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Production of Reserpine in Somatic Embryos of *Rauwolfia serpentina* Cultured in Bioreactors by the Induction of Elicitor (Methyl Jasmonate)

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Abstract: This study was concentrated on the production of reserpine in embryogenic suspension cultures of *Rauwolfia serpentina* by exposing them to different concentrations (50-500 µM) of methyl jasmonate (MJ) during the culture period. In the bioreactor cultures, reserpine content increased significantly by elicitation of MJ, however, the fresh weight, dry weight and growth ratio of embryos was strongly inhibited by increasing MJ concentrations. The highest total reserpine (7.3 fold increment) yield was obtained with 200 µM MJ treatment. There was 1.4, 3.4 and 14.9 fold increase in the reserpine production respectively with such elicitation treatment. These results suggest that MJ elicitation is beneficial for reserpine accumulation in the embryogenic cell suspension cultures.

Key words: *Rauwolfia serpentina* · Reserpine · Bioreactors · Elicitor · somatic embryos

INTRODUCTION

Reserpine is an indole alkaloid, antipsychotic and antihypertensive drug that has been used for the control of high blood pressure and for the relief of psychotic behaviors, although because of the development of better drugs for these purposes and because of its numerous side-effects, it is rarely used today. The antihypertensive actions of Reserpine are a result of its ability to deplete catecholamines (among the others) from peripheral sympathetic nerve endings. These substances are normally involved in controlling heart rate, force of cardiac contraction and peripheral resistance. Reserpine depletion of monoamine neurotransmitters in the synapses is often cited as evidence to the theory that depletion of the neurotransmitters causes subsequent depression in humans. Moreover, reserpine has a peripheral action in many parts of the body, resulting in a preponderance of the cholinergic part of the nervous system (GI-Tract, smooth muscles vessels).

Reserpine was isolated in 1952 from the dried root of *Rauwolfia serpentina* (Indian snakeroot), (which had been known as *Sarpaganda* and had been used for centuries in India for the treatment of insanity, as well as fever and snakebites. Its molecular structure was elucidated in 1953 and natural configuration published in 1955. Reserpine is rarely used in the management of hypertension today. Reserpine is a second-line adjunct agent for patients who are uncontrolled on a diuretic when cost is an issue. It is also used to treat symptoms of dyskinesia in patients suffering from Huntington's disease. In some countries reserpine is still available as part of combination drugs for the treatment of hypertension, in most cases they contain also a diuretic and/or a vasodilator like hydralazine. These combinations are currently regarded as second choice drugs. The daily dose of reserpine in antihypertensive treatment is as low as 0.1 to 0.25 mg.

The use of reserpine as an antipsychotic drug had been nearly completely abandoned, but more recently it made a comeback as adjunctive treatment, in combination with other antipsychotics, so that more refractory patients get dopamine blockade from the other antipsychotic and dopamine depletion from reserpine. Doses for this kind of adjunctive goal can be kept low, resulting in better tolerability. Originally, doses of 0.5 mg to 40 mg daily were used to treat psychotic diseases. Doses in excess of 3 mg daily often required use of an anticholinergic drug to combat excessive cholinergic activity in many parts of the body as well as parkinsonism. For adjunctive treatment, doses are typically kept at or below 0.25 mg twice a day. Reserpine may be used as a sedative for horses.

Rauwolfia serpentina is a plant of Apocynaceae family wide spread in tropical and sub-tropical Asia and Africa. In India, *Rauwolfia serpentina* leaves are commonly used to treat high blood pressure and mental

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disorders including schizophrenia and were particularly popular for that purpose in the West from 1954 to 1957. Indian system of medicine recommends *Rauwolfia serpentina* alkaloids reduce blood pressure, depress activity of central nervous system and act as hypnotics and snakebite [1]. According to World Health Organization (W.H.O) still about 80% of the world's populations rely mostly on plant based drugs. Low cost and easy availability, these factors has generated a renewed interest in plant medicine in the last decade.

The traditional practitioners in India prescribe the leaves to the patients without regard to any possible adverse effects in the view of its many uses. In the line of the pharmacological validation of this plant, the toxicological evaluation of *Rauwolfia serpentina* revealed that the drug is safe and is not toxic up to 40g/Kg in rats [2]. It is known to contains a number of bioactive chemicals, including ajmaline, deserpidine, rescinnamine, serpentinine, reserpine and yohimbine [3,4]. So, the plant tissue culture process has been looked at as a potential alternative for the more efficient mass propagation method. Recently, induction of somatic embryogenesis has been reported [5]. Somatic embryos were successfully cultivated in bioreactors and germinated somatic embryos are used as raw material for medicinal purposes, but the accumulation of physiologically active reserpine in germinating embryos was low.

Elicitation has been proved to be effective way to increase secondary metabolite production. A number of elicitors and precursors such as methyl jasmonate (MJ) have been used successfully for enhancing production of secondary metabolites such as saikosaponins, taxoids, plaxitaxel and baccatins, ginsenosides during cell cultures of many plant species [6-12]. In recent years, we have been searching for a strategy that could significantly affect the accumulation of reserpine by focusing on commercially valuable reserpine as a research target. In the present study, MJ was employed in embryogenic suspension of *Rauwolfia serpentina* in order to examine the impacts on reserpine production.

MATERIALS AND METHODS

Induction of Somatic Embryogenesis and Maintenance of Stock Cultures of Embryos: Young leaves (2 cm in length) of *Rauwolfia serpentina* were collected from *in vitro* grown plants and cut into 5 x 5 mm pieces, cultured on Murashige and Skoog medium [13]; pH 5.8; with 1 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 3% (w/v) sucrose and 0.2% (w/v) gel rite and cultures were maintained in dark at 25°C. Embryogenic callus was developed from the leaves within twelve weeks after culture. Embryogenic callus was maintained on MS liquid medium supplemented with 1 mg L^{-1} 2,4-D, 3% (w/v) sucrose and 0.2% (w/v) gel rite by sub-culturing once in four weeks.

Embryogenic Cell Suspension Culture: Embryogenic cells of *Rauwolfia serpentina* were transferred to MS liquid medium supplemented with 1 mg L^{-1} 2,4-D and suspension cultures were sub-cultured at every two weeks interval. To induce somatic embryos, 2 weeks old embryogenic cell clumps were filtered through a sterile 212 µm stainless steel sieve to remove the larger clumps. The suspension was allowed to settle for 5 min for easier removal of the used medium. About 500 mg of cell clumps was transferred to 100 mL MS liquid medium without 2,4-D in 300 mL Erlenmeyer flasks. The cultures were incubated at 100 rpm on a ratory shaker (Sub zero, India) in dark at 25°C. At the end of four weeks of culture, the content of flask was passed through different stainless steel sieves to separate different stages of embryos (>800 µm=cotyledonary; 600 µm=torpedo; 420 µm=heart; <420 µm globular). Cotyledonary embryos were used as explants for establishing subsequent cultures.

Establishment of Large Scale Suspension Cultures in Bioreactors: Ten grams of cotyledonary somatic embryos were transferred to 3 L bioreactor with 2 L MS liquid medium with 3% (w/v) sucrose and 4 mg L^{-1} GA_3. The pH of the medium was adjusted to 5.8 before autoclaving by using 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. The volume of input air was adjusted to 0.1 v/v (air volume/culture volume) per min. Cultures were kept under a 16 hrs photoperiod at 35 µmol m^{-2} s^{-1} photosynthetic photon flux. In an elicitation experiment different concentrations of methyl jasmonate (0, 50, 100, 150, 200, 300 or 400 µM) was added to the cultures on the day of inoculation. The fresh and dry weights were recorded after 6 weeks of culture. Dry weight was determined after drying the biomass for 24 hrs at 60°C.

Determination of Reserpine: Germinated somatic embryos were dried and powdered (2 g) with a blender and extracted with 60% aqueous methanol (2 x 50 mL) for 30 min. each at 60°C and filtered through filter paper. The combined extract, was evaporated to dryness in vacuum and washed with 50 ml of ether. The insoluble fraction was dissolved in water and extracted with

n-butanol (water saturated). The organic phase was evaporated to dryness, dissolved in (10 mL) high performance liquid chromatography (HPLC) grade methanol and filtered through 0.45 µm membrane filter (Millipore, India) filter. Reserpine were quantified by HPLC (Systronics, India) by following the procedure described previously [14]. Reserpine was separated using a flow rate 0.8 mL min^{-1} with water (solvent A) and acetonitrile (solvent B) as the mobile phase. The elution programme was: initially 90:10 (A:B) with isocratic elution for 5 min followed by linear gradient to 80:20 in 22 min, linear gradient to 60:40 in 15 min, isocratic for 5 min, linear gradient to the starting conditions (90:10) in 5 min and isocratic for 5 min (equilibration time). Quantification was based on ultraviolet absorption at 216 nm. The peak areas corresponding to reserpine from the samples, with same retention time as authentic reserpine. Retention time is 28.20 for reserpine.

RESULTS AND DISCUSSION

The growth and secondary metabolite accumulation by the embryos of *Rauwolfia serpentina*, cultivated in bioreactor cultures are presented in Table 1, Fig-1 and Table 2, Fig-2. The embryos in the untreated cultures reached 102.65 g L^{-1} fresh weight and 11.32 g L^{-1} dry weight. Growth of embryos was significantly affected by the application of MJ. There was slight increment in fresh weight of embryos at 150µM MJ (104.66 g L^{-1}) when compared to the control (Table 1 and Figure 1).

However, the fresh weight, dry weight and growth ratio were decreased with increasing MJ concentration. On the other hand, reserpine content was significantly enhanced by the addition of MJ. Amount of total reserpine increased with increasing MJ concentration and reached a maximum at 200 µM MJ representing 7.3 fold (649.95 µg g^{-1} DW) increases over control.

Table 1: The effect of MJ on *Rauwolfia serpentina* embryogenic suspension after six weeks of bioreactor culture

	Biomass		
MJ Concentration (µM)	Fresh weight (FW)	Dry weight (DW)	Growth ratio[z]
	(g L^{-1})	(g L^{-1})	
0	102.65a[y]	11.32a	20.36
50	103.16a	10.60a	19.01
100	104.66a	10.10b	18.05
150	102.52a	9.52c	16.96
200	99.25b	9.29c	16.52
250	95.28b	9.18c	16.31
300	88.81c	8.58d	13.30
350	85.16	8.06d	12.54
400	61.37	5.61d	8.26
450	47.19	4.21e	6.37
500	29.30d	3.91e	5.49

yMean separation within column by Duncan's multiple range test at $p < 0.05$.

zGrowth ratio is the quotients of the dry weight after cultivation and the dry weight of the inoculum.

Table 2: The effect of MJ on accumulation of reserpine in somatic embryos of *Rauwolfia serpentina* cultured in bioreactors[z]

MJ concentration (µM)	Reserpine (µg g^{-1} DW)
0	88.70
50	235.74
100	271.90
150	437.20
200	649.95
250	566.45
300	476.33
350	416.29
400	361.48
450	251.91
500	201.30

yMean separation within column by Duncan's multiple range test at $p < 0.05$.

zData was taken after 6 weeks of culture.

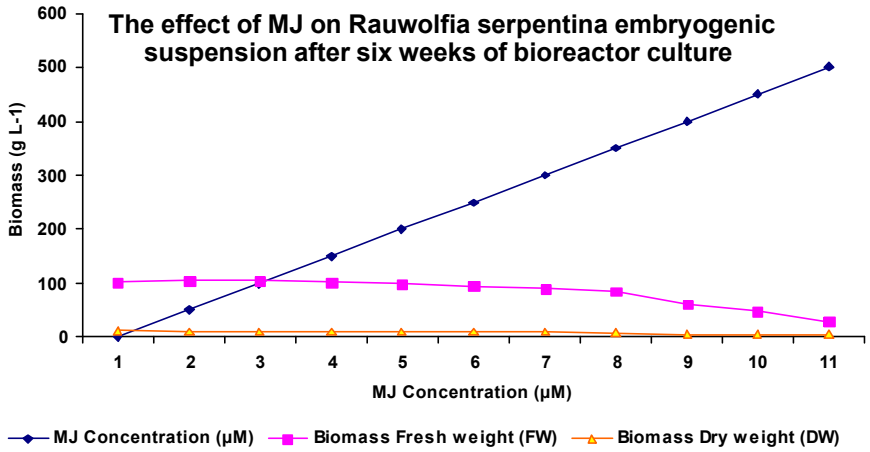

Fig. 1: Effect of MJ on *Rauwolfia serpentina* embryonic suspension after six weeks of bioreactor culture

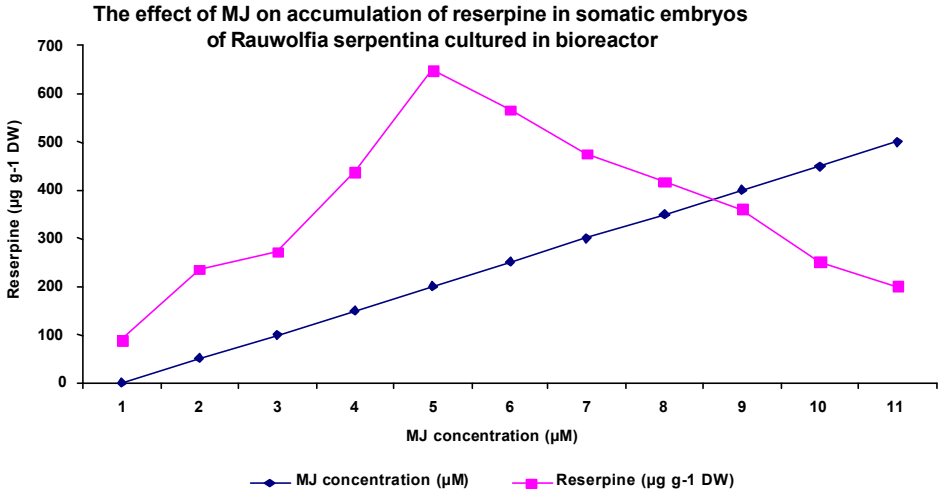

Fig. 2: The effect of MJ on accumulation of reserpine in somatic embryos of *Rauwolfia serpentina* cultured in bioreactors[z].

The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses [15].

Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures. The most frequently used elicitors in previous studies were fungal carbohydrates, yeast extract, MJ and chitosan. MJ, a proven signal compound, is the most effective elicitor of taxol production in *Taxus chinensis* Roxb. [16] and ginsenoside production in *Panax ginseng* [8,10,11,12] cell/organ culture. In the present study, the effect of different concentrations of MJ on embryogenic cell growth and reserpine accumulation was tested and results reveled that addition of 200 µM MJ was suitable for optimum accumulation of reserpine. However, addition of MJ at higher concentration (above 100 µM) was detrimental for biomass accumulation. Similar to the present results, MJ inhibited the cell growth and promoted the secondary metabolite production with cell/adventitious root cultures of *Bupleurum falcatum* L. [9], *Taxus* spp. [6,7] and *Panax ginseng* [11,12]. Accumulation of reserpine was observed during elicitation experiments (Table 2).

Reserpine content was highest in 200 µM MJ treatment produced by the suspended somatic embryos (Figure-2). Similar to the present observation differential accumulation of secondary compounds have been reported during cell/organ cultures of *Panax ginseng* [11, 12].

The results from this study demonstrate that MJ elicitation strategy was quite useful to improve the yield of reserpine in embryogenic cell cultures of *Rauwolfia serpentina*. The biomass produced in the bioreactor cultures may be used as source of medicinal raw material for the extraction of reserpine.

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