Bio-elicitation of β-carboline alkaloids in Cell Suspension Culture of *Peganum harmala* L.

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Abstract

Background: Sustainable and commercial production of secondary metabolites is a critical issue when dealing with its clinical application. Efforts are still being made to look for biotic or abiotic elicitors with more efficient and universal effects on the improvement of secondary metabolites.

Objective: In order to evaluate the suitability of different biotic elicitors on *P. harmala* L. cell suspension cultures was established to enhance the β-carboline alkaloids (harmaline and harmine) production.

Methods: The elicitation of cell suspension cultures of *Peganum harmala* L. was done by adding various fungal mycelium homogenates (*Aspergillus flavus, Alternaria alternate, Coriolus versicolor, Fusarium oxysporum, Mucor sp, Penicillium notatum,* and *Rhizopus stonifer*), Casein hydrolysate and *Saccharomyces cerevisiae* at different concentrations. The cell cultures of *P. harmala* L. were subcultured on MS medium with optimal treatment of biotic elicitor. CAMAG analytical HPTLC system was used for estimation of harmaline and harmine after extraction of β-carboline alkaloids.

Results: The maximum harmine production (91.2±1.8 µg g^{-1} DW) was observed at 1000 mg l^{-1} *S. cerevisiae* in cell suspension culture of *P. harmala* L. (1.68 fold over than the control). Also the results showed that supplement of 75-100 mg l^{-1} casein hydrolysate in cell cultures media increased biomass of cell culture and harmaline and harmine production (1.61 and 1.46 times over than the control, respectively).

Conclusion: The conclusion of the research showed that by applying biotic elicitors, we can reach to higher secondary metabolites (harmaline and harmine) in cell suspension culture of *P. harmala* L. We suggest future investigation on using other elicitors like bacterial extract or signal transduction compounds in cell suspension culture of *P. harmala* L. in order to increase the production of different kind of secondary metabolites.

Keywords: ***Peganum harmala* L., Biotic elicitors, Cell suspension culture, Harmaline, Harmine**

Background

Peganum harmala L. (Zygophyllaceae) is a native herb which can be found in dry area from east of Mediterranean Sea to north of India. This plant is known as "Espand'' in Iran, "Harmel" in North Africa and "African Rue", "Mexican Rue" or "Turkish Rue" in United States [1]. The seeds of *P. harmala*, are used as an anti-hemorrhoids and central nervous system stimulating agent in folk medicine [2, 3]. In traditional medicine, seeds of *P. harmala* L. were used as powder, decoction, maceration or infusion for fever, diarrhea, abortion and subcutaneous tumors. It is also widely used as a remedy for dolorous events (rheumatic pain, painful joint and intestinal pain) [4]. It is also used for treatment of asthma, jaundice, lumbago and many other human ailments [5, 6]. *P. harmala* traditionally has been used in Iran as an antiseptic and disinfectant agent by burning its seeds [7].

Some of reported pharmacological effects of *P. harmala* may be attributed to its β-carboline alkaloids, mostly harmine, as well as harmaline, harmalol, harman, peganine, isopeganine, dipeganine, deoxypeganine and quinazolin derivatives such as vasicine, vasicinone and deoxyvasicinone [49, 50] that have a wide spectrum of pharmacological actions in various areas. These consist of anti-spasmodic, anti-pyretic [8, 9], anti-cancerous and anti-tumor [4, 10], central nervous system effects [11], Cardiovascular actions [12], hallucinogenic [13], central monoamine oxidase inhibition [14], binding to various receptors including 5-HT and the benzodiazepine binding receptors [15], platelet aggregation inhibitory [16] and immunomodulatory effects [17]. Previous studies have reported that the alkaloids extract of *P. harmala* causes significant anti nociceptive effect in both phases of the formalin test in mice [18].

Plant cell culture systems represent a potential renewable source of valuable medicinal compounds, flavours, fragrances, and colorants. The principle advantage of this technology is to provide continuous and reliable source of plant pharmaceuticals. So it could be used for the large scale culture of plant cells from which these metabolite can be extracted.

Plant cell suspension cultures are attractive alternative for large-scale production of plant natural products, particularly secondary metabolites. General strategies for optimization of secondary metabolite production in plant cell culture, such as elicitation, immobilization, cell wall permeabilization and feeding of organic supplements and precursors, have been successfully used for improving products yield in many cases [19]. The reasons responsible for the diverse stimulating effects of elicitors are complicated and could be related to the interactions between elicitors and plant cells, elicitor signal transduction and plant defense responses [20]. However, efforts are still being made to look for biotic or abiotic elicitors with more efficient and universal effects on the improvement of secondary metabolites [21]. The main objective of this study was to investigate the appropriateness of different biotic elicitors function on *P. harmala* L. cell suspension cultures in an effort to enhance the β-carboline alkaloids (harmaline and harmine) production.

Materials and Methods

Plant materials

The Samples of *P. harmala* L. were collected from natural population of Birjand (South Khorasan Province, Iran) and used as a source of leaflet explants. The young leaflets were washed five times with sterilized distilled

water and then disinfected for 5 minutes in 0.1% $HgCl_2$. Explants were rinsed five times with sterile distilled water and transferred to MS solid medium [22] supplemented with 1.0 mg l^{-1} BAP/ Kin and in combination with 0.5 mg l^{-1} NAA/IAA/2,4-D.

The callus induction was initiated on leaflet surface after 2 weeks. The calli were subcultured every four weeks in the test tubes of solid MS supplemented with 1.0 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 3% (w/v) sucrose. Media pH was adjusted to 5.7 before autoclaving. The calli were incubated at 27°C and illuminated with cool, white fluorescent light for 16 h light and 8 h darkness. After four weeks, calli were transferred to 50 ml MS liquid medium supplemented with 1.0 mg l^{-1} BAP, 0.5 mg l^{-1} NAA in a 250-ml Erlenmeyer flask as the stock of suspension culture. Cell cultures were grown on a rotary shaker at 100 rpm with a 16-h photoperiod (30 µmol m^{-2} s^{-1}) at 25±2 °C. Cell cultures were maintained by subculturing to fresh medium using 5 ml cell suspension inoculums every three weeks. Treatments were various fungal mycelium homogenates, Casein hydrolysate and *S. cerevisiae* at different concentrations. The treatments were set up in a factorial experiment based on completely randomized design with 6 replications. Means differing significantly were compared using Duncan's Multiple Range Test (DMRT) at the 5% probability level.

Growth kinetics of cell culture

Measurements of the fresh and dry cells weight were carried out in suspension cultures at weekly intervals. The cell biomasses were separated from the liquid media by centrifugation at 8000 rpm for 10 min. The liquid medium was pipetted out and the cell pellet was dried between sheets of filter paper until no moisture was released from the cell pellet. The fresh weight of pellet was determined by using analytical balance. The cell biomasses were dried in an oven at 60°C till constant weight was obtained. Duplicate samples were taken from three independent shake flasks. Values based on these six samples were calculated as mean ± S.E. The dried samples were used for alkaloids estimation.

Biotic elicitor preparation

The strains of *Aspergillus flavus, Alternaria alternate, Coriolus versicolor, Fusarium oxysporum, Mucor sp., Penicillium notatum, Rhizopus stonifer* and *Saccharomyces cerevisiae* were obtained from Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The fungal cultures were established on Potato Dextrose Agar (PDA) medium and *S. cerevisiae* on MGYP medium [Malt extract (0.3 g), Glucose (1.0 g), Yeast extract (0.3 g), Peptone (0.5 g), Distilled water (100 ml), Agar (2.0 g), pH=6.4-6.8]. The biotic cultures were maintained in the laboratory at 25±2°C at 8 hours photoperiod with diffuse lighting and 16 hours darkness.

Twenty days old fungal mycelia were autoclaved for 10 min at 120°C and separated by filtration from the nutrient media and washed with sterilized distilled water for five times. The mycelia mass were weighed and dried in an oven at 50°C for 72 hours and were stored at 4 °C, until treatment. Treatments of the cell suspensions of *P. harmala* L. with the fungal mycelia (250, 500, 750, 1000 mg l^{-1}), *S. cerevisiae* (250, 500, 750, 1000, 1250 mg l^{-1}) and casein hydrolysate (Sigma) (25, 50, 75, 100 mg l^{-1}) were performed 7 days after subculture when the cells were in the log phase of growth (Figure 1). Also cell suspensions

culture on MS medium without elicitor were used as control. Appropriate concentration of casein hydrolysate was dissolved in distilled water and then added to the medium before setting pH and sterilization. Moreover, the cell cultures were subcultured on MS medium with optimal treatment of casein hydrolysate (75 mg l^{-1}) and biotic elicitor (1000 mg l^{-1} *S. cerevisiae*). Treated and control cell suspensions were then harvested by vacuum filtration on day 15 post-elicitation, weighed for growth analysis and stored at -21°C until determination of alkaloids.

Extraction and quantification of secondary metabolites

The dried powdered cell cultures and seeds of *P. harmala* L. were used for obtaining the crude extract by soaking 1.0 g of the dried biomass in 50 ml methanol at 50°C in water bath for 1 h. The extracts were combined and evaporated to dryness. The residue was dissolved in 50 ml HCl (2%) and filtered through Whatman No. 1 filter paper. The filtrate was extracted two times with 20 ml petroleum ether. The aqueous acid layer was basified (pH: 10) with NH_4OH and extracted four times with 50 ml chloroform. The chloroform layer was combined and evaporated to dryness, and then the residues were dissolved in 25 ml methanol [23]. The solution of alkaloid extract was passed through 0.45 mm filter and 0.2 µl extract was directly injected into the HPTLC (High Performance Thin Layer Chromatography). For estimation of β-carboline alkaloid (Anchrom lab, Mumbai, 400081, India) CAMAG analytical HPTLC system was used. The results were obtained as a mean value of three separate injections. Harmine (Sigma; H-8646) and harmaline (Sigma; H-2256) were obtained from Sigma chemicals and were used as standards. Aluminum sheets of silica gel $60F_{254}$ (Merck) were also been applied. The chromatograms were developed in the mobile phase chloroform: methanol: 25% ammonia (5:4:1) dried and sprayed. Alkaloids were analyzed by using CAMG TLC Scanner 3 in UV-254 and UV-366 nm (Figure 2 & 3). The peaks corresponding to harmine and harmaline were confirmed by comparison with the commercial standard the crude extract samples. The alkaloids content in the crude extract was determined by comparing the peak areas with those of standard harmine and harmaline.

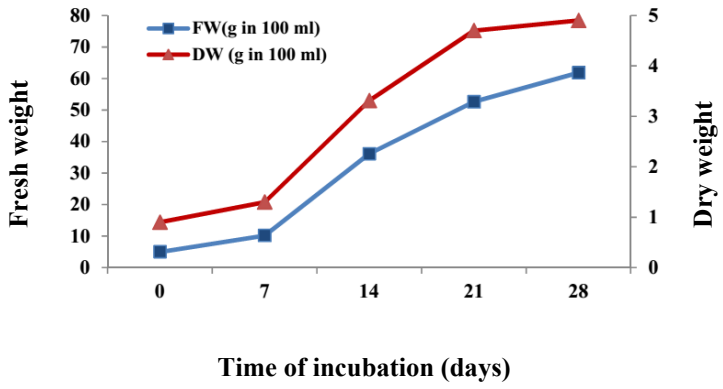

Figure 1- Growth of cell suspensions culture of *P. harmala* L. on MS + 5.0 µM BAP + 2.5 µM NAA

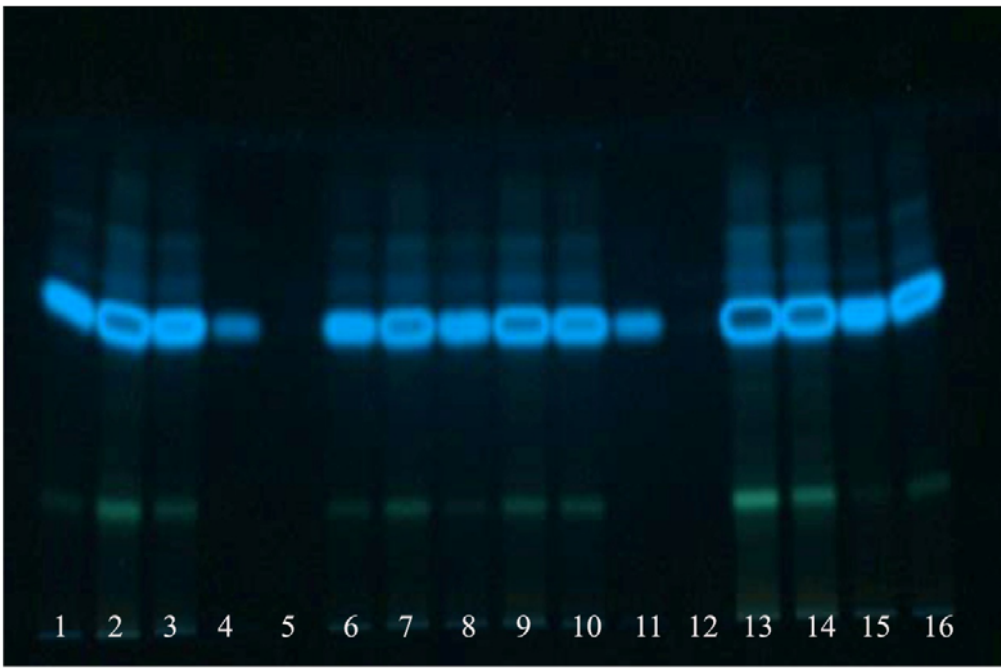

Figure 2- HPTLC fingerprint profile for the quantitative analysis of harmaline from cell culture of *P. harmala* L. on media containing biotic elicitors and Casein hydrolysate at 366 nm for cell grown on MS + 5.0 µM BAP + 2.5 µM NAA + 750 mg l^{-1} elicitors derived. Lanes: No.1: *Aspergillus flavus* No.2: *Saccharomyces cerevisiae,* No3: *Mucor sp.,* No4: Standard of harmaline, No.5: Not detected, No.6: *Alternaria alternate,* No.7: *Fusarium oxysporum*, No.8: *Penicillium notatum,* No.9: *Rhizopus stonifer*, No.10: *Coriolus versicolor,* No.11: Standard of harmaline, No. 13-16 from MS + 5.0 µM BAP + 2.5 µM NAA + Casein hydrolysate (100, 75, 50, 25 mg l^{-1}, respectively)

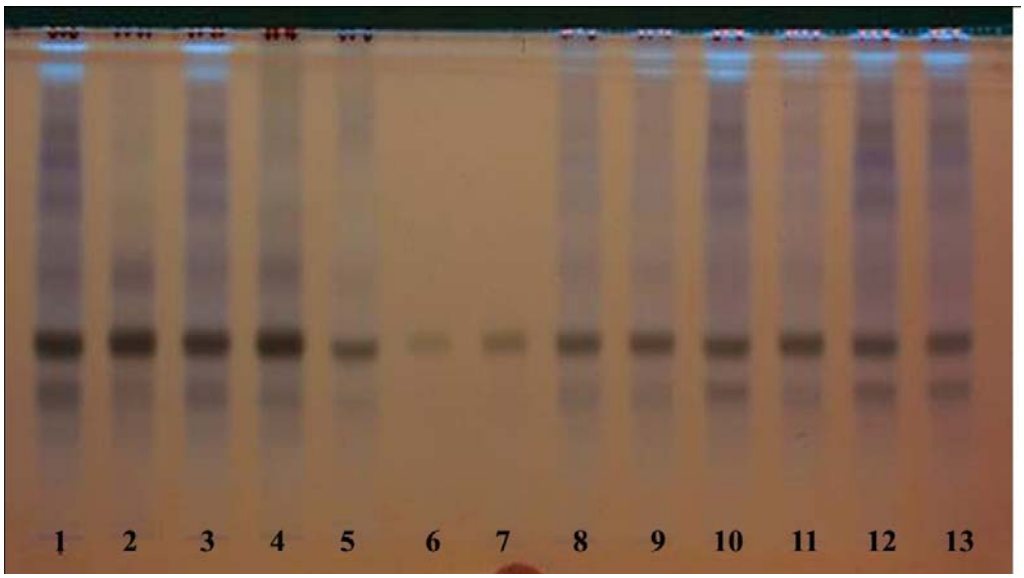

Figure. 3- HPTLC fingerprint profile for the quantitative analysis of harmine from cell culture of *P. harmala* L. on media containing biotic elicitors and Casein hydrolysate at 245 nm for cell grown on MS + 5.0 µM BAP + 2.5 µM NAA + 750 mg l^{-1} elicitors derived. Lanes: No.1: *Aspergillus flavus,* No.2: *Rhizopus stonifer, No.3*: Casein hydrolysate at concentration of 100 mg l^{-1}, No.4: *Saccharomyces cerevisiae,* No.5 *Fusarium oxysporum,* No 6, 7: Standard of harmine, No.8: *Penicillium notatum,* No.9: *Alternaria alternate,* No.10: *Coriolus versicolor,* No. 11-13: Casein hydrolysate (75, 50, 25 mg l^{-1}, respectively).

Results

Effects of fungal mycelia and *S. cerevisiae*

Adding of homogenate elicitors such as *Aspergillus flavus, Alternaria alternate, Coriolus versicolor, Fusarium oxysporum, Mucor* sp., *Penicillium notatum* and *Rhizopus stonifer* in the medium, declined the dry weight of cell culture, however it increased the total alkaloids as compared with control (Table 1). The dry weight of cell biomass gradually decreased by increasing concentration of fungal elicitors. While inclusion of *S. cerevisiae* elicitor increased up to 1000 mg l^{-1}, The maximum cell biomass reached to 5.64 ± 0.7 g in 100 ml (Figure 4).

The maximum accumulation of harmaline (71.2 ± 1.7 µg g^{-1} DW) was detected in the presence of 750 mg l^{-1} of *A. flavus*, which was about 1.69 times more than the control. The maximum harmine (91.2 ± 1.8 µg g^{-1} DW) was observed at 1000 mg l^{-1} *S. cerevisiae* which was about 1.68 times more than the control. Addition of higher concentration of *A. flavus* (1000 mg l^{-1}) and *S. cerevisiae* (1250 mg l^{-1}) in the medium slightly declined the accumulation of harmaline and harmine in the culture. The harmaline and harmine content showed slight increase in the presence of homogenate of *A. alternate, C. versicolor, P. notatum* and *R. stonifer*, however, inclusion of *F. oxysporum* was inhibitory for harmaline accumulation in the cell suspension culture. The results showed that among the various fungal mycelium homogenates and *S. cerevisiae*, the highest harmaline content was obtained in cultures elicited by 500 mgl^{-1} of *A. flavus* and the highest harmine was achieved in cultures containing 1000 mg l^{-1} of *S. cerevisiae*.

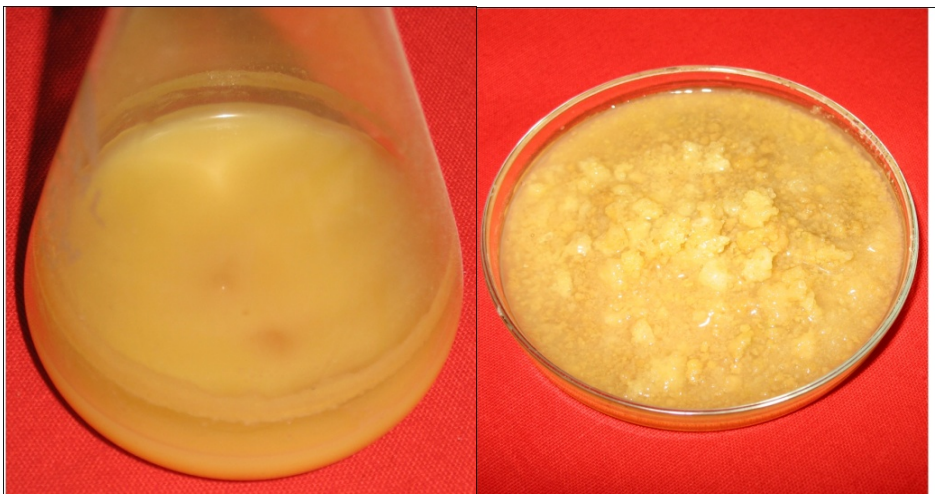

Figure 4- The cell biomass obtained from cell cultures of *P. harmala* L. on MS + 5.0 µM BAP + 2.5 µM NAA + 1000 mg l^{-1} *Saccharomyces cerevisiae*

Table 1- Effect of biotic elicitors on growth and secondary metabolites in cell culture of *P. harmala* L.

MS + 5.0 µM BAP + 2.5 µM NAA + Biotic elicitors (mg/l)		DW (g in 100 ml)	Secondary metabolites (µg/g DW)	
			Harmaline	Harmine
Control	0.0	5.32±0.7 b	42.3±1.3 lm	54.5±1.6 m
Aspergillus flavus	250	4.94±0.8 de	61.2±1.7 de	59.0±1.5 k
	500	4.72±0.5 ef	57.8±1.6 g	70.1±1.3 f
	750	4.46±0.6 hi	71.2±1.7 a	73.4±1.6 d
	1000	4.66±0.7fg	65.6±1.7 c	81.3±1.6 b
Alternaria alternate	250	4.94±0.4 d	32.3±1.6 o	57.8±1.4 l
	500	4.83±0.7 de	39.0±1.8 n	63.4±1.6 hi
	750	4.24±0.5 jk	47.8±1.8 k	62.3±1.8 ij
	1000	3.65±0.5 op	41.2±1.5 m	67.8±1.6 g
Coriolus versicolor	250	3.97±0.4 mn	38.9±1.7 n	47.8±1.4 q
	500	4.33±0.9 j	43.4±1.6 lm	43.4±1.5 s
	750	3.65±0.6 q	53.4±1.8 i	50.1±1.7 op
	1000	3.14±0.5 r	49.0±1.5 j	47.8±1.4 r
Fusarium oxysporum	250	3.88±0.6 no	41.2±1.4 m	51.2±1.7 p
	500	3.73±0.7 op	39.0±1.7 n	53±0.6 mn
	750	3.17±0.4 r	47.8±1.6 k	45.6±1.4 r
	1000	2.94±0.5 s	43.4±1.5 l	47±0.09 p
Mucor sp.	250	5.14±0.7 c	49.0±1.7 j	63.4±1.7 hi
	500	4.76±0.5 ef	56.7±1.5 gh	82.3±1.4 b
	750	4.87±0.7 de	57.8±1.3 g	72.3±1.6 de
	1000	4.08±0.6 lm	61.2±1.7 de	74.5±1.5 c
Penicillium notatum	250	4.56±0.8 gh	47.8±1.6 k	51.2±1.4 no
	500	4.75±0.5 ef	52.3±1.4 i	63.4±1.6 h
	750	4.17±0.7 kl	60.1±1.7 ef	71.4±1.3 ef
	1000	3.64±0.6 q	62.3±1.5 d	70.1±1.6 f
Rhizopus stonifer	250	4.63±0.8 fg	52.3±1.7i	62.3±1.6 ij
	500	4.85±0.4 de	59.0±1.6 f	68.9±1.7 g
	750	4.47±0.6 hi	56.7±1.7 g	73.4±1.5 d
	1000	4.13±0.4 kl	69.0±1.5 b	70.1±1.3 f
Saccharomyces cerevisiae	250	4.47±0.5 hi	59.0±1.6 f	61.2±1.5 j
	500	5.24±0.3 bc	57.8±1.7 g	79.0±1.9 c
	750	5.15±0.6 c	62.3±1.4 d	83.4±1.5 b
	1000	5.64±0.7 a	68.9±1.6 b	91.2±1.8 a
	1250	5.25±0.4 bc	62.3±1.4 d	78.9±1.4 c
Seeds			66.7±1.4 e	82.3±1.3 c

D.W: Dry Weights represent the mean ± SE calculated on 6 replications. Data of secondary metabolites were expressed by mean±SE (n = 3). Harmaline and harmine values were compared separately

Effect of casein hydrolysate and combination of effective treatments

Incorporation of casein hydrolysate in the medium promoted the growth and total alkaloids accumulation. The dry weight of cell biomass was gradually increased with increase in concentration of casein hydrolysate. Addition of 100 of casein hydrolysate produced the maximum dry weight of the cell culture, which was a 1.64- fold more than control (Figure 5). The maximum accumulation of harmaline and harmine was detected in the presence 75 mg l^{-1} of casein hydrolysate (Table 2, Figure 6)

The treatment by a combination of effective biotic elicitor derived from *S. cerevisiae* (1000 mg l^{-1}) and casein hydrolysate (75 mg l^{-1}) showed a slight increase of harmaline and harmine in cell culture but it was not significantly different as compared with individual treatment of *S. cerevisiae* and casein hydrolysate.

Figure 5- Influence of 100 mg l^{-1} Casein hydrolysate on cell cultures of *P. harmala* L. (MS + 5.0 µM BAP + 2.5 µM NAA)

Table 2- Effect of casein hydrolysate on growth and accumulation of secondary metabolites in cell cultures of *P. harmala* L.

Organic supplement	D.W g in 100ml medium		Harmaline (µg/g D.W)	Harmine (µg/g D.W)
Control	0	5.32±0.7 ^{e}	42.3±1.3 ^{f}	54.5±1.6 ^{f}
Casein hydrolysate (mg/l)	25	6.15±0.5 ^{d}	49.0±1.5 ^{e}	59.0±1.3 ^{e}
	50	6.94±0.8 ^{c}	56.7±1.4 ^{d}	67.8±1.6 ^{d}
	75	8.71±0.8 ^{a}	68.9±1.6 ^{a}	79.0±1.9 ^{b}
	100	8.73±0.9 ^{b}	57.8±1.7 ^{cd}	71.2±1.7 ^{c}
Seeds			66.7±1.4 ^{b}	82.3±1.3 ^{a}

D.W: Dry Weights represent the mean±SE calculated on 6 replications. Data of secondary metabolites were expressed by mean ± SE (n = 3). Harmaline and harmine values were compared separately.

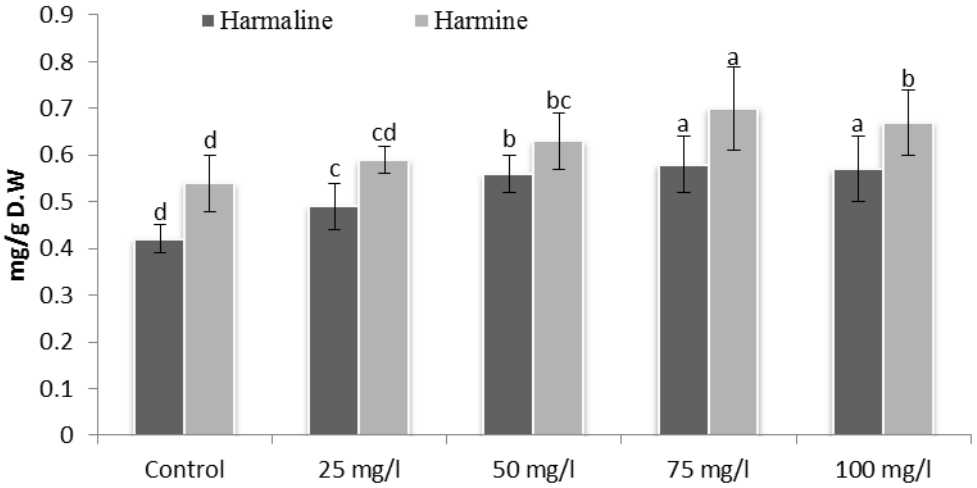

Figure 6- Effect of different concentration of casein hydrolysate on the harmaline and harmine content of cell cultures of *P. harmala* L. The values represented (mean ± SE) of three samples. Treatment means with similar letter are not significantly different as per DMRT at *p*=0.05. Harmaline and harmine values were compared separately

Discussion

Fungal elicitors have been widely employed to increase natural product formation in plant cell cultures and this strategy has been effective in stimulating the production of many chemical classes of secondary metabolites, such as terpenoids [24], coumarin derivatives [25], alkaloids [26, 27], and flavonoids [28]. Besides its biotechnological importance, elicitation has also been employed to dissect plant–microbe interactions and plant defenses responses as well as signaling pathways involving elicitors [29].

Stress in plant acts as an external constraint which limits the rate of dry matter production in all or part of the vegetation. Carvalho and Curtis [27], Zhao *et al.,* [30] also reported that the growth index of cell suspension culture of *Catharanthus roseus* was decreased by various fungal elicitors. They reported that the fungal elicitors derived from 12 different fungal strains (*Fusarium solani*, *Absidia cristata*, *Penicillium spimulorum*, *Rhixepus japonicus*, *Mucor fragilis*, *Mucor rouxianus*, *Aspergillum niger*, *Penicillium citrium*, *Verticillium dahliae*, *Pythium irregulare*, *Armillaria mellea*, *Ustilaginodia verens*) caused decline in growth index of cell suspension by 18–37% as compared to control and they stimulate indole alkaloid accumulation which ranged from 2-to 5-fold higher than the control. Similar results on accumulation of indole alkaloid by adding biotic elicitors in *Catharanthus roseus* have been reported by Eilert *et al*., [31]; Di Cosmo *et al*., [32]; Rijhwani and Shanks, [21]. Rokem *et al*., [33] reported that elicitors of *A. niger, F. oxysporum* and *Rhizopus arrhizus* increase the total amount of diosgenin from 10% to 72%. Moreover, endophytic fungi and its elicitors caused rapid response and action in cultured *Catharanthus roseus* suspension cells. The cell membrane became damaged, and the redox state changed at the cellular level.

The specific signal transduction pathways were activated in the process of releasing and

clearing free radicals. Some specific secondary metabolic pathways, such as phenylpropanes metabolic pathways, were opened or strengthened. Additionally, enzyme expression in the path strengthened. After treatment with endophytic fungi and elicitor, PAL and TDC activity showed a notable increase in *C. rouseus*. Meanwhile, the alkaloid content of the cells improved significantly [52].

Treating *Euphorbia pekinensis* suspension cultures with endophytic fungal elicitor from *Fusarium* sp. resulted in accumulation of isoeuphpekinensin and euphol, activated defense-related enzymes, and increased biomass. The results of this study showed that the biomass of culture after elicitation increased to 19.35%, whereas the isoeuphpekinensin and euphol contents were 5.81 and 3.56 times greater than the controls, respectively [53].

According to Moinuddin *et al*., (2013), the elicitation treatment with fungal elicitors (*Aspergillus niger* and *Penicillium expansum*) was observed to be most suitable for eliciting andrographolide production in cell suspension culture of *Andrographis paniculata*. *A. niger* extract (1.5 ml with 10 days treatment duration) revealed 6.94 fold increase in andrographolide content (132 µg) which was higher than the control (19 µg). *Penicillium expansum* elicitor (0.6% with 8 days treatment duration) could reveal 6.23 fold enhancement in andrographolide content (81.0 µg) over control (13 µg) [51].

In the present study, the addition of *S. cerevisiae* (1000 mg l^{-1}) in the medium was most effective biotic elicitors for promotion of growth and accumulation of secondary metabolites in the *in vitro* culture. Similar to this is the positive effect of yeast extract on stimulations of secondary metabolites production which were reported on *in vitro* raised material of *Beta vulgaris* [34], *Silybum marianum* [35, 36], *Medicago truncatula* [37], *Salvia miltiorrhiza* [38], *Brugmansia candida* [39], *Datura stramonium* [40] and *Morinda elliptica* [41]. According to Lu *et al*., [42], the culture of *Panax ginseng* treated with 3 g l^{-1} yeast extract resulted in 20-fold increase in the saponin content of the suspension culture as compared to control. The cell-free extracts of *Aspergillus niger*, *Saccharomyces cerevisiae*, *Agrobacterium rhizogenes*, *Bacillus subtilis* and *Escherichia coli* were employed as sources of biotic elicitors to study the effect on gymnemic acid production in the suspension cultures of *Gymnema sylvestre* and all the elicitors have shown a positive response [54].

The *Mucor* sp. elicitor treatment was one of the best stimulator of secondary metabolites in cell culture of *P. harmala* L. Similar results indicating positive effect of this genus of fungi reported by Wang *et al*., [43]. They reported that among the fungal elicitors tested, the *Mucor sp.* elicitor promoted the biomass and total carotenoids yield most remarkably in *Xanthophyllomyces dendrorhous.*

Thus from the results of the present study in *P. harmala* L. and earlier results, it is suggested that biotic elicitors such as *S. cerevisiae* and fungal mycelial biomass can be employed as elicitor to stimulate the biosynthesis of important pharmaceutical alkaloids (harmaline and harmine).

The effect of casein hydrolysate on the growth of callus, induction of somatic embryogenesis and production of secondary metabolites in *T. terrestris* L. have been studied by Nikam *et al*., [44]. They reported that the supplementation of casein hydrolysate at the concentration of 25 - 100 mg l^{-1} stimulated the growth of callus and induction of somatic embryogenesis. The harmaline and harmine content of embryogenic callus of

T. terrestris L. showed about 9-18% increase when casein hydrolysate was added up to 75 mg l^{-1} to the medium as compared to control at the fifth week of culture.

Comparable result of increased saponin content has been reported in cell culture of *Panax ginseng* in media supplemented with casein hydrolysate [45]. They reported 0.5 g/l of casein hydrolysate combined with 0.2 mM sorbitol enhanced saponin yield 3.5-fold over the control. Mukherjee *et al*., [46] reported that adding casein hydrolysate enhanced forskolin production in genetically transformed cultures of *Coleus forskohlii* and the optimum concentration was 0.5 - 1.0 g l^{-1} for growth and also was 2.0 mg l^{-1} for secondary metabolite production. Enhanced artemisinin production was noted on MS medium supplemented with 0.5 g l^{-1} casein hydrolysate in cell cultures of *Artemisia annua* [47].

Effects of plant growth regulators (PGRs) and organic elicitors (OEs) on *Coleonema pulchellum in vitro* micropropagation, secondary product production and pharmacological activities were evaluated by Baskaran *et al*., 2014 [55].

Plant growth regulators and organic elicitors had a significant effect on the synthesis and accumulation of phenolic compounds and flavonoids. In particular, casein hydrolysate (CH) as well as a combination of glutamine and benzyladenine induced high levels of total phenolics and flavonoids during *in vitro* culture. Cytokinins and organic elicitors had a significant effect on diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and antibacterial activities of Coleonema *pulchellum* extracts [55].

The increase of secondary metabolites content in cell culture cultivated on media with the addition of casein hydrolysate might be due to its sterols or amino acids content [48]. The results are partially consistent with the report that *in vitro* culture of *Solanum laciniatum* was able to enhance the solasodine and chlorophyll content significantly with the addition of 100 - 200 mg l^{-1} casein hydrolysate to media.

Conclusion

In conclusion, by applying biotic elicitors in cell suspension culture of *P.harmala* L., higher secondary metabolites like harmaline and harmine can be obtainable, though little is known about the biosynthesis of them. The unknown participating enzymes have to be characterized before expanding the study to the effects of elicitation in *P. harmala* L. cell suspensions. It is still unclear whether distinct elicitors acted on the same or on distinct signaling pathways. Therefore, more work is needed to better understand the effects of various elicitors on the partitioning between the secondary metabolites in *P. harmala* L. such results could be explained by the distinct potential signaling pathways and secondary metabolites for medicinal casein hydrolysate which seems to be the most promising elicitors for such an application.

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