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Response of ultraviolet-B induced antioxidant defense system in a medicinal plant, *Acorus calamus*

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Abstract: *Ultraviolet-B (UV-B) radiation generates an oxidative stress in plant cells due to excessive generation of reactive oxygen species (ROS). ROS can denature enzymes and damage important cellular components. In the present study, an important medicinal plant Acorus calamus (Sweet flag) was subjected to two doses of supplemental UV-B radiation (sUV-B): sUV_1 (+1.8 kJ m^{-2} d^{-1}) and sUV_2 (+3.6 kJ m^{-2} d^{-1}) to evaluate the relative response of antioxidant defense potential. Stimulation of activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) was observed at initial growth period while the activities of CAT and SOD decreased at later age of sampling. sUV-B induced lipid peroxidation (LPO) was observed showing alteration of membrane properties. No definite trend of change was observed for ascorbic acid (AsA), while increaments in thiol, proline, phenol and protein contents were observed due to sUV-B. Results suggested that sUV-B radiation may stimulate the enzymatic and non-enzymatic defense system of Acorus plants, showing its better adaptation at lower dose of sUV-B.*

Key words: *Reactive oxygen species, Acorus calamus L., Supplemental UV-B, Antioxidants*
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Introduction

A reduction in the thickness of the stratospheric ozone layer induced by anthropogenic emissions of pollutants, such as chlorofluorocarbons (CFCs), detected over the past several decades has resulted in increase in level of biologically effective ultraviolet-B radiation (UV-B) on Earth surface (Stolarski *et al*., 1992; Madronich *et al*., 1998). Exposure of plant tissue to ultraviolet-B (UV-B: 280-315 nm) radiation accelerates the level of reactive oxygen species (ROS), such as $^1O_2$, O_2^-, H_2O_2 and $^{\cdot}OH$ and can cause oxidative damage to proteins, lipids and nucleic acids. Enhanced production of ROS in plant tissues exposed to supplemental level of UV-B (sUV-B) has detrimental effects on enzyme activities and gene expression, which ultimately leads to cellular damage and programmed cell death (Mackerness *et al*., 2001).

Plants have evolved protective defense mechanisms including enzymatic and non-enzymatic antioxidants and production of secondary metabolites to counteract the destructive effect of ROS (Jansen *et al*., 2008). The main enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT) glutathione peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR), whereas non-enzymatic portion comprised of low molecular weight antioxidants *i.e.* proline, thiol, ascorbic acid and glutathione (Blokhina *et al*., 2003). Plants might use and scavenge ROS and various metabolites (glutathione, ascorbate *etc.*) to regulate gene expression and plant function.

The information concerned with role of antioxidants in plant protection against ROS in UV-B stress condition are mainly based on agricultural crops and annual plants (Costa *et al*., 2002; Agrawal and Rathore, 2007; Jain *et al*., 2004; Yannarelli *et al*., 2006). To our knowledge, very little is known about antioxidant defense mechanism in medicinal plant against UV-B stress. Indian tetraploid variety of *Acorus calamus* L. is an aromatic herb, have been known since long for medicinal value of its volatile oil, possessing anti-spasmodic, carminative and anthelmintic properties, used for treatment of various abdominal, respiratory and nervous disorders (Shukla *et al*., 2002; Raina *et al*., 2003). Present study was aimed to evaluate the effects of varying doses of supplemental UV-B on antioxidant defense system of *A. calamus* (sweet flag) helping plants to make appropriate adjustment against environmental stress.

Materials and Methods

Plant material and experimental design: Rhizomes of sweet flag (*Acorus calamus* L., Indian teteraploid variety) were obtained from nursery of Horticulture Department, Banaras Hindu University, Varanasi and transplanted in experimental plots (1× 1 m^2 area) in the Botanical garden, Department of Botany, Banaras Hindu University, Varanasi, (25° 18'N latitude, 82°03'E longitude, about 76 m elevation above mean sea level) situated in the eastern Gangetic plains of India. During the experimental period, average minimum and maximum temperature ranged from 16.7 to 36.8°C, and relative humidity 53 to 85%. Photosynthetic active radiation (PAR) ranged between 1100 and 1200 µ mol m^{-2} s^{-1}.

sUV-B treatment: sUV-B radiation was artificially provided by 40 W flurescent lamps (UVB-313, Q panel Co, Cleveland, OH, USA). UV lamps (120 cm long) were fitted in mobile adjustable frame (30

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cm apart). For treatment, plants were exposed to two doses of supplemental Ultaviolet-B radiation (sUV-B): sUV_1 and sUV_2 of +1.8 and +3.6 kJ m^{-2} d^{-1} biologically effective UV-B ($UV\text{-}B_{BE}$) above ambient, simulating 5 and 10% depletion, respectively in stratospheric ozone at Varanasi. Control plants receiving only ambient level of biologically effective UV-B radiation (9.6 kJ m^{-2} d^{-1}) as weighted by Caldwell (1971) generalized plant action spectrum normalized at 300 nm. Cellulose diacetate (transmission down to 280 nm) and polyester filter (absorbed radiation below 320 nm) were used for sUV-B treated and control plants, respectively. These filters were replaced frequently at every week to avoid photodegradation effect caused by UV-B. Exposure of plants to sUV-B was provided after establishment of plants for 3 hr d^{-1} (10.00 to 13.00 hr) at solar noon period.

The spectral irradiance from the lamps was measured by an Ultraviolet Intensity meter (UVP Inc., San Gabriel, CA, USA) and $UV\text{-}B_{BE}$ values were determined by Spectropowermeter (Scientech, Boulder, USA).

Biochemical analysis: Random sampling of fresh leaves was done in triplicates from replicate plots of each treatment at 40, 70 and 100 days after transplantation (DAT) for analysis of the various parameters.

Peroxidase (POX) and catalase (CAT) were determined by using the method of Britton and Mehley (1955) and Aebi (1984), respectively. Superoxide dismutase (SOD) activity was assayed according to method of Fridovich (1974). Ascorbate peroxidase (APX) and glutathione reductase (GR) activity was measured spectrophotometrically using the methods described by Nakano and Asada (1981) and Anderson (1996), respectively. Leaf samples were homogenized in oxalic acid and Na EDTA extraction solution for ascorbic acid content determination as described by Keller and Schwager (1977). Thiol content was determined by Ellman's reagent (DTNB) using the method described by Fahey *et al*. (1978). Phenol content was estimated by the method of Bray and Thorpe (1954). Determination of proline was performed by ninhydrin test as described by Bates *et al*. (1973) and protein was estimated by following the method of Lowry *et al*. (1951).

Lipid peroxidation (LPO) in the leaf tissues was determined in terms of malondialdehyde (MDA) content by thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968).

Statistical analysis: Differences between means were evaluated for significance by using Duncan's multiple range test (DMRT) ($p<0.05$). All the data on various antioxidants were analyzed by two ways ANOVA to test the individual and interactive effects with sUV-B and plant age. All the statistical tests were performed using SPSS software (SPSS Inc., version 14.0).

Results and Discussion

The activities of antioxidant enzymes SOD, CAT, APX, GR and POX increased at initial stage of sampling under both the doses of sUV-B (Fig. 1,2). The observed trend was in

Table - 1: F- ratios and level of significance of multivariate ANOVA test to determine the effects of sUV-B (T), plant age (*A*) and their interactions for various biochemical parameters of *Acorus* plants

Parameters	Plant age (A)	sUV-B (T)	A x T
SOD	108.27***	98.37***	32.65**
CAT	74.08***	42.08***	21.73***
POX	212.42***	72.54***	16.75*
APX	89.05***	25.30**	40.08**
GR	42.21***	14.28*	9.84^{ns}
Ascorbic acid	89.43***	34.53***	17.82**
Thiol	87.09***	98.34**	30.74**
Proline	122.86***	35.29**	25.18*
Phenol	22.09***	28.57*	6.13^{ns}
Protein	16.78**	72.50**	4.26^{ns}
LPO	331.62***	107.68***	57.84***

Significance levels are indicated by: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, ns = Not significant, SOD = Superoxide dismutase, CAT = Catalase, POX = Peroxidase, APX = Ascorbate peroxidase, GR = Glutathione reductase, L.P.O. = Lipid peroxidation

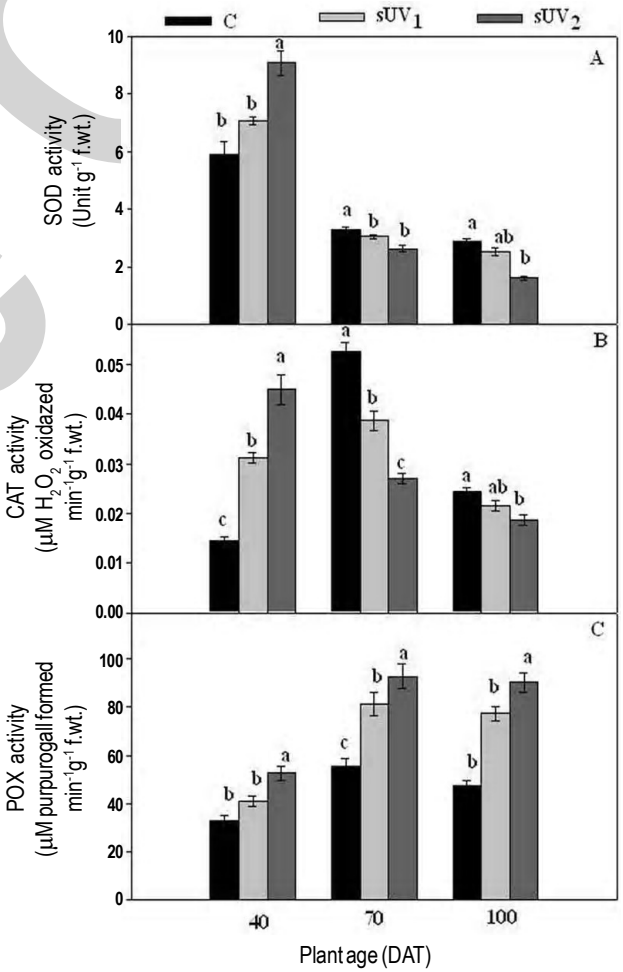

Fig. 1: Effect of different doses of sUV-B on (A) SOD, (B) CAT and (C) POX activity of *Acorus* plants (mean ± S.E.) at 40, 70 and 100 days after transplantation (DAT). Values followed by the different letter within same group indicate statistically significant difference ($p< 0.05$)

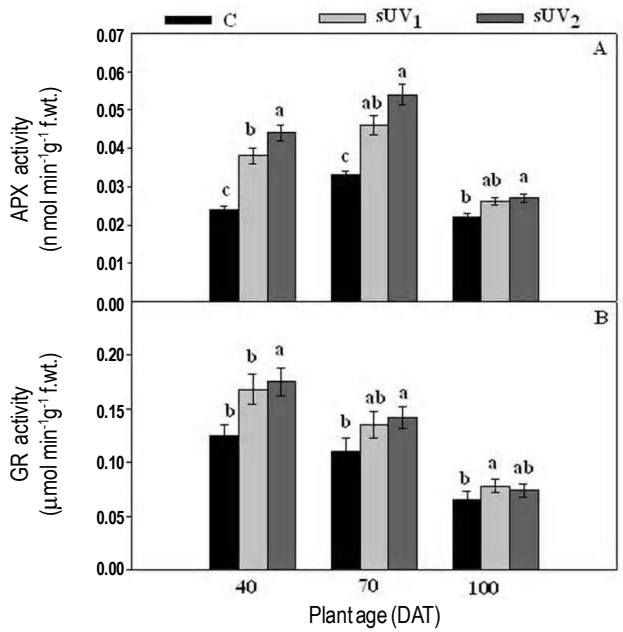

Fig. 2: Effect of different doses of sUV-B on (A) APX and (B) GR activity of *Acorus* plants (mean ± S.E) at 40, 70 and 100 days after transplantation (DAT). Values followed by the different letter within same group indicate statistically significant difference ($p < 0.05$)

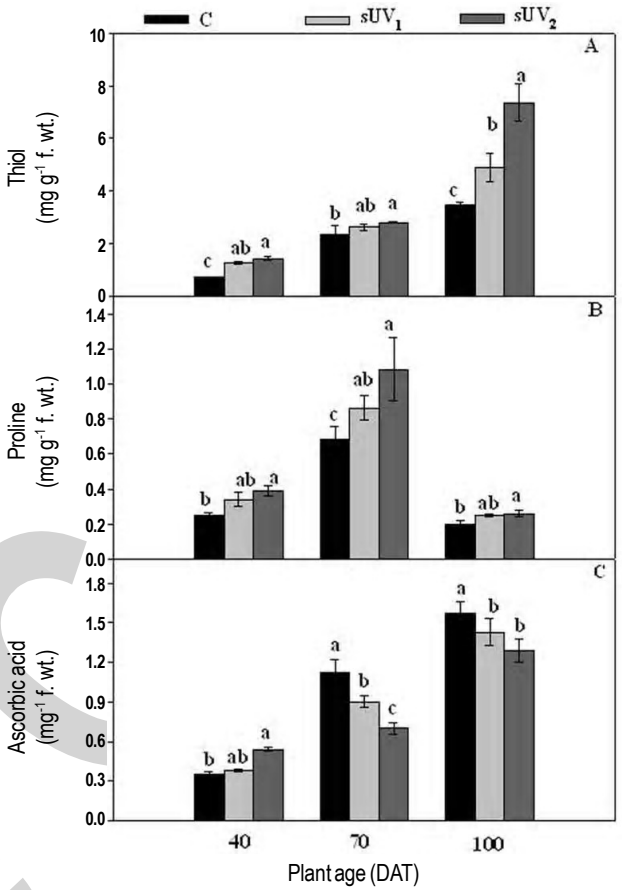

Fig. 3: Effect of different doses of sUV-B on (A) Thiol (B) Proline and (C) Ascorbic acid contents of *Acorus* plants (mean ± S.E) at 40, 70 and 100 days after transplantation (DAT). Values followed by the different letter within same group indicate statistically significant difference ($p < 0.05$)

agreement of earlier studies, reporting induction of the activities of these enzymes under UV-B to detoxify excess ROS as reported in *Cucumis sativus* (Jain *et al*., 2004), *Triticum aestivum* and *Vigna radiata* (Agrawal and Rathore, 2007) and *Cassia auriculata* (Agarwal, 2007). Antioxidative enzymes like catalase, peroxidase, ascorbate peroxidase and glutathione reductase are involved in ascorbate-glutathione cycle, for detoxification of excess H_2O_2 produced under various stresses (Noctor and Foyer, 1998).

In the present study, SOD activity increased initially in sUV-B treated plants, causing dismutation of superoxide radicals ($^{\bullet}O_2$) into H_2O_2 (Kondo and Kawashima, 2000). Activity of SOD was greatly induced by sUV-B exposure (20 under sUV_1 and 53.7% at sUV_2) at 40 DAT while at later stage of sampling, reduction in SOD activity was observed under sUV_2 by 19.7 and 44.1% at 70 and 100 DAT, respectively as compared to their respective controls (Fig. 1). Catalase activity increased initially by 117.4 and 212.5% under sUV_1 and sUV_2, respectively at 40 DAT, but declined significantly at later stages. At 70 and 100 DAT, CAT activity reduced maximally by 30.2 and 22.6%, respectively under UV_2 (Fig. 1). Foyer *et al*. (1994) reported that catalase is not a stable enzyme and it is susceptible to photoinhibition and degradation, so decrease in its activity is frequently observed under stress (Shim *et al*., 2003).

sUV-B exposure induced POX activity at all the sampling ages with their respective increments of 66.3 and 90.6% under sUV_1 and sUV_2 respectively at 100 DAT as compared to controls (Fig. 1). Multivariate analysis results showed that individual factors of age, sUV-B and the interactions between them significantly affected SOD, CAT and POX activities (Table 1). Both CAT and POX showed enhanced activity under sUV_2, supporting the findings of earlier studies on various plants (Yannarelli *et al*., 2006; Costa *et al*., 2002; Jain *et al*., 2004; Xu *et al*., 2008).

APX activity increased significantly under sUV-B at all the ages, with increments of 83.3 and 22.7% under sUV_2 at 40 and 100 DAT, respectively when compared to their controls (Fig. 2). GR activity showed stimulation of 34.4 and 40% at 40 DAT and 20 and 13.8% at 100 DAT at respective dose of sUV_1 and sUV_2 (Fig. 2). Results of two-way ANOVA showed that interaction of age × treatment was significant for APX, while non-significant for GR (Table 1). Results showing an increment in APX activity was in agreement with the findings of earlier studies made on *Hordeum vulgare* (Zancan *et al*., 2008), *Glycine max* (Xu *et al*., 2008), *Vigna unguiculata* and *Crotalaria juncea* (Selvakumar, 2008). Increasing trend of GR activity was also consistent with other studies performed under UV-B stress (Costa *et al*., 2002; Jain *et al*., 2004; Xu *et al*., 2008). Induction of APX and GR due to sUV-B indicates a preferential synthesis/activation of these enzymes, playing a crucial

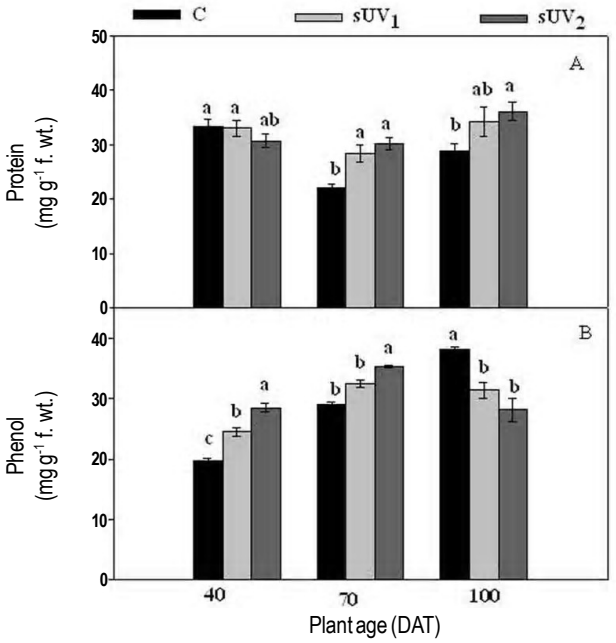

Fig. 4: Effect of different doses of sUV-B on (A) Protein and (B) Phenol content of *Acorus* plants (mean ± S.E.) at 40, 70 and 100 days after transplantation (DAT). Values followed by the different letter within same group indicate statistically significant difference ($p < 0.05$)

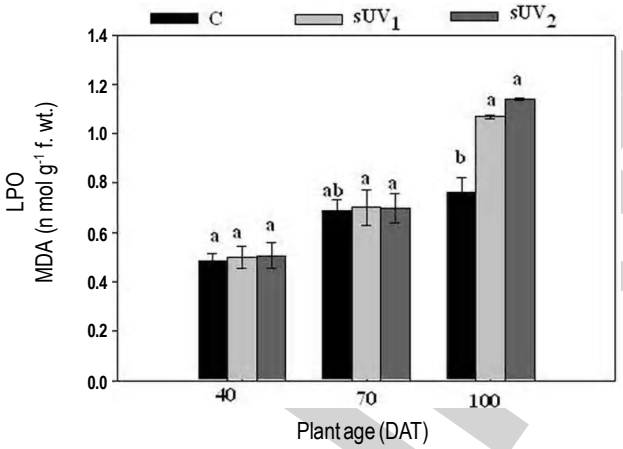

Fig. 5: Effect of different doses of sUV-B on Lipid peroxidation of *Acorus* plants (mean ± S.E) at 40, 70 and 100 days after transplantation (DAT). Values followed by the different letter within same group indicate statistically significant difference ($p < 0.05$)

role in scavenging of H_2O_2 *via.* the ascorbate-glutathione cycle (Noctor and Foyer, 1998).

Results of Two-way ANOVA showed that individual effects of age, sUV-B as well as interaction between age × sUV-B were significant for ascorbic acid, thiol and proline (Table 1). An increase in ascorbic acid content was observed due to sUV-B exposure at initial stage of sampling, showing a significant increment of 54.3% with sUV_2 as compared to control ones, whereas at later stages a decline was observed (Fig. 3). Ascorbic acid (AsA) acts as an antioxidant, reacting directly with hydroxyl radical, singlet oxygen and super oxide radicals. Increase in ascorbic acid in plants at early age after sUV-B exposure was also manifested in several studies suggesting its induction due to UV-B stress (Costa *et al*., 2002; Nasibi and Kalantari, 2005). The reduction in ascorbic acid at later stages of observations could be explained due to increased activity of APX after UV-B exposure resulting into more consumption of ascorbic acid for effective quenching of oxyradicals. Decline in ascorbic acid under UV-B stress was also reported by Agrawal and Rathore (2007) in wheat and mung bean. Thiol and proline contents increased by 40.9 and 25% under sUV_1 and 112.4 and 30% at sUV_2, at 100 DAT (Fig. 3). Thiols as antioxidant play a crucial role in stabilizing the protein structure, function and regulation of enzyme activity and in controlling the activity of transcription factors. It is quite possible that increased content of thiols under UV-B stress might be involved in more efficient quenching of oxyradicals. Proline accumulation was also higher under UV-B stress condition, which might protect the plant cells against peroxidative process (Pardha Saradhi *et al*., 1995). Increment of proline under UV-B stress is in consistent with data of Carletti *et al.* (2003) on maize. Under sUV-B irradiation, an increment in proline and thiol content was observed in pea (Singh *et al.,* 2009).

Results of two-way ANOVA showed that protein and phenol content were significant due to age, sUV-B but interaction of age × sUV-B was not significant (Table 1). Foliar protein content increased under sUV-B, showing increments of 18.7 and 25.3% under sUV_1 and sUV_2 respectively at 100 DAT (Fig. 4). Increment of protein under sUV-B might be due to the synthesis of stress proteins and other related enzymes. Similar trend of increment in protein content was reported in *Brassica napus* under UV-B stress (Nasibi and Kalantari, 2005). Total phenol content increased, particularly under sUV_2 (44.5 and 21.2%) when compared with their controls at 40 and 70 DAT, respectively. However, at 100 DAT, reduction in phenol was observed in sUV-B exposed plants (Fig. 4). Phenol plays important role in plant protection by shielding UV-B irradiation to sensitive photosynthetic mesophyll tissue. Reductions in total phenol contents at later age of experiment were contradictory to earlier works showing its higher accumulation under UV-B stress (Caldwell *et al*., 1983). Reduction in phenol content at later stage might be due to higher activity of the phenol oxidizing peroxidase consuming the plant phenolics in plant defense against UV-B stress (Jansen *et al*., 2001).

Oxidative damage to cellular membrane due to oxyradicals was measured in terms of lipid peroxidation (LPO) that assessed via increase in MDA content. At initial age, LPO did not vary significantly at sUV_1, depicting no damage to membrane integrity at lower dose. Similar findings were reported by Giordano *et al.* (2004) on *Gunnera magellanica*, observing no damage to lipid at low or moderate level of sUV-B. At later stage of sampling, an increment of MDA content by 28.2 and 44.7%, respectively in sUV_1 and sUV_2 was observed (Fig. 5). Significant interaction due to age × sUV-B was detected on LPO phenomenon (Table 1). Increase in LPO under UV-B stress was also reported in earlier studies made on *Helianthus annuus* (Costa *et al*., 2002; Yannarelli *et al.*, 2006) and

Pisum sativum (Agrawal and Mishra, 2009). Increment in MDA content under high dose of sUV-B clearly suggest that it caused oxidative damage to cell membrane despite of stimulation of antioxidant defense system as it was not sufficient enough to counteract the stress due to excess generation of ROS.

Results conclude that exposure of *Acorus* plants to sUV-B treatment significantly affected the secondary metabolites with activation of antioxidative defense system (both enzymatic and non enzymatic antioxidants), indicating their crucial role in detoxifying the excess ROS generated due to sUV-B. The damage due to these radicals at later age as indicated by increase in LPO indicated that activation of various antioxidants did not provide complete protection to *Acorus* plants under the given sUV-B stress.

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