Int J Pharm Bio Sci 2014 April ; 5(2): (P) 548 - 558

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

THE INFLUENCE OF CADMIUM AS ABIOTIC ELICITOR ON THE PRODUCTION OF PHYTOESTROGENS IN HAIRY ROOT CULTURES OF *PSORALEA CORYLIFOLIA*

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ABSTRACT

Environmental pollution especially with heavy metals poses serious problem on product synthesis of medicinal plants cultivated in the field. Accordingly, the purpose of this study was to assess *in vitro* effects of cadmium as abiotic elicitor on phytoestrogens production by *Psoralea corylifolia* hairy roots. Transformed hairy roots were developed by genetic transformation using *Agrobacterium rhizogenes*. The stable, transformed and fast growth hairy roots demonstrated high growth rate 7.23% dry weight in growth regulators free Murashige and Skoog's medium enriched with 8 µM of cadmium. Hairy roots were tested with low and high concentrations of cadmium. Correlation of *in vitro* cadmium exposure and phytoestrogens production showed that low percentage of cadmium and exposure period was stimulated product synthesis, while high levels of cadmium in nutrient medium and more exposure period impaired the product synthesis. Elevated concentrations of cadmium and increased exposure time decreased the hairy roots growth by five-fold compared to devoid of cadmium in the medium. Cadmium concentrations in cells and medium were determined by Atomic Absorption Spectrophotometer. Maximal cadmium concentrations and treatment period harmfully effects on phytoestrogens production. HPLC revealed that hairy roots tested with 8 µM of cadmium in medium produced phytoestrogens daidzein 1.74% dry weight and genistein 0.23% dry weight on day 2. The results showed that the hairy root growth decreased as a concentration of cadmium increased and related to time-dependent manner**.** The present results revealed that abiotic elicitor cadmium at optimum concentrations stimulated the production of phytoestrogenic isoflavones daidzein and genistein in *P. corylifolia* hairy roots.

KEYWORDS: *Psoralea corylifolia,* hairy roots, cadmium , phytoestrogens, daidzein, genistein

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INTRODUCTION

Cultivation of crop plants being a source of food, many plant species especially medicinal plants are cultivated as source of phytomedicines. According to the World Health Organization, 80% of the world population in developing countries depends on traditional medicines obtained from natural plants for primary healthcare needs[1]. Natural plant resources are the primary source of structurally diverse natural compounds exhibiting different bioactive properties led to develop innovative and effective drug molecules[2]. Thus, medicinal plants remain as unexploited sources for identification and isolation of important phyto-biomolecules. Medicinal plantcultivation in the fields is common practice for extraction of bioactive compounds. In the conventional, pharmaceutical industry produce lifesaving drugs from compounds extracted and isolated from harvested plant materials or plant derived compounds as starting materials to synthesise semi-synthetically[3]. More than 25% of the pharmaceutical drugs used in the world today are derived from plant origin[4-5]. The present demand of medicinal plants is about $14 billion a year and the projected demand by the year 2050 is $5 trillion and their trade in India is estimated to about $100 million per year[6]. Cadmium represents one of the major industrial soil pollutants, which is not only human and animal toxic but it may negatively influence also plant growth at relatively low concentrations. Cadmium is a common, non-vital, toxic metal and is a cumulative toxin[7]. The total amounts of cadmium in most agricultural soils normally do not exceed 0.4–0.5mgkg^{-1} and higher values reflect the impact of human activities. Consequently, the cadmium concentration in plants usually ranges from 0.05 to 0.2mgkg^{-1} DW[8]. According to the World Health Organization[9], heavy metal contamination of medicinal plants should be monitored to ensure their safety. Medicinal plants may contain high levels of toxic metals when cultivated in open field[10-11]. Recent studies have shown the effects of heavy metals in the production of bioactive compounds. The effects of environmental contamination with a common inorganic pollutant, nickel, on the synthesis and accumulation of hypericin, hyperforin and pseudohypericin in *Hypericum perforatum*[12]. Such changes caused by heavy metals could have serious implications on the quality, safety and efficacy of natural products prepared from medicinal plant species[12]. In the present study, we propose to demonstrate the possibility of *P. corylifolia* hairy roots accomplished with genetic competence to uptake of cadmium from culture medium. Our idea was to use these systems as a model to remove cadmium from a culture medium so that the root biomass can be used in remediation of cadmium. Hairy roots of many medicinal plants, e.g., *Catharanthus roseus, Armoracia rusticana, Panax ginseng and Alyssum species* have been used to remove toxic metals such as Cu2+, Cr6+, Ni2+,Pb2+, and Zn 2+, uranium from aqueous solutions by a process known as rhizofiltration[13-16]. Transformed roots due to their highly branched nature have a large surface area in comparison with control roots. The hairy roots can also be scaled up in bioreactors and the biomass developed can be used for potential applications like rhizofiltration[17]. Several workers studied the effect of cadmium on different physiological and biochemical parameters of various plants [18-22] , but no information is available on the study of cadmium uptake and simultaneous association with secondary metabolites by *P. Corylifolia* hairy roots. Keeping these facts in mind, present study has been undertaken to understand the effect of cadmium as abiotic elicitor on phytoestrogens production in hairy roots of *P. corylifolia*.

MATERIALS AND METHODS

Plant Materials

Psoralea corylifolia plants were grown in the Experimental Filed Station, Bhabha Atomic research Centre, Mumbai, India. Seeds were scarified by immersion in concentrated sulphuric acid for 60 min followed by washing under running tap water for 20 min. The seeds were surface disinfected in 70% ethanol for 2 min followed by immersing in aqueous solution of $HgCl_2$ (0.1% w/v) for 3 min and subsequently washed 5 to 6 times with sterile distilled water. Surface sterilized seeds were

cultured on half-strength Murashige and Skoog's medium[23] enriched with 3% sucrose for germination. The pH of the medium was adjusted to 5.8 by 0.1 N NaOH or 0.1 N HCl prior addition of 0.8% agar. Culture medium was autoclaved at 121^{0}C at 15 psi for 20 min. Cultures were kept on photo-simulation tissue culture racks under 16 h photoperiod (40 μMol $m^{-2}s^{-1}$, cool white fluorescent tubes, Phillips, Holland) at 25±1^{0}C.

Agrobacterium rhizogenes cultures and hairy roots induction

Agrobacterium rhizogenes strain LBA 9402 was grown on solid YMB medium[24] supplemented with 50 mg/l rifampicin at 28^{0}C for 48 h. Three-week-old stem segment of *in vitro* seedling was used for infection. Infected stem fragments were placed on the growth regulator free MS medium under similar culture conditions. After three days of cultivation, shoot segments were placed on MS medium supplemented with 500 mg/l filter sterilized cefotaxime. Hairy roots originated from the wounding sites were carefully excised and cultured on fresh MS medium with reduced concentrations of cefotaxime till complete eradication of bacteria. Hairy roots were maintained on growth regulator free MS medium containing 30 g/l sucrose. Non-transformed root cultures were initiated from *in vitro* seedlings and maintained on growth regulator free MS medium.

Confirmation of transformation

Total genomic DNA of hairy roots of *P. corylifolia* was isolated by using Dellaporta's method described elsewhere[25] . Plasmid DNA from *A. rhizogenes* strain LBA 9402 was extracted by alkaline lysis[26] . Polymerase Chain Reaction (PCR) amplification of the isolated DNA samples were carried out using primers specific for ORF-13 coding sequence of T_L DNA and *mas*1' sequence of T_R DNA of pRiLBA9402. The primers used for amplification of ORF-13 coding sequence were (+) 5' CAG CTT CTA AAT GTG GAG GCC and (-) 5' CCT TGC CGA TTG CCA GTA TGG C. These primers defined a 498 bp domain of ORF-13 coding sequence of T_L DNA of $pR1A_4$[27] . For amplification of *mas* 1' sequence, primers used were (+) 5' CGG TCT AAA TGA AAC CGG ACG and (-) 5'GGC AGA TGT CTA TCG CTC GCA CTC C which defined 970 bp domain of the mas 1' coding sequence of T_R DNA of $pR1A_4$[28] . DNA amplification was performed on the Eppendorf thermal cycler. Each reaction mixture (25 μl) consists of 50ng of plant genomic DNA (or 25 ng of plasmid DNA), 2.5 μl of 10x *Taq* DNA polymerase buffer, 50 μM each of dNTPs, 0.2 μM primers and 0.5U *Taq* polymerase. The amplification programme involves an initial denaturation step at 94^{0}C for 2 min following 30 cycles of 30 sec at 94^{0}C, 30 sec at 58^{0}C and 1.5 min at 72^{0}C with a final extension of 7 min. amplification products were examined by electrophoresis on 1.5% (w/v) agarose gel in 1X TBE buffer, stained with ethidium bromide and visualized under UV light.

Growth studies

Approximate 150 to 160 mg of hairy roots was inoculated in 50 ml MS medium in 250 ml Erlenmeyer flasks and kept on a gyratory shaker at 60 rpm. The rate of growth was determined from 2 to 5 days. Hairy roots were harvested on every fifth day to determine fresh weight and dry weight and details have been described elsewhere[29] . In brief, harvested hairy roots blotted briefly and fresh weight was determined. The hairy roots then dried in an oven at 65^{0}C for 48 h and the final dry weight was recorded. The rate of growth of the tissue was determined as percentage dry weight. The growth index was calculated using formula $GI = W_f - W_i$, where W_f is a fresh wt of a culture after harvest and W_i is the fresh wt of inoculum. The doubling time of each culture was calculated using the formula.

$T_D = \ln 2 (t_1-t_0) / \ln (GI)$

where t_1-t_0 corresponds to period between inoculation and the stationary phase. All experiments were replicated three times and each experimental point four Erlenmeyer flasks were used for each treatment.

Analyses of cadmium content in hairy roots tissue

Hairy roots were collected after the end of phytoremediation for the analyses of cadmium. The treated hairy roots were washed thoroughly in tap water followed by distilled water. Subsequently these hairy roots were dried in an oven for 24 hr at 80^0 Celsius. Dried materials were powdered with a Wiley mill (Model 4276-M, Thomas Scientific, USA) to pass a 20 mesh sieve. Dried powdered

tissues (1 gm) were digested with 20 ml of 70% HNO_3 and $HClO_4$ (5:1) in a fume hood. The digested tissue samples were diluted to 10 ml with double distilled water MQ and impurities were removed by filtration (0.2 µ filter followed by centrifugation at 10,000xg for 5 min. Blank samples were prepared simultaneously. These solutions were stored in a refrigerator at 4 ◦C until the analysis was carried out. Determination of cadmium contents in the digests was done by Atomic Absorption Spectroscopy (GBC Model 932 B+ Australia). The wavelength used for quantification was: cadmium 228.8 nm. The chemicals used for analysis were Merck analytical grade (spectroscopic grade). Quality control measures were taken to assess the contamination and reliability of the data. Blank and drift standards were run after every five reading to calibrate the instrument.

Extraction of phytoestrogenic isoflavones daidzein and genistein

Harvested biomass was dried in an oven at 65^0C for 24 h and powdered by Wiely Mill and followed by extracted with 3M H_2SO_4, and sonicated (33 KH_z) for 5 min. Subsequently, the extract was kept in a water bath at 100^0C for 45 min finally an equal volume of ethanol was added to the sample. Samples were vortexed for 2 min and centrifuged at 14000x*g* for 5 min. The supernatant was transferred to clean glass vials and directly analysed by High Performance Liquid Chromatography (HPLC).

Quantification of phytoestrogenic isoflavones daidzein and genistein by HPLC

The HPLC was performed on Jasco Liquid Chromatograph (Mode2080 plus, Japan) equipped with auto sampler injector (Model No. Jasco AS-2055, Japan) with a 25 µl loop and a variable wavelength detector (Model No. UV-2075, Japan). Separations were performed on GL Sciences Inc C_{18} (250 mm x 4.6 I.D) column. The daidzein and genistein were determined by using acetonitrile:water (50:50 v/v) as a mobile phase. The flow rate was 0.7 ml/min and the elution was monitored at 250 nm. Data collection and integration were accomplished using BORWIN software (Japan). This method is sensitive and accurate with good reproducibility. Peak identification carried out by using authentic samples of daidzein and genistein (Sigma, USA).

Statistical analysis

The influence of various treatments on growth and phytoestrogenic isoflavones content was analysed by one-way analysis of variance (ANOVA). Values are mean of three replicates from three experiments. The data were analysed statistically by analysis of variance (ANOVA) and the difference between means of the samples was analysed using least significant difference (LSD) at a probability levels of 0.05.

RESULTS AND DISCUSSION

Hairy roots growth rate

Hairy roots were successfully induced by infection with *A. rhizogenes* LBA9402 to stem *in vitro* seedlings of *P. corylifolia*. Hairy roots emerged from the infected sites within 12–15 days with 30% transformation frequency and maintained on growth regulator free MS medium. Transformed hairy roots showed rapid growth and tendency of profuse branching and active elongation (Fig. 1). The effect of various concentrations of heavy metal cadmium salts ($CdCl_2$) on cell growth and content of phytoestrogens daidzein and genistein by *P. corylifolia* hairy roots was studied. Their addition condition was optimized separately in our experiments. In brief, 15-day-old hairy roots were transferred to 50 mL of growth regulator free MS medium in a 250 ml Erlenmeyer flask and kept on a gyratory shaker at 80 rpm at 25^0C. After 10 days, hairy roots were transferred to MS medium fortified with different concentrations of cadmium. Three sets of experiments were carried out independently for lower and higher concentrations of cadmium. No considerable shift in pH (5.6) was observed by the addition of cadmium chloride solution, irrespective of different concentrations of cadmium chloride.

Figure. 1
Psoralea corylifolia hairy root cultures

Molecular analysis

Hairy roots were analyzed by PCR for confirmation of integration of T-DNA in the plant genome. PCR amplification with specific primers was used to show the presence of TR-DNA of the Ri plasmid in the genomic DNA of hairy roots of *P. corylifolia*. DNA isolated from hairy roots Growth of Hairy root Cultures treated with cadmium induced by LBA 9402 when amplified with primers specific for ORF 13 and mas10 sequence showed the expected fragments. PCR analysis confirms the successful integration of T-DNA in the genome of transformed roots, while DNA from untransformed roots (used as control) did not show any amplification (Fig. 2).

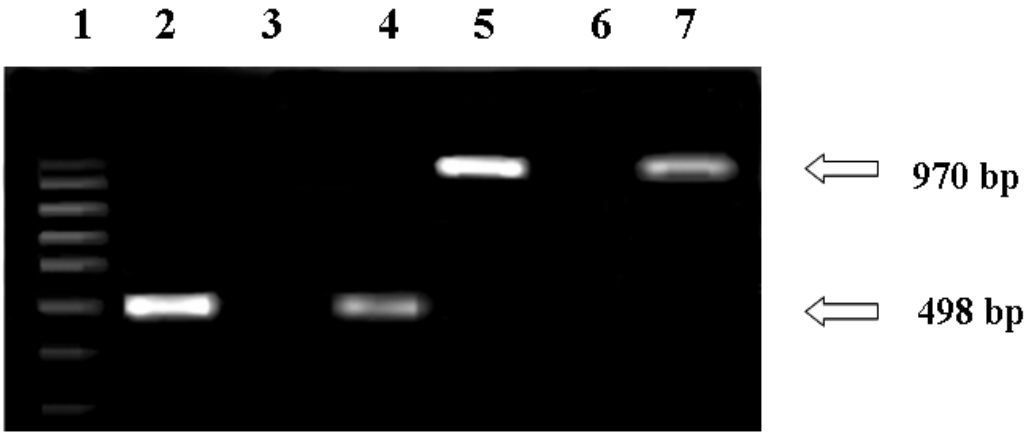

Figure. 2
PCR amplification of DNA from transformed roots of P. corylifolia. Lane 1: 100 bp DNA ladder (marker), Lane 2 and 5: DNA from Plasmid pRi9402, Lane 3 and 6: DNA from non-transformed roots, Lane 4 and 7: DNA from transformed roots.

Uptake of cadmium and growth rate at low concentrations by hairy root cultures

The kinetic profiles of growth rate and content of phytoestrogens (daidzein and genistein) were recorded over a 5-day period. As shown in Fig. 3, low concentrations of cadmium did not inhibit the cell growth. Hairy roots were grown better in medium containing 8 µM cadmium and yielded 7.23% dry weight on day 2 and relatively higher to control. The presence of cadmium in medium showed an inhibitory effect on cell growth during 4 to 5 days incubation period. The dry weight of the control and cadmium treatment reached the

maximum values on day 2 and relatively decreased thereafter. Overall, hairy roots grown in MS medium tested with different concentrations of cadmium (1, 2, 4, 6, and 8 µM) had not reduced the growth and yields relative to the control. Increased growth period with similar concentrations of cadmium impaired the growth rate and reduced the growth rate by 42%. In the cadmium-uptake studies of *P. corylifolia* hairy roots, low cadmium concentrations were used. It was found that uptake of cadmium by *P. corylifolia* hairy roots at low concentrations was 91.44% for 8 µM concentrations tested for 2 days of incubation (Fig. 4). Minimum cadmium-uptake was observed when *P. corylifolia* hairy roots tested with 4 µM concentration of cadmium.

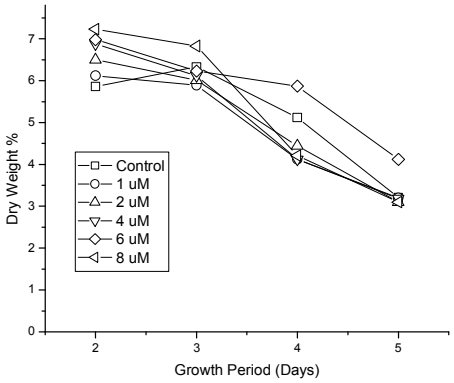

Figure. 3
Rate of growth of P. corylifolia hairy roots on dry weight basis tested with low concentrations (1 to 8 µM) of cadmium

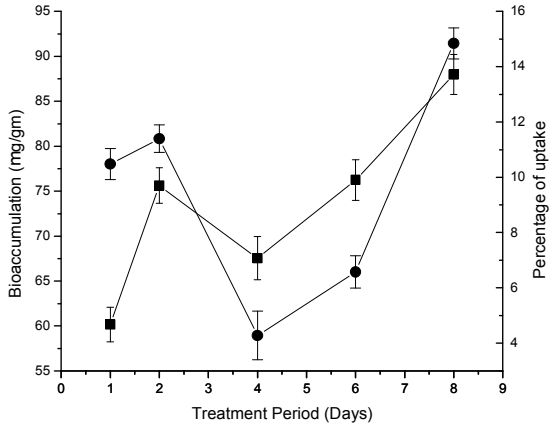

Figure. 4
Bioaccumulation and percentage of cadmium retained in the medium at low concentrations over the period of treatment in days by P. corylifolia hairy roots. Bioaccumulation (●---●); Percentage of uptake (■---■).

Uptake of cadmium and growth rate at high concentrations by hairy root cultures

To study the uptake of high concentrations of cadmium, five different concentrations of cadmium, 10, 50, 100, 200 and 300 µM, were tested. A linear uptake relationship was observed with the increased concentrations of cadmium when compared with dry weight basis. Hairy roots growth was reduced gradually with increased concentration of cadmium and relatively to time dependent matter (Fig. 5). The present results revealed

that the increased concentrations of cadmium hampered the growth of hairy roots. It was found that the bioaccumulation of cadmium was low at 10 µM cadmium compared to that of 8 µM tested on day 2. Uptake of cadmium by hairy roots of *P. Corylifolia* is 0.7% at 100 µM concentration of cadmium in medium (Fig 6). It can be concluded that higher concentrations of cadmium reduced the uptake rate of cadmium and also affects growth of hairy roots. Cell growth decreased proportionally with increasing cadmium concentration and treatment period.

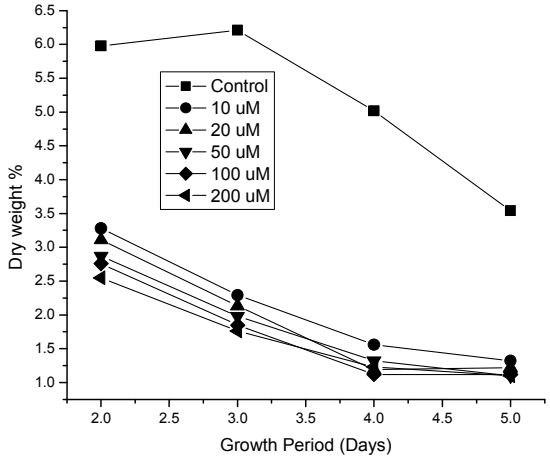

Figure. 5
Rate of growth of P. corylifolia hairy roots on dry weight basis tested with high concentrations (10 to 200 µM) of Cadmium

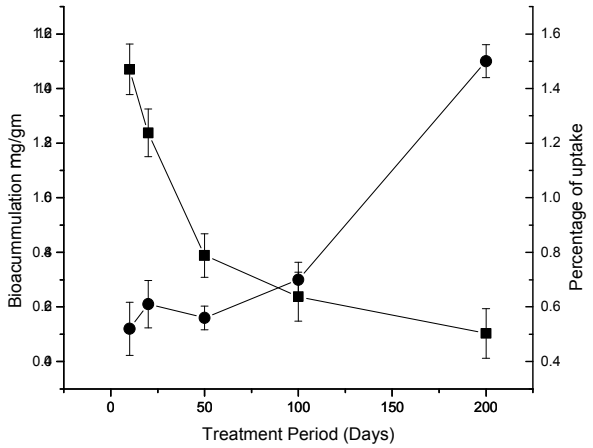

Figure. 6
Bioaccumulation and percentage of cadmium retained in the medium at high concentrations over the period of treatment in days by P. corylifolia hairy roots. Bioaccumulation (●---●); Percentage of uptake (■---■).

Effect of cadmium ions on phytoestrogens production

The role of cadmium on phytoestrogens production by *P. corylifolia* hairy roots was evaluated. Growth and phytoestrogens production was directly associated with time dependant manner. Hairy roots tested with high concentrations of cadmium for long period unfavourably effected on cell growth and product synthesis. Among them,

cadmium 8 µM, exerted the most effective induction on phytoestrogens biosynthesis. The effect of 2 µM and 4 µM cadmium on the content of phytoestrogens was almost identical to that of control. However, increased concentrations and incubation period reduced the product synthesis. Optimum concentrations of cadmium (8 µM) and exposure time (2 days) influenced the phytoestrogens production compared to that of control. The maximum phytoestrogens by cadmium as abiotic elicitor produced phytoestogenic isoflavones daidzein 1.74% dry weight (Fig. 7) and genistein 0.23% dry weight (Fig. 8), which was obtained on day 2. Accordingly, the maximum daidzein and genistein production was about 21 and 18 fold respectively that of control.The present results revealed that high concentration of cadmium and long incubation period reduced the phytoestrogens production by *P. corylifolia* hairy roots. In this study, *P. corylifolia* hairy roots were developed for the cadmium hyper-accumulator, to investigate whether the high concentrations cadmium tolerance and stimulate phytoestrogens production or whether cadmium intervene as signal transduction to enhance the product synthesis. The hairy roots exposed to cadmium were determined for fresh weight and dry weight to assess the rate of cell growth. The reported data showed that on day 2 incubation periods following high concentration cadmium treatment, the cell growth significantly decreased by 2 fold in comparison with the untreated hairy roots. This finding is in agreement with previous report[30-31] . Correlation between phytoestrogens production and bioaccumulation of cadmium by *P. corylifolia* hairy roots was differed according to the concentrations of cadmium and incubation period. But this correlation was significantly improved when optimum incubation period and cadmium concentration tested. Kotrba et al.,[32] observed that low concentrations of cadmium in nutrient medium did not cause dramatical decrease of growth and branching of hairy roots of *Solanum nigrum and* none or very slight growth was observed in the presence of higher Cd concentration. Based on results of Fig 7 and 8, it is possible to state that low concentration of cadmium and minimum treatment period influence the phytoestrogens production and marked variations in cells growth. This finding was also supported by Rai et al.,[33] ; Toppi et al.,[34] ; Pitta–Alvarez et al.,[35] . They have reported decreased concentration of cadmium in nutrient medium did not affect on cells growth and production of secondary metabolites.

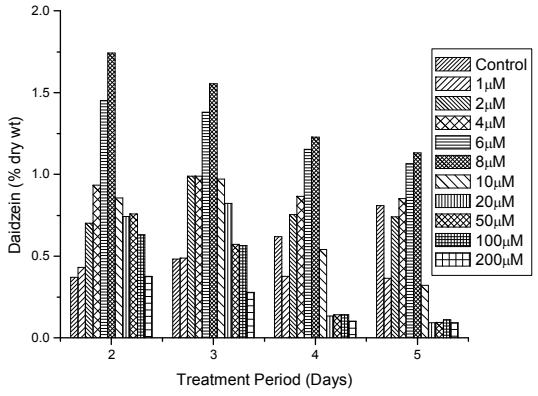

Figure. 7
Effect of abiotic elicitor cadmium in different concentrations and for different exposure time on the production of daidzein in hairy root s of P. corylifolia

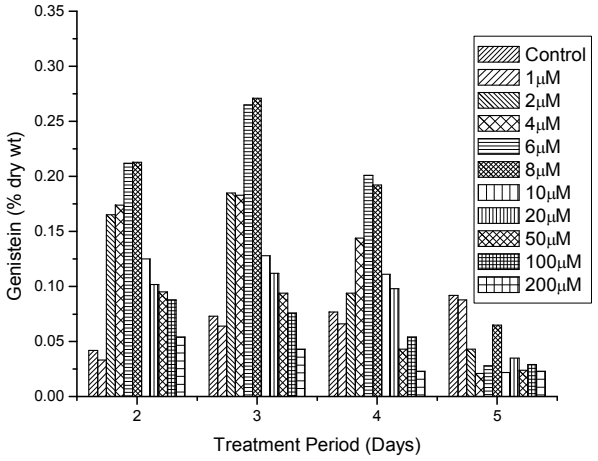

Figure.8
Effect of abiotic elicitor cadmium in different concentrations and for different exposure time on the production of genistein in hairy root s of P. corylifolia

CONCLUSION

To date, there have been no published attempts to use cadmium as signal transduction to increased phytoestrogens production by *P. corylifolia* hairy roots study. From ongoing studies it has been concluded that Cd used as abiotic elicitor causes significant increased in phytoestrognes production at 8 µM Cd and marked variations in cells growth. The phytoestrogens daidzein and genistein are anticancer drugs and demand in pharmaceuticals industry is increasing constantly. The production of such important anticancer drug under stress conditions could be scaled up in a bioreactor to increase the production capacity.

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