties of cowpea (Kokilavani and Rajendiran, 2014a, 2014b, 2014c, 2014d, 2014e, 2014f, 2014g, 2014h, 2014i, 2014j, 2014k, 2014l, 2014m, 2014n, 2014o). Changes in the leaf area and dry mass indicated that cell elongations as well as cell contents were affected and growth inhibitions are part of general UV-B effects (Britz and Adamse, 1994).

UV-B exposed leaves on the long run exhibited various kinds of abnormalities (Plate 2). The leaves became generally pale which at times occurred in patches. The yellowing intensified and became discretely chlorotic.

Browning developed in patches indicating necrosis of the underlying tissues during later stages. Necrotic lesions appeared in older leaves which have received UV-B over a long time. The leaves were also tiny, exhibited bronzing and became silvery and brittle. Similar abnormalities were reported in black gram (Kokilavani and Rajendiran, 2013), cucumber (Kokilavani and Rajendiran, 2014b) and several varieties of cowpea grown under *in situ* UV-B exposure (Kokilavani and Rajendiran, 2014a; Kokilavani and Rajendiran, 2014b; Kokilavani and Rajendiran, 2014c; Kokilavani and Rajendiran, 2014e; Kokilavani and Rajendiran, 2014f; Kokilavani and Rajendiran, 2014g; Kokilavani and Rajendiran, 2014j; Kokilavani and Rajendiran, 2014k; Kokilavani and Rajendiran, 2014l; Kokilavani and Rajendiran, 2014m; Kokilavani and Rajendiran, 2014n; Kokilavani and Rajendiran, 2014o; Kokilavani and Rajendiran, 2015a and Kokilavani and Rajendiran, 2015b).

UV-B exposure reduced root length significantly by 43.60 to 46.36 % on all stages of growth till 30 DAS (Table 3 to 4; Plate 1). Shoot length of UV-B stressed plants decreased by 54.93 % within 15 DAS and continued so till 30 DAS with 35.06 % reduction. S / R ratio was decreased by UV-B stress by 58.77 % on 15 DAS. S / R ratio continued to decrease by 20.01 % below control on 30 DAS. Fresh weight of roots increased with age in all treatments.

But the biomass accumulation in root was inhibited by UV-B treatment by 36.09 % on 15 DAS, the maximum reduction being on 30 DAS 44.66 %. A decrease of 47.67 % in shoot fresh weight of UV-B treated plants was observed.

The same trend was maintained till 30 DAS of growth (31.67 %). The trends observed in root and shoot biomass pattern were reflected at the whole plant level too with severe inhibitions on 15 DAS (43.46 %) under UV-B and little improvement on 30 DAS (38.60 %). A gradual reduction in the root biomass content starting from 32.73 % on 15 DAS and reaching 78.10 % on 30 DAS was caused by UV-B treatment. UV-B exposure suppressed dry weight of shoot by 33.33 % on 15, reaching a maximum of 81.05 % on 30 DAS over control. Plant dry weight increased with age but after UV-B stress, it fell below control by 31.52 % on 15 DAS and 70.36 % on 30 DAS.

Inhibition of growth indicated by reductions in root and shoot length and biomass content due to UV-B stress were apparent at all stages. Such inhibitions are characteristic of UV-B stressed legumes as in *Vigna unguiculata (*Kulandaivelu *et al.,* 1989), *Phaseolus vulgaris* (Mark and Tevini, 1997), *Vigna mungo* (Rajendiran and Ramanujam, 2000a) and *Vigna radiata* (Rajendiran and Ramanujam 2003) and ten varieties of cowpea (Kokilavani and Rajendiran, 2014o). The stunting of UV-B stressed plants is attributed to destruction of endogenous IAA whose photo-oxidative products may be inhibitory (Kulandaivelu *et al.,* 1989; Tevini and Teramura, 1989) as indicated by a decrease in IAA content concomitant with a corresponding increase in IAA oxidase activity in rice leaves (Huang *et al.,* 1997).

The relative growth rate (RGR) was lowered in UV-B irradiated plants which showed a reduction of 25.67 % below control on 15 DAS. RGR reduction continued with age as it reached 74.32 % on 30 DAS (Table 3 to 4). Similar inhibitions of RGR by UV-B were observed by Jain *et al.,* (1999) in mungbean and in ten varieties of cowpea (Kokilavani and Rajendiran, 2014o).

In vitro Studies

The unstressed spleen amaranth seeds responded *in vitro* germination and the seedlings grew well in cuture media. However, the UV-B stressed dry and wet seeds responded differently from the control seeds as they did not germinate under *in vitro* condition (Table 5; Plate 3). Similar results were reported by Rajendiran *et al.,* (2014a, 2014b) after experimenting with the *in vitro* regeneration of UV-B stressed seeds in ten varieties of cowpea.

Table 1: Changes in foliage of 15 DAS *Amaranthus dubius* Mart. ex Thell. in control and UV-B irradiated plants – *In situ*

Treatment	Number of leaves	Total leaf area (cm^2)	Leaf area index	Specific leaf weight (g^{-2})	Fresh weight of foliage (g)	Dry weight of foliage (g)
Control	10.33	113.31	10.920	0.061	0.552	0.519
UV-B	5.66	31.26	6.924	0.058	0.338	0.357

Table 2: Changes in foliage of 30 DAS *Amaranthus dubius* Mart. ex Thell. in control and UV-B irradiated plants – *In situ*

Treatment	Number of leaves	Total leaf area (cm^2)	Leaf area index	Specific leaf weight (g^{-2})	Fresh weight of foliage (g)	Dry weight of foliage (g)
Control	12.66	171.51	11.759	0.063	1.173	0.542
UV-B	6.66	47.56	9.772	0.060	0.456	0.395

Table 3: Changes in growth parameters of 15 DAS *Amaranthus dubius* Mart. ex Thell. in control and UV-B irradiated plants – *In situ*

Treatment	Plant height (cm)	Root length (cm)	Shoot length (cm)	Shoot / root ratio	Root fresh wt. (g)	Shoot fresh wt. (g)	Plant fresh wt. (g)	Root dry wt. (g)	Shoot dry wt. (g)	Plant dry wt. (g)	Relative growth Rate
Control	17.6	8.86	8.43	0.68	0.455	0.661	1.660	1.669	0.493	1.490	0.10
UV-B	8.5	5	3.5	0.28	0.132	0.346	0.943	0.331	0.330	1.020	0.07

Table 4: Changes in growth parameters of 30 DAS *Amaranthus dubius* Mart. ex Thell. in control and UV-B irradiated plants – *In situ*

Treatment	Plant height (cm)	Root length (cm)	Shoot length (cm)	Shoot / root ratio	Root fresh wt. (g)	Shoot fresh wt. (g)	Plant fresh wt. (g)	Root dry wt. (g)	Shoot dry wt. (g)	Plant dry wt. (g)	Relative growth Rate
Control	32	19.26	12.73	1.06	0.929	2.660	2.195	1.011	1.557	1.633	0.32
UV-B	17.43	10.33	8.26	0.84	0.514	1.817	1.361	0.221	0.295	0.484	0.13

Table 5: Changes in growth parameters of 15 DAI *Amaranthus dubius* Mart. ex Thell. in control and UV-B irradiated dry seeds – *In vitro*

Treatment	Plant height (cm)	Root length (cm)	Shoot length (cm)	Shoot / root ratio	Root fresh wt. (g)	Shoot fresh wt. (g)	Plant fresh wt. (g)	Root dry wt. (g)	Shoot dry wt. (g)	Plant dry wt. (g)	Relative growth Rate
Control	6	3	6.3	0.96	0.118	0.114	0.232	0.104	0.100	0.205	0.04
UV-B	-	-	-	-	-	-	-	-	-	-	-

Figure 1: On 15 DAS

Figure 2: On 30 DAS

Plate 1: The control and supplementary UV-B stressed plants of *Amaranthus dubius* L. Mart. ex Thell. (1: Control, 2: UV-B)

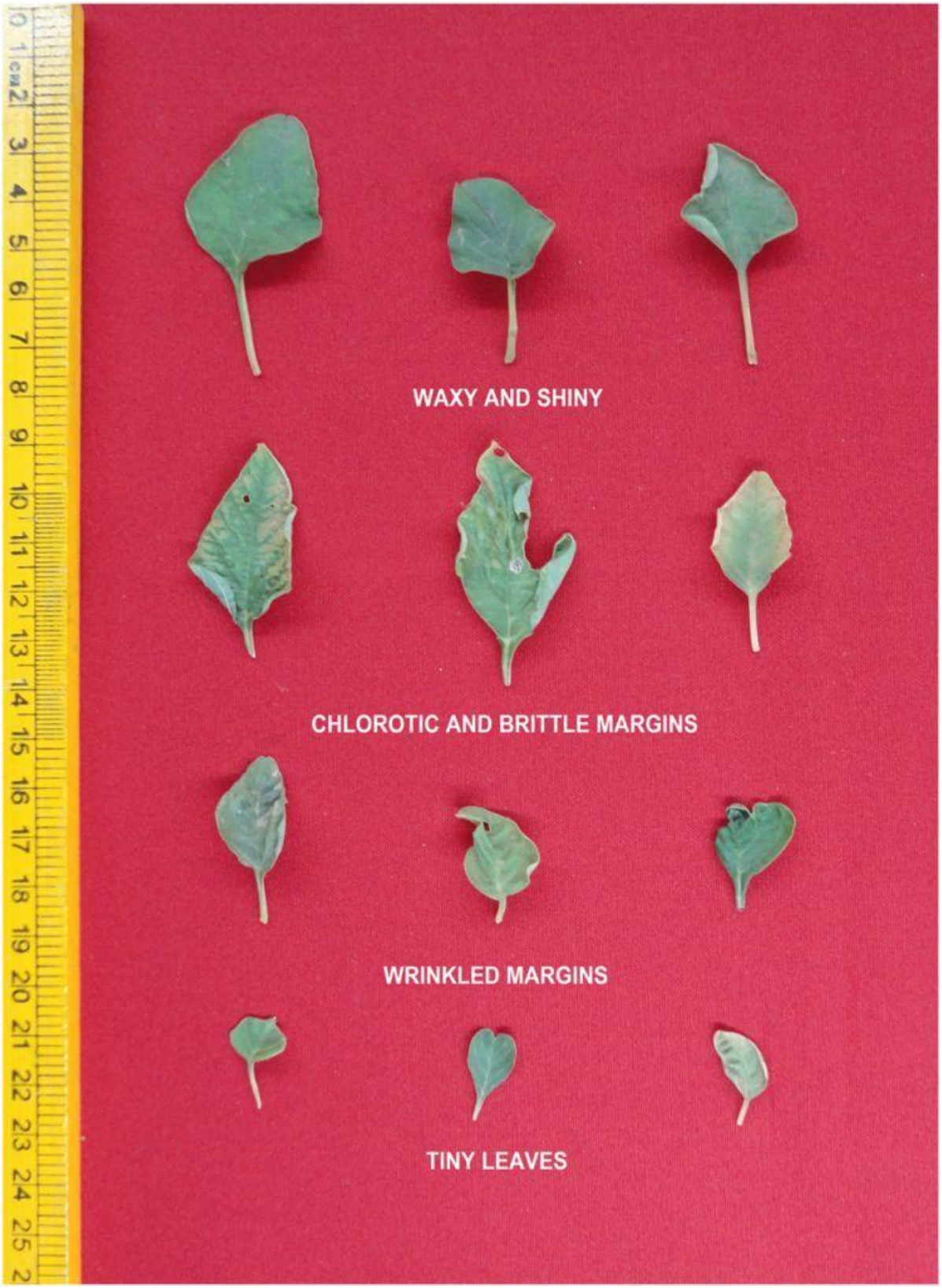

Plate 2: Types of foliar injury caused by elevated UV-B radiation in *Amaranthus dubius* Mart. ex Thell. on 30 DAS

1 DAI Figure 1: Control

Figure 2: UV-B on dry seed

Figure 3: UV-B on soaked seed

7 DAI Figure 4: Control

Figure 5: UV-B on dry seed

Figure 6: UV-B on soaked seed

Plate 3: *In vitro* seed germination and growth of *Amaranthus dubius* L. Mart. ex Thell. in control and UV-B irradiated dry and soaked seeds. (DAI - Days after inoculation)

7 DAI: Stem explants Figure 1: Control

Figure 2: UV- B

7 DAI: Leaf explants Figure 3: Control

Figure 4: UV- B

Plate 4: *In vitro* callus proliferation from stem and leaf explants of control and UV-B irradiated *Amaranthus dubius* L. Mart. ex Thell. plants. (DAI - Days after inoculation)

Proliferation of callus occurred in stem explants of spleen amaranth. Callus induction was observed only in control stem explants of spleen amaranth. However, axillary bud regeneration took place both in control as well as in stem explants harvested from *in situ* supplementary UV-B irradiated plants. The regeneration of axillary buds was delayed by one or two days in explants harvested from *in situ* UV-B irradiated plants compared with those of control (Plate 4, Figure 1 to 2). Similar experiments were carried out by Rajendiran *et al.*, (2014c) with *in vitro* regeneration of stem explants harvested from *in situ* grown UV-B stressed ten varieties of cowpea. Callus proliferation did not occur both in control leaf explants as well as in leaf explants harvested from *in situ* supplementary UV-B irradiated crops (Plate 4, Figure 3 to 4). This is in accordance with Rajendiran *et al.*, (2014d) who tried *in vitro* regeneration of leaf explants harvested from *in situ* grown UV-B stressed ten varieties of cowpea.

Taking into consideration of all the parameters studied *viz.*, growth under *in situ* condition and the responses of seeds and explants under *in vitro* culture, the present study recommended that out of the explants of spleen amaranth taken for screening, stem explants are considered to be best suited for germplasm conservation and regeneration.

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