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Research Article

ELICITOR INDUCED PRODUCTION OF SHATAVARINS IN THE CELL CULTURES OF *ASPARAGUS RACEMOSUS*

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ABSTRACT
Asparagus racemosus is one of the important monocot medicinal plants, which is in great demand for its steroidal saponins called shatavarins. In the current study, *A. racemosus* cell cultures were tested for elicitation with biotic elicitors derived from *Fusarium oxysporium* and *Rhiizopus stolonifer* and abiotic elicitors UV and Salicylic acid for the production of Shatavarins. Two elicitation points were selected 0 d and 13d. Biotic elicitors were used as 50µg equivalents of glucose and UV irradiation of 5 min and SA at 100 and 200 µM respectively. The outcomes of the study indicated that biotic elicitors showed mild elicitation effect after 25 d with 0day and 13d treatments with maximum shatavarins levels of 14.44 ± 0.16 mg g^{-1} DW for *Fusarium* and 22.48 ± 0.61 mg g^{-1} with *Rhizopus*. Abiotic elicitors gave higher accumulation of shatavarin with 0 d treatment and were more effective than biotic elicitors in terms of shatavarin accumulation. SA gave maximum accumulation of 22.63± 0.12 mg g^{-1} and UV gave an accumulation of 24. 22± 0.16 mg g^{-1} of shatavarin respectively. Out of all the elicitor's studies, UV was found to be the superior elicitor in the induction of shatavarin accumulation.

Key Words: *Asparagus Racemosus, Elicitation, Steroidal Saponins, Shatavarins.*

INTRODUCTION
Plant secondary metabolites are important sources for pharmaceuticles, flavors and several important biochemicals. These compounds get accumulated in plants when they are subjected to various kinds of stresses such as biotic and abiotic elicitors or signal molecules. In the current study the impacts of certain elicitors have been reported on *Asparagus racemosus* (local name Shatavari) cell cultures. It is an indigenous monocot medicinal plant of India which produces steroidal saponins called shatavarins (Figure 1). It is routinely used in indigenous medicines and is one of the extensively exported medicinal formulations from India for menopausal and fertility related problems. *A. racemosus* is particularly drawing attention as it is a well known immunomodulant, galactogauge, adaptogen, antitusive, anticarcinogen, antioxidant, antidiarrhial and in general a tonic for both the sexes (Gaitonde *et al.*, 1969; Joglekar *et al.*, 1967; Oketch-Rabah, 1998; Rao, 1952; Rice, 1988; Shao *et al.*, 1997; Thatte *et al.*, 1987). Owing to the seasonal and physiological constrains, the large scale commercial plantation of this plant has not been possible. Wasteful collection practices have compounded the problems even further (Pise *et al.*, 2011) and have lead this plant get recognized as 'vulnerable' (Warner *et al.*, 2001). This is one of those several medicinal plants for which sustainable conservation methods are required on a priority basis (Rao, 1952; NMPB 2002; Saxena *et al.*, 2008).
Plant tissue culture has been successfully employed for several plants facing similar problems including *A. racemosus* (Pise *et al.*, 2011). *Asparagus racemosus* cultures have been reported to produce shatavarins and have been studied extensively (Pise *et al.*, 2012). Elicitation of plant cells in culture represents a useful tool to improve the production of phytochemicals. Plants respond to attack of pathogens, insects and other abiotic stresses by activating several biosynthetic processes (Vasconsuelo and Boland, 2007). Keeping the foregoing in view, biotic and abiotic elicitors were screened for elicitation of shatavarin production in *in vitro* cultures of *A. racemosus.*

MATERIALS AND METHODS

Initiation of suspension culture

Cell cultures of *A. racemosus* were initiated from the friable calli grown on solid media as reported by Pise *et al.,*2012. 4 g of callus tissue was inoculated in 40 ml MS liquid media in a 100 ml flasks supplemented with 1.0 mg l^{-1} NAA and 2,4-D and 0.5 mg l^{-1} BAP + 2 g l^{-1} casein hydrolysate (CHL) + 0.005% pectinase Suspension cultures were maintained at 25 $\pm$ 2 **^{o}C** on a rotary shaker at 80 rpm. Further subculture involved replacement of 10 ml of suspension with the fresh medium of same composition, for a period of three months regularly, at an interval of 7 d to obtain a viable and established cell culture. At the end of thirteenth subculture, they were treated as experimental flasks and were maintained for a period of 30 days. Flasks were sacrificed for growth and shatavarin assay at a regular interval of 5 d. In all, 3 replicas were kept for each evaluation point and the full 30d passage cycle was repeated twice.

Preparation of Elicitors

The elicitor was prepared from *Fusarium oxysporium* and *Rhizopus stolonifer* as reported previously (Hu et al. 2003d; Lee *et al.,*1981). Crude elicitors from both the fungi were prepared by autoclaving washed fungal cell walls and the elicitor concentration was determined by the anthrone method with glucose as the standard (Ayer et al. 1976). The *Fusarium* time course was conducted during the 12th passage by adding 2.5 ml of a 5 mg ml^{-1} aqueous solution of a *Fusarium* cell wall preparation for a final concentration of 50µg glucose equivalents ml^{-1} (Schumacher *et al.,*1987). Similarly, a 50µg equivalent glucose / ml of *Rhizopus* elicitor were used to elicit cell cultures of *A. racemosus* on 0d and 13d of culture cycle. UV elicitation was performed during the 12th passage. Cultures were strained from culture media and spread onto 150 ml plates containing; 50 ml MS agar. Treatment plates were irradiated in a UV box for 5 min at 8000 J m^{-2} while control plates received no exposure. Plates were then held on an illuminated shelf at 24ºC until harvested.

Extraction and estimation of Saponin

Extraction and quantification of total saponin from the dried plant roots, *in vitro* cells and the spent media were carried out as by the previously published method (Mathur *et al.,* 1994). Briefly, cells and the media were extracted separately with methanol (1:2) overnight and the procedure was repeated 4 times. All the extracts were pooled and concentrated by evaporating at 60 ºC on a rotary evaporator to dryness. The dried residue was redissolved in 10 ml of H_2O and further extracted with n-butanol. The n-butanol fraction was finally concentrated to dryness on a rotary evaporator under reduced pressure and redissolved in 5 ml of methanol and stored. Quantitation of shatavarins was carried out as reported by pise et al in 2012.

Statistical analysis

A completely randomized design was used in all the experiments. Experiments were repeated twice. ANOVA and mean separation were carried out using Duncan's multiple range tests. Significance was determined at the 5% level. For the quantitative analysis of the phytochemicals, data were collected from three independent experiments and were presented as mean ± standard deviation (SD)

RESULTS

Effect of biotic elicitors

The fungus *F. oxysporium* and *R. stolonifer* were selected for this study as biotic elicitors. A 50µg glucose equivalents ml^{-1} was introduced in the cell cultures on 0 d and 13 d of the culture cycle. Both the elicitors were found to have mild elicitation effect on the *Asparagus* cultures when compared to the non-elicited controls. Maximum shatavarin accumulated on 20 d (14.44 ± 0.016mg / g of DW) when the cultures were treated with elicitor on the 13 d (Figure2). Rhizpus showed a delayed elicitation compared to *Fusarium*. Maximum shatavarin accumulated on 25 d of culture cycle with 0 d treatment. The levels remained constant throughout the stationary phase. Compared to elicitation on 0 d, elicitation on the 13 d was found to be more suited for shatavarin production (Figure 3) and was found to be better suited for elicitation than Fusarium in terms of shatavarin accumulation. Results of the experiments showed that

biotic elicitors did not show much of an impact on the elicitation phenomenon, on the chosen day of treatments.

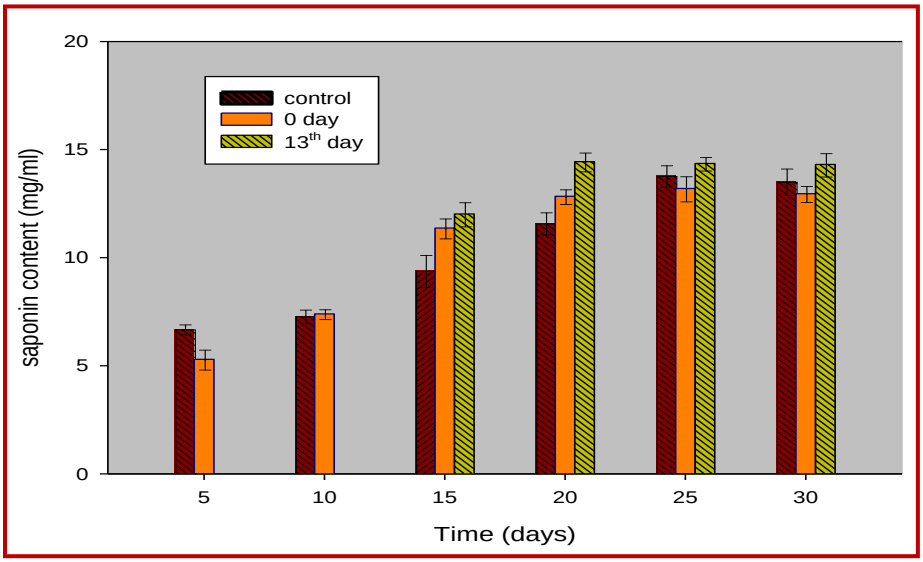

Figure 2: Elicitation of shatavarin production by *F. oxysporium* in *A. racemosus* cell cultures. Cell cultures were treated with 50µg equivalent of glucose ml^{-1} elicitor on 0d and 13d of culture cycle and shatavarin accumulation was determined. Each value represents mean ± SD for five determinations.

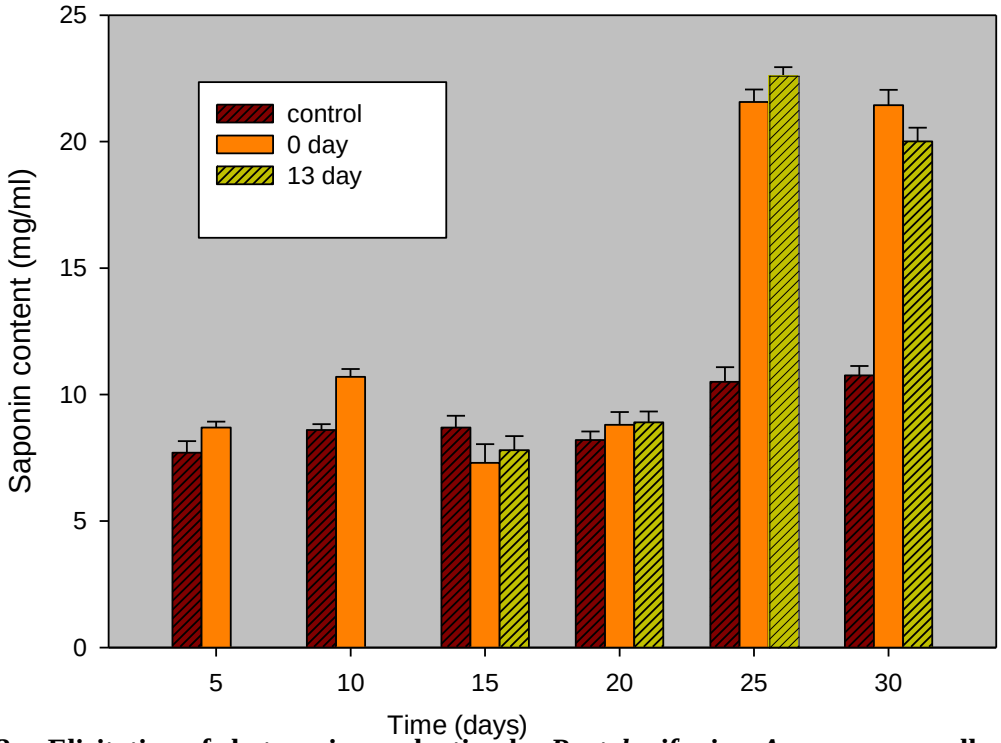

Figure 3: Elicitation of shatavarin production by *R. stolonifer* in *A. racemosus* cell cultures. Cell cultures were treated with 50µg equivalent of glucose ml^{-1} elicitor on 0d and 13d of culture cycle and shatavarin accumulation was determined. Each value represents mean ± SD for five determinations.

Effect of abiotic elicitors

Salicylic acid (SA) is a naturally occurring phenolic compound found in many plants. SA supplied exogenously affects various physiological and biochemical processes. It is known to act as a signaling molecule in plant disease resistance, flowering and thermogenesis (Lee et al, 2001). It affects growth and accumulation of secondary metabolites in cell cultures, in a dose dependent manner. In our study, two concentrations 100 µM and 200 µM were used for elicitation. Out of the two doses, 200 µM gave better elicitation both on 0 d and 13d treatments (Figure4). The amount of Shatavarin increased from 15.2 ± 0.053 mg/ g on 20 d to a maximum of 22.32 ± 0.033 mg/g on 25d with 0 d treatment.

UV rays (250-340nm) when used for elicitation , a preliminary study to fix the time duration for UV exposure was carried out during which cells were exposed to UV radiations for 2, 5, 10 and 15 min respectively. Cell viability when checked 24 h after the irradiation showed that irradiation with UV for 2 min and 5 min did not cause cell death (98% cell survival as visualized by florescein diacetate staining); however, irradiation for longer than 5 min caused 80 – 100 % cell death (data not shown). We have therefore used 5 min of UV-B as the standard irradiation time for all further experiments. Figure 5 indicates elicitations in both 0 d and 13 d treatments but the former was more suited for Shatavarin production. A maximum of 24.77 ± 0.0152 mg/ g was observed on the 25 d of culture cycle.

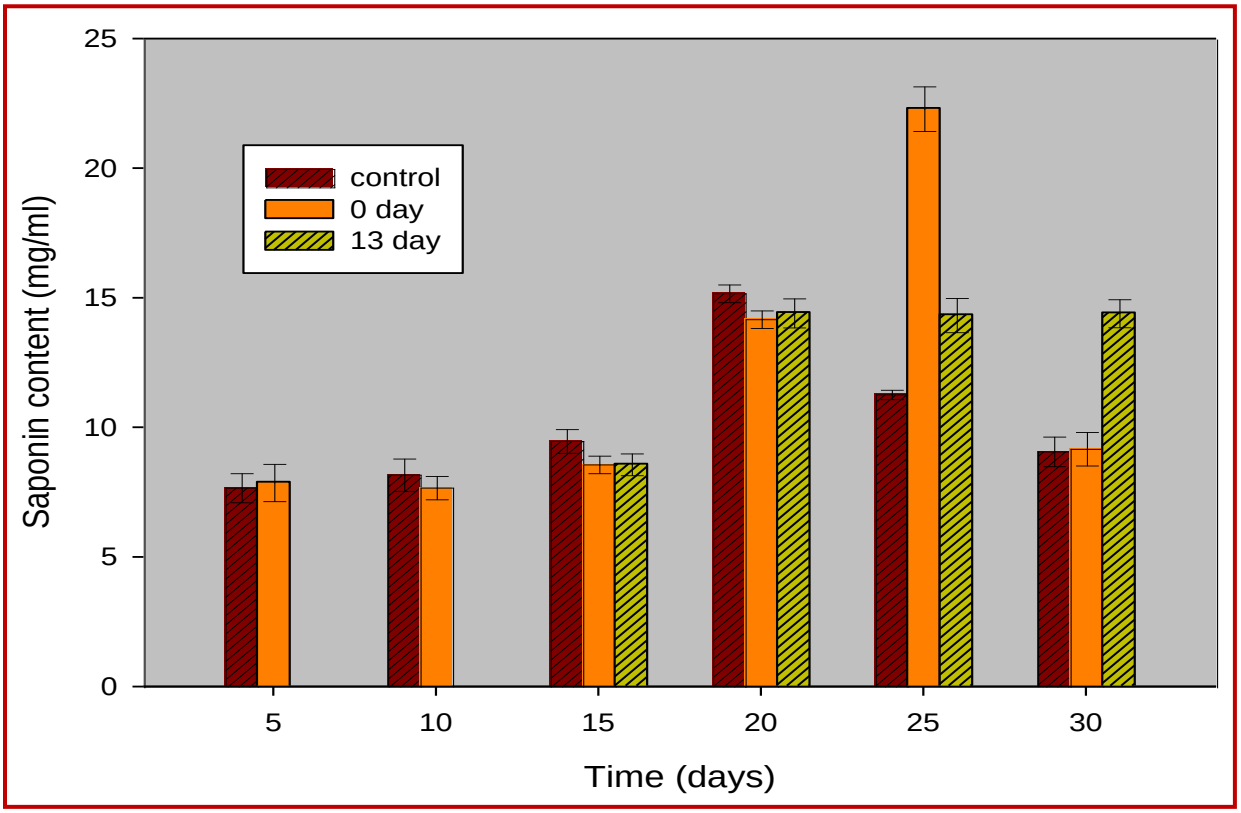

Figure 4: Elicitation of shatavarin production by salicylic acid in *A. racemosus s* cell cultures. Cell cultures were treated with 50µg equivalent of glucose ml^{-1} elicitor on 0d and 13 d of culture cycle and shatavarin accumulation was determined._Each value represents mean ± SD for five determinations.

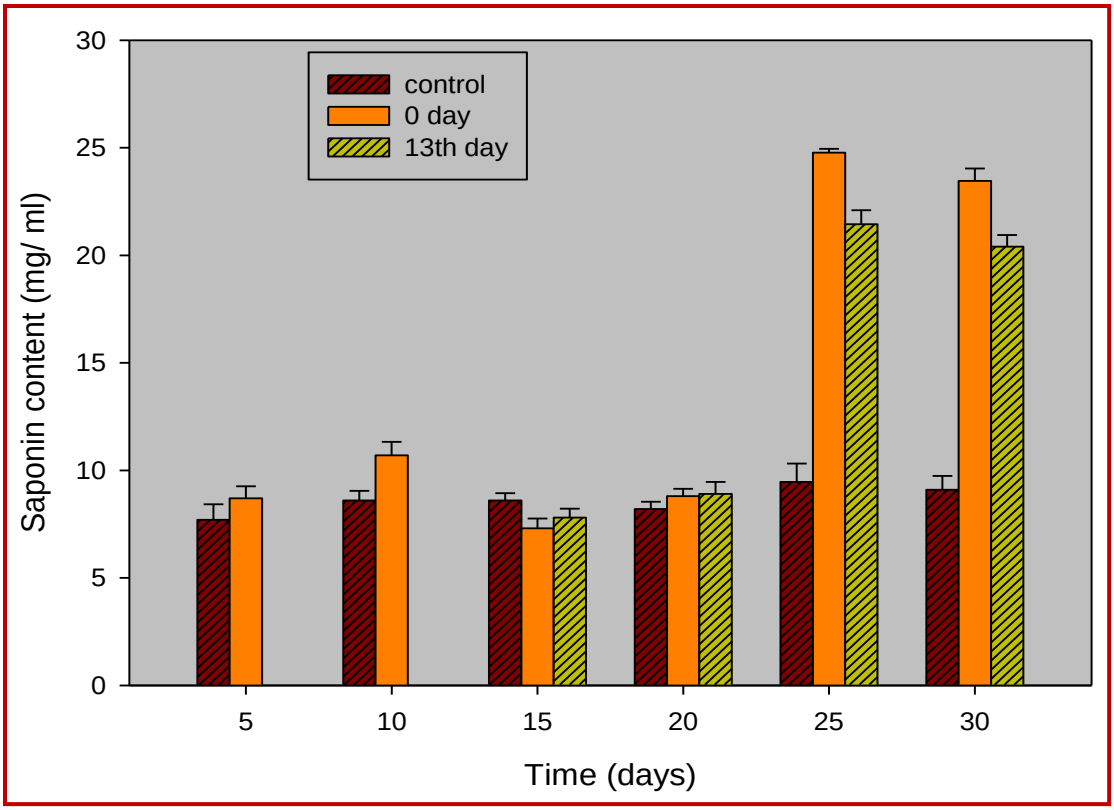

Figure 5: Elicitation of shatavarin production by UV rays irradiation of *A. racemosus* cell cultures. Cell cultures were irradiated with UV rays for 5 min and then shatavarin contents were evaluated at an interval of 5d in a 30d culture cycle

DISSCUSSION

In the current study the above four probable elicitors were tested and standardized for the elicitation of shatavarin production in cell cultures of *A. racemosus*. All the tested elicitors showed varied effects on the elicitation of saponin by *A. racemosus* cell cultures. Fusarium was selected for the current study as it is one of the major known pathogens of *Asparagus* genera. They are known to cause crown and stem rot problems in *Asparagus* (Cohen and Heald, 1947; Graham, 1955; Manning and Vardaro, 1977). Natural plants are found to harbor non virulent strains of *Fusarium* as endophytes. These endophytes probably induce resistance against virulent strains of *Fusarium*. This has been observed both *in vitro* and *in vivo* studies carried out by Domicone and Manning in 1982. Probably this could be one of the probable reasons for lower elicitation potential of *Fusarium oxysporium* as presence of fusarium spores were found in the cultured tissue also. All the tested elicitors were found to increase the accumulation of shatavarin in *A. racemosus* cell cultures but the biosynthetic dynamics of the culture differed with the type of elicitor to a large extent. Few of the major conclusions which could be the study are;

- *A. raracemosus* cell cultures were more responsive at the beginning of log phase when elicited with biotic elicitors. With the abiotic elicitors such phase preference was not noticed.
- Salicylic acid and UV irradiation for 5 min were found to be significantly superior elicitors for *A. racemosus* cultures than the tested biotic elicitors ($p < 0.05$).
- For biotic as well as abiotic elicitors, growth parameters remained stable under the tested elicitor dose.

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