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Medium alkalinization and induction of phenylalanine ammonia lyase are involved in the early responses of UV-B mediated hyperproduction of shatavarin

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A B S T R A C T

Shataverins are the steroidal saponins of *Asparagus racemosus.* They have widespread use in traditional Indian and Chinese medicinal system. In this paper we report that the cell cultures of *A. racemosus* hyperproduce shatavarins upon time dependent exposure to UV-B light. When irradiated for 5 minutes with UV, the pH of the culture medium shot up by 1.01 units within 10 minutes of irradiation. A significant increase in the activity of the enzyme Phenylalanine ammonia lyase was also observed in UV treated cells (353 µkat/Kg Protein) compared to the control (142 µkat/Kg Protein) with increased production of shatavarin. Therefore, medium alkalinization and induction of Phenylalanine ammonia lyase seems to be the early responses of UV-B perception by this monocot plant indicating presence of UVR8 like receptor.

Introduction

Shataverins are the steroidal saponins produced by *Asparagus racemosus*. It helps the plant to cope up with various biotic and abiotic stresses. Commercially, *A. racemosus* is widely used in Ayurveda as immunomodulant, galactogauge, adaptogen, antitusive, anticarcinogen, antioxidant, and as a general tonic (Gaitonde and Jetmalani, 1969; Joglekar *et al.*, 1967; Oketch-Rabah, 1998; Rao, 1952; Rice, 1988; Thatte *et al.*, 1987). All these medicinal properties are due to shataverins. Elicitations are considered to be an important strategy for hyper production of secondary metabolites *in vitro.* In this regard, we have previously reported a simple and standardized medium for callus and cell culture of *A. racemosus.* Various elicitors were tested for elevated synthesis of shataverins in cell cultures (Pise *et al.*, 2011; Pise *et al.*, 2012; Pise *et al.*, 2013). During the elicitation studies it was found that UV-B irradiation induces more than twenty fold hyperproduction of shatavarins in cell cultures. UV-B mediated signal transduction is known to induce stress and expression of defense genes in many

plants (Ballare *et al.,* 1991; Ballare *et al.,* 1995; Frohnmeyer *et al.,* 1999) and more specifically in Arabidopsis thaliana (Christie and Jenkins, 1996; Kim *et al.,* 1998; Boccalandro *et al.,* 2001; Brosche *et al.,* 2002; Ulm *et al.,* 2004; Brown *et al.,* 2005; Favory *et al.,* 2009). Plants have a specific receptor called UVR8 for UV-B perception. This receptor is well characterized and is a conserved protein in most of the dicot plants through many species. With the identification of UVR8, a new era has begun regarding our understanding of plant UV-B responses, and the relationship of UV-B to plant photomorphogenesis in general. The signalling through this receptor causes activation of photomorphogenesis and activation of defence genes.

In the current study we report the probability of UVR-8like receptor on the cell surface of *A. racemosus* cells and medium alkalinization and induction of Phenylalanine ammonia lyase, EC 4.3.1.24, (PAL) activity to be early responses in UV-B mediated hyperproduction in cell cultures of *A. racemosus*.

Materials and Methods

Plant material, callus and cell culture of A. racemosus

The authors have reported a detailed methodology for the callus induction and cell culture of *A. racemosus.* Briefly, nodal explants of field grown *A. racemosus* plants were collected after confirming the authenticity.

A voucher specimen (MP/312) has been deposited at the University herbarium maintenance section. Callus cultures and suspension cultures were initiated using a modified MS media supplemented with naphthalene acetic acid (NAA), 2.4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyl aminopurine **(**BAP). *A. racemosus* suspension-cultured cells were cultivated under constant light as described previously (Pise *et al.,* 2012; Pise *et al.,* 2013). Cells were subcultured weekly and 5day old cultures were used for experiments after subculture.

UV Irradiation

Irradiation of the cells was carried out with a UV-B lamp (Minera lights, UVM 57, Sangabriel, California) and a UV meter were used to for the experiments. The distance between leaf surfaces and the UVB source was 2 ± 0.5 cm to get average standard UVB irradiance of 5mW·cm22 resulting in a dose of 15±0.5 kJ·m22 after an irradiation period of 5 min. Different doses were obtained by varying the exposure time (0–15 min).

Medium Alkalinization Response (AR)

Suspension cultures were maintained as described in the previous section and 5-6 day old cells were divided into control and experimental groups. Cells were equilibrated for 1 hour on a rotary shaker after transferring to multiwell plates (1.5 ml/well) under ambient light n temperature conditions. After equilibration period, the pH of the medium reached a starting pH of 4.8 ± 0.2. Experimental cells were treated with UV for 5 min and extracellular pH changes were measured with a glass combination electrode ($Ag/AgCl_2$, model 15 pH meter, Elico) for 120 min. Suramin (1Mm) in water were supplied to the cells 10 min prior to the UV exposure.

Phenylalanine ammonia lyase assay

The enzyme assay procedure involved extraction of enzyme from frozen tissue by homogenizing 1 gm tissue in 1 m1 ice - cold extraction buffer. Poly-venylpyrroledone, 0.5% (PVP) was added at the time of extraction to

remove the phenolic components. The extract was centrifuged at 10,000 rpm and clear supernatant was used as enzyme source. Experimental sets were prepared and incubated at 40°C for 1hour. Then absorbance was recorded at every 30 minutes interval up to 2 hours at 290 nm. The assay was done in triplicate. The enzyme activity of PAL was expressed as µKat/Kg protein (Lamb, 1979).

Results and Discussion

Alkalinization of *A. racemosus* cell-suspension medium in response to irradiation

When irradiated for 2 or 5 minutes with UV, the pH of the culture medium shot up by 0.9 units within 10 minutes of irradiation (Fig.1). The pH of the medium returned to baseline by 300 min. This indicates UV-B irradiation for 5 min induces medium Alkalinization in *A. racemosus* cell cultures.

Inhibition of Alkalinization Response (AR) by Suramin

To test whether this perception of UV is receptor mediated like other reported plants, cell cultures were treated with increasing concentrations of suramin and subsequently irradiated with UV for 5-10 min. It was found that Suramin inhibited the UV induced alkalinization of the growth medium for all exposure times of UV irradiation (Fig 2) with IC50 of 540µg/ ml. At 1200 µg/ ml 70% AR got subsided indicating dose dependency of the phenomenon. This indicates presence of systemin like receptors on the cell surface of *A. racemosus* which perceives UV-B radiation.

Induction of PAL activity in UV treated cells of *A. racemosus*

To assess whether UV irradiation actually triggeres the expression of biosynthetic genes in *A. racemosus*, the activity of Phenylalanine ammonia lyase (PAL), which is a key enzyme of the saponin biosynthesis pathway was assayed. Figure 3 indicates that PAL enzyme was significantly induced in UV irradiated cells.

Inhibition of UV elicited saponin synthesis by suramin

AR and PAL could be induced by UV-B in *A. racemosus* cells and these responses got inhibited by suramin, the effect of suramin on accumulation of shatavarin was examined. *A. racemosus* cells were pretreated with 1mM suramin concentrations and subsequently irradiated with UV for 5 min, the UV induced accumulation of Shatavarin got inhibited by suramin (Fig. 4). Heparin, a structurally similar polysulphonated molecule, could not inhibit the processes induced by UV in cultured cells of *A. racemosus*. Therefore it can be said that inhibition by suramin is not through non specific binding.

Medium alkalinization is considered as a marker of elicitor response in studying elicitor-binding sites in plant cells. AR is thought to result from elicitor induced depolarization of the plasma membrane and subsequent K^+/H^+ exchange with Ca^{2+} influx/Cl^- efflux. It is known that receptor proteins that bind elicitors generate signals that are transmitted to the sites of gene expression via different components, such as Ca^{2+}/ion fluxes, medium alkalinization, cytoplasmic acidification, oxidative burst, jasmonate and nitric oxide etc. (Boller, 1995; Zhao *et al.*, 2005). As per the recent reports UV-B is absorbed by a UVR8 receptor (Roberts and Harmon, 1992; Rizzini *et al.*, 2011) which in turn are linked with COP1 and HY5 (Kliebenstein *et al.*, 2002; Heijde and Ulm, 2012; Heijde and Ulm, 2013) like transcription factors which

ultimately lead to induction of defense genes in Arabidopsis thaliana, Maize and many other plants. In the light of above knowledge we propose that UV-B mediated AR response in cell cultures of *A. racemosus* might be linked to hyper production of shatavarin. Suramin is known to inhibit signalling pathways by binding the cell surface components such as the systemin receptor in *Lycopersicon peruvianum* and *Catharanthus roseus* suspension cultured cells (Yalamanchili and Stratmann, 2002).

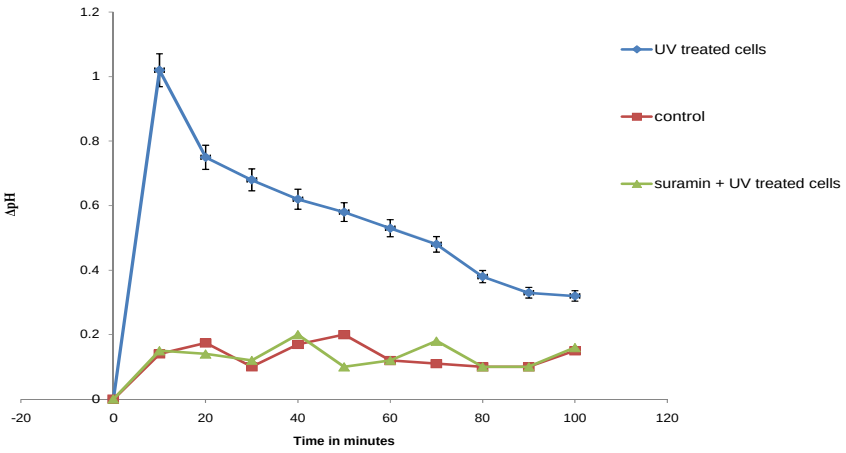
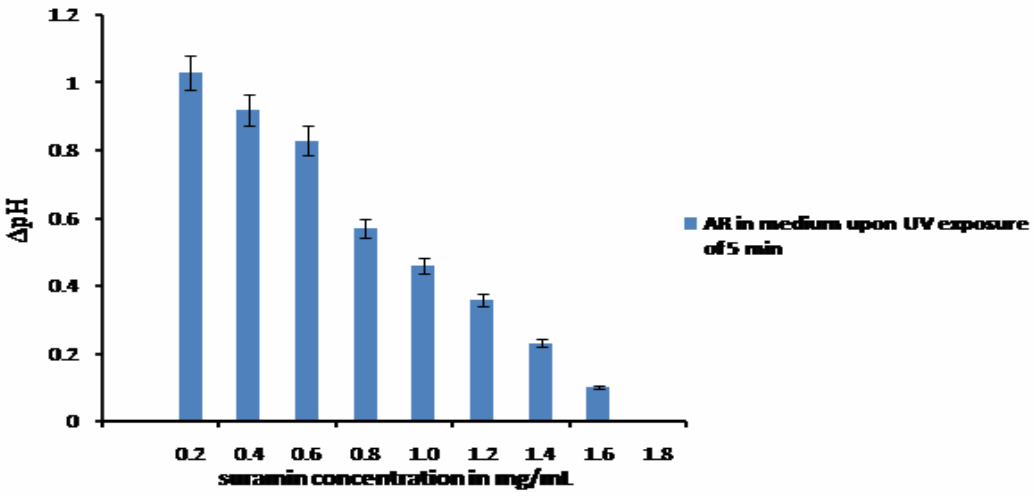


Fig. 1: Medium alkalinization in A. racemosus cell culture upon UV-B exosure for various time period.

Fig. 2: AR in medium upon UV exposure of 5 min



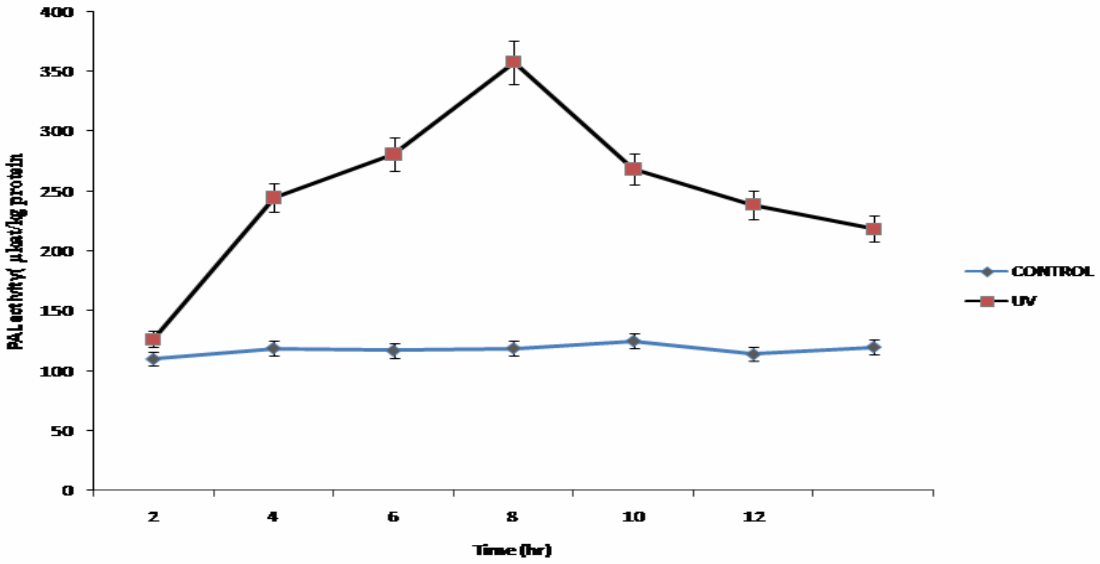


Fig 3 Increased Pal activity in UV-B induced cell cultures

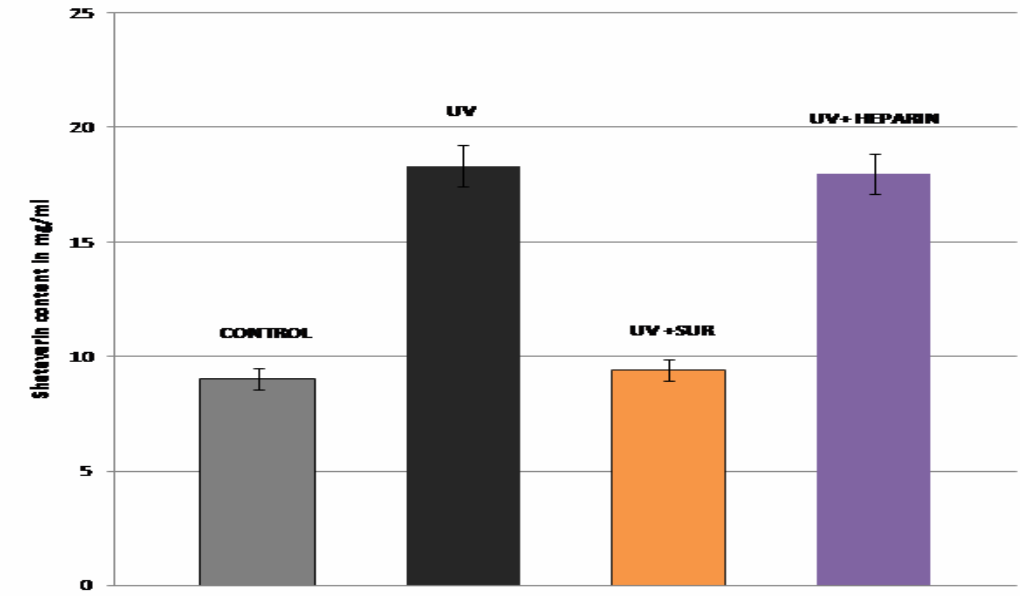


Fig. 4 Effect of suramin on shatavarin synthesis

Heparin, which is similar to suramin in possessing polysulfonated groups, had no effect on alkalinization of the medium induced by UV irradiation further strengthening the involvement of UV-B receptor on the cell surface. Absorption UV-B light by UVR8 receptor and its downstream signalling controls phytomorphogenesis, phototropism and biosynthesis of secondary metabolites.

UV-B is known to stimulate accumulation of specific flavenols, flavenol glycosides in the vacuoles of epidermal and sub epidermal cells for protecting the plants from UV-B irradiation stress (Emiliani *et al.,* 2013). The flavenol pathway is mainly regulated by genes encoding biosynthetic enzymes like PAL, Chalcone synthase, Chalcone isomerase etc (Emiliani *et al.,* 2013; Hectors *et al.,* 2012). The significant induction of PAL within 10 min of UV-B irradiation strongly suggests that UV-B perception is able to induce biosynthestic genes in *A. racemosus* cell.

Conclusions

The above results strongly points towards existence of UVR8 or a similar kind of receptor. The downstream pathway involves medium Alkalinization, PAL induction and shatavarin hyperproduction as is a linked phenemonon. However further research is needed to characterize this receptor, molecular events like transcription factors and other biosynthetic genes. In future the authors plan isolate the receptor from *A. racemosus* and to study the transcriptome after UV irradiation as this will enhance the knowledge regarding UV-B signaling in the monocot medicinal plant *A. racemosus*.

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