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Establishment of callus and cell suspension culture of *Sophora alopecuroides* Linn. for the production of oxymatrine

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ABSTRACT

Oxymatrine is one of the most important biologically active compound and is present in *Sophora alopecuroides* L. The present investigation focuses on the development of an efficient tissue culture method to induce callus and cell suspension culture of *S. alopecuroides* by studying the effect of jasmonic acid and nitric oxide on cell suspension culture. Callus induction efficiency is high in axenic leaf explants grown in MS medium supplemented with 1.0 mg/L Kinetin (Kin), 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The cell suspension culture was developed using the same callus induction medium without agar. The maximum cell number and dry weight of suspension culture were obtained by the 9th day of incubation. The synthesis of oxymatrine is higher in jasmonic acid and nitric oxide (200 μ MJA and 50 μ MNO) combination (11.91 μ g/g) when compared to the non-elicited control (8.3 μ g/g) of callus.

KEYWORDS: *Sophora alopecuroides* Linn., callus culture, cell suspension culture, oxymatrine, jasmonic acid, nitric oxide

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INTRODUCTION

Sophora is a perennial herb belonging to the *Fabaceae* family and is used in Mongolian traditional medicine to treat heart disease, bacterial infection, fever, and rheumatism [1]. Mongolian *Sophora* has two species viz., *S. flavescens* and *S. alopecuroides* Linn. The *S. alopecuroides* extracts contain quinolizidine alkaloids and its main constituents are matrine and oxymatrine [2]. Due to the unique oxygen structure of oxymatrine it can strengthen the heart, resist arrhythmia and virus, treat jaundice, cures inflammation, aches, and also lowers the blood pressure. Earlier studies showed that the antitumor activity of the compound oxymatrine restricts the growth of tumor cells by inhibiting the DNA synthesis and restraining the enzyme activity in the tumor cells [3]. Secondary metabolites are produced from the plant cells and by tissue culture system which might be useful in the large scale production of desirable secondary metabolites [4]. Properties of callus tissue are one of the important factors to establish cell cultures. These features will get exhibited during the formation of a single or cluster of cells in the cell suspension culture which also increases the quality of cell suspension culture. Also, the compounds can also be synthesized by the cells dispersed in the liquid medium [5]. Cell multiplication is much higher and more effective by changing the media constitutions [6]. However, the

production of secondary metabolites is limited in the case of cell culture whereas the biosynthesis of the secondary metabolites in plants is due to its stress factors and its response [7]. Synthesis and accumulation of secondary metabolites are due to the compounds called elicitors which are produced at the time of stress and enhance the accumulation of secondary metabolites [8]. There are different types of elicitors found to alter the metabolism for enhancing secondary metabolite synthesis in the cell cultures [9]. Based on the previous study, the elicitors viz., Jasmonic acid (JA), and Nitric oxide (NO) are selected on the enhancing ability of the compound oxymatrine. The present investigation focuses on developing an efficient procedure for the establishment of callus and cell suspension cultures, to determine the oxymatrine production from these cultures with the influence of jasmonic acid and nitric oxide. Cell viability and culture biomass of *S. alopecuroides* is also taken up in the present study.

MATERIALS AND METHODS

Inoculation of Explants

The seeds of *S. alopecuroides* were obtained from Bayankhongor province, Mongolia. Seeds were surface sterilized by treating it with 70% ethanol followed by 1.0% of NaOCl with 2-3 drops

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of Tween-20 for three and ten minutes respectively. The seeds were then rinsed in the sterile distilled water for 5 minutes under aseptic condition. After the sterilization process, the seeds were germinated in half-strength MS medium (Murashige and Skoog) without any plant growth regulators. pH was adjusted to 5.8 and was solidified with 0.7% agar. The autoclaving was performed with 121°C for 25 min. Incubation was done under a photoperiod of 16/8h light/dark cycle at $25 \pm 2^\circ\text{C}$ in the growth chamber. After four weeks of incubation, the seedlings were cut down into 3-4 cm having 1-2 nodes. The selected nodal explants were used for collection of leaf explants that were ainooculated for callus proliferation in MS medium having 0.7 and 3 percent agar and sucrose respectively.

Callus Induction

Seedlings grown under the *in vitro* condition were used for collecting the leaf explant to establish callus. Seedlings with 4 cm height were cut into four parts and the abaxial side of the leaf is placed in the culture medium. At two different concentrations, the growth regulators such as 2,4-D and Kinetin were tested for callus induction. Four weeks after inoculation the frequency of the callus induction and its appearance were tested and the newly developed callus was sub cultured once every 28 days.

Initiation and Establishment of Cell Suspension Culture

The obtained friable callus tissues from the MS solid medium supplied with 1.0 mg/L 2,4-D and 1.0 mg/L Kinetin were used for cell suspension cultures. Friable callus from 28 days old was transferred to 250 mL conical flask containing 50 mL MS liquid medium with 100 mg/L of glutamine, 3% sucrose without agar. The suspension culture was placed in a rotary shaker at 80 rpm under the culture room condition.

Growth Curve of Cell Suspension Cultures

After 21 days from the initiation of cell suspension culture, the growth curve was estimated to optimize the period for attaining maximum cell growth. 20 mL aliquots from cell suspension culture were taken and transferred to the conical flask having 50 mL MS liquid media added with 1.0 mg/L 2,4-D and 1.0 mg/L Kinetin. The cultures were incubated uniformly at 80 rpm under a rotary shaker. Quality of the culture was tested by taking the dry weight (DW) and the cell number and viability was tested once in three days at the incubation period of 21 days. To

detect the presence of a single cell (Fig. 1c) or small cell clusters (Fig. 1d) and its morphological appearance observations were recorded through a microscope.

Measurement of Cell Growth and Oxymatrine Production in Cell Suspension Culture

In plants, Sodium nitroprusside (SNP) is a frequently used NO donor [19]. The working concentration of 100 mM of jasmonic acid was prepared by diluting the stock concentration (100 mg/mL) prepared with dimethyl sulfoxide. Nitric oxide (100 mM) solution was prepared using distilled water. Using membrane filter the solutions prepared were filtered and sterilized.

Jasmonic acid and Nitric oxide were added to the individual conical flask to get a final concentration of 50, 100, 200 μM . Elicitors were added to the culture after the 9th day of growth under aseptic condition and the same was incubated at the rotary shaker.

Nine-day old cell cultures of *S. alopecuroides* were treated with the elicitors chemicals *viz.*, NO, and JA which were filtered and washed with 50mL fresh medium to avoid the chemicals for 48 h. After that, the cultures were re suspended in fresh medium. Suspension cultures were harvested on the 9th day of post elicitation to monitor the influence of elicitors on biomass growth and oxymatrine production.

Estimation of Oxymatrinecontent using HPLC

The oxymatrine (alkaloids) extracted from cell cultures were analyzed using high performance liquid chromatographic (HPLC) method as reported by [10]. After drying, the samples are extracted in a solution of Trichloromethane - Methanol - Ammonium (40:10:1) with ultrasonic treatment for 30 min. All samples were filtered and then 10 ml filtrate was again filtered through a 0.45 μm membrane filter to obtain the pure solution for HPLC. For the standard solution, 7.5 mg of oxymatrine was added to methanol and finally made up to 150 $\mu\text{g}/\text{mL}$ of oxymatrine. Subsequently, 2, 4, 6 and 8mL of the solution was diluted using methanol to obtain 30, 60, 90 and 120 $\mu\text{g}/\text{mL}$ oxymatrine standard solutions and the standard curve was constructed (Fig.1). The HPLC Shimadzu SPD-M20A, Japan with a C18 column was used for the quantification of oxymatrine. The mobile phase comprised of 3% phosphoric acid-water in the ratio of 80:20. The injection volume was 40 μl , the flow rate was 1mL/min, UV wavelength was set at

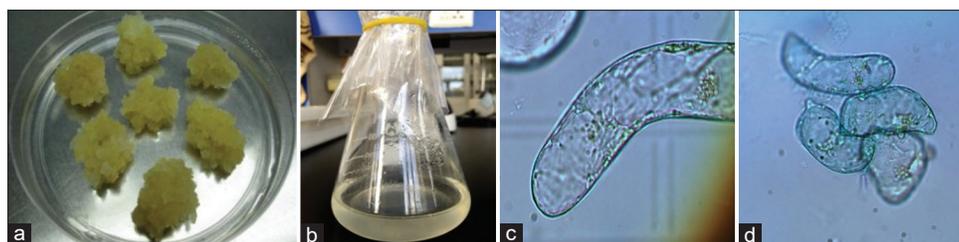


Figure 1: Cell suspension cultures of *S. alopecuroides* established in MS liquid medium supplemented with 1.0 mg/L Kin, 1 mg/L 2,4-D. a friable callus initiation from leaf explants of *S. alopecuroides* b, Maintaining of cell suspension cultures c, enlarged single-cell d, small cell cluster.

220 nm and the column temperature was 30°C. The results were represented as mean ± SD. The standard curves of oxymatrine obtained by measuring the absorbance at 220 nm were linear in the concentration range of 30 µg/mL to 120 µg/mL ($y = 14701x + 9611.3$, $r = 0.9851$)

RESULTS

Initiation of Callus and Cell Suspension Cultures

The friable as well as fast-growing callus from leaf explants were observed in MS solid medium supplemented with different concentrations of hormones. The MS medium in combinations of Kinetin (0.5-3.0 mg/L) and 2,4-D (1.0-6.0 mg/L) was used for the callus initiation studies. The combinations Kinetin and 2,4-D in MS solid medium induced callus but showed different morphological appearance and growth rate. Also, no callus induction was found on MS medium basal (devoid of growth regulators) MS medium supplemented with either kinetin or 2,4-D alone. With friable yellowish calli along with higher callus growth (75-100%) was observed in MS solid medium provided with 0.5 mg/L Kin, 1 mg/L 2,4-D and 1.0 mg/L Kin, 1 mg/L 2,4-D (Table 1). Cell suspension culture was initiated with the callus obtained from the MS medium provided with 1.0 mg/L Kin, 1 mg/L 2,4-D (Fig. 1a). Callus formed on this medium recorded were yellowish, friable, and showed more rapid growth.

The obtained friable callus was transferred to the liquid medium similarly as like callus growth medium without agar. After incubation in the rotary shaker, with the continuous agitation process, clumps were formed in 7-10 days which later established in to free cell and small cell aggregates on MS medium supplemented with 1.0 mg/L Kin and 1 mg/L 2,4-D.

As incubation of cultures got extended for 10-15 days, the medium became whitish turbid and optically dense with abundant free cells and small cell aggregates (Fig. 1b). The cell suspension cultures were further proliferated by sub-culturing aliquots to fresh liquid medium supplemented with the same hormone combinations.

The growth curve of cell suspension is obtained by studying the time required for the culture to attain the maximum level of dry weight (DW). The cell number and cell viability at three-day intervals up to 21 day culture period were measured. The cells started dividing after a short lag phase of 3 days and moved onto the exponential phase for 3-6 days and attained maximum cell biomass on the 9th day of incubation (Fig. 2). After the 9th day of incubation in MS liquid medium with 1.0 mg/L Kin, 1 mg/L 2,4-D, the culture showed reduced biomass and cell number.

Effect of Elicitor and Production of Oxymatrin in Cell Suspension Culture

The effects of elicitors jasmonic acid (JA) and sodium nitroprusside (SNP) on cell growth and oxymatrine content were studied in cell suspension cultures. The cell suspension cultures incubated for 9 days at 50–200 µM for 48h were hand-harvested

Table 1: Effect of different PGRs on callus induction of leaf explants in MS solid medium

No	Kin	2,4-D	Callus formation (%)	Callus Morphology
1.	0.5		0	-
2.	1.0		0	-
3.	3.0		0	-
4.		1.0	0	-
5.		3.0	0	-
6.		6.0	0	-
7.	0.5	1.0	75	yellowish, friable
8.	1.0	1.0	100	yellowish, friable
9.	3.0	1.0	100	green, compact
10.	0.5	3.0	0	-
11.	1.0	3.0	0	-
12.	3.0	3.0	50	brownish, compact
13.	0.5	6.0	0	-
14.	1.0	6.0	50	brownish, compact
15.	3.0	6.0	0	-

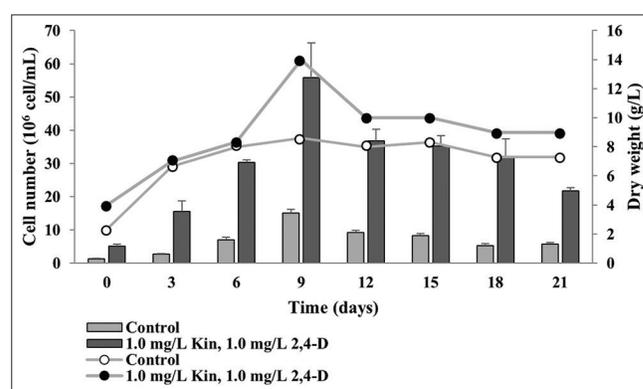


Figure 2: Biomass values of the cell suspension cultures observed from day 0 to 21

for the determination of oxymatrine. The results showed that the oxymatrine production of cells treated with 200 µM JA increased (16.1 µg/g) than the control (10.3 µg/g).

The amount of oxymatrine in cell cultures treated with 200 µM SNP (17 µg/g) was higher (6.7 µg/g) when compared to JA control. The Oxymatrine productions in the cells treated with 200 µM JA and 50 µM SNP (18.6 µg/g) were the highest. However, the cell treated with 200 µM JA or 50 µM SNP separately had lower oxymatrine production (17 µg/g and 16 µg/g) than the combination (Fig. 3).

Cell suspension cultures treated with a combination of 200 µM JA and 50 µM SNP had an increased level of oxymatrine levels (8.3 µg/g) than the non-elicited callus cell suspension culture (Fig. 5). The increased concentration of elicitor revealed that reduced viability of cell cultures and vice-versa (Fig 4). This reflects the loss of viability and more oxymatrine production which may be due to the defense response of cells influenced by the elicitor.

DISCUSSION

The leaf explants derived from *in vitro* shoots grown from sterilized seeds (Figure 6) were found to be a good source for

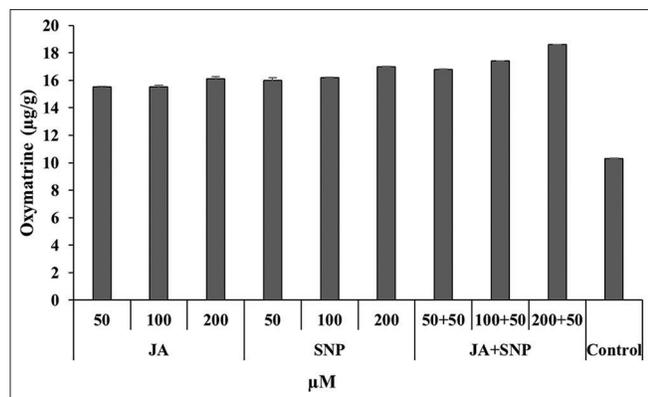


Figure 3: Effect of JA and SNP on oxytmatine production in cell suspension cultures

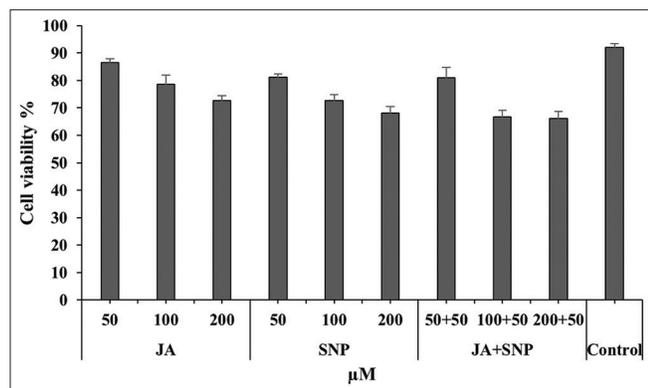


Figure 4: Effect of JA and SNP on the viability of cells of suspension cultures

inducing fast proliferating friable type of callus. The PGRs play a significant role in cell growth and differentiation in the presence of both auxins and cytokinins in the nutrient media which facilitate callus induction and proliferation. The secondary metabolite productivity of callus cultures can be altered by phytohormone supplementation and could be used as a source for rapid and increased production. The positivity of the addition of 2,4-D in media for callus initiation in *Sophora* species was already well established [11,12]. Cao *et al.* (2010) reported that 2,4-D was found to be a stronger auxin than NAA and also Kinetin produced callus at a greater frequency than BA. They also observed that the callus tissue was more friable in the presence of 2,4-D than in presence of NAA. Cao *et al.* (2010) found that amount of cytokinin affected the compactness of the cultures; as greater compactness related to increased concentrations of cytokinin.

Due to the friable structure of callus, the initiation of cell suspension was conducted on the medium supplied with 1.0mg/L Kin, 1 mg/L 2,4-D. However, the efficient manipulation of the cultures requires knowledge about the growth habit of the culture which identifies the onset and the duration of various growth phases: lag, exponential or log, linear, progressive deceleration, and stationary [13]. Many of the studies revealed that the age of the subculture plays a significant role in the synthesis of bioactive compounds [14]. Hence it is necessary to understand the days of the subculture through growth kinetics.

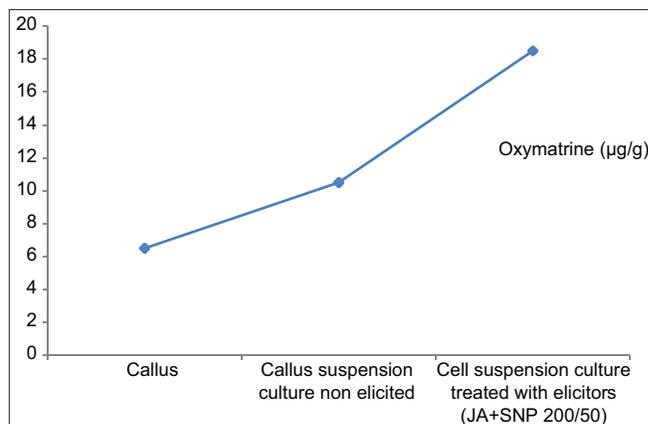


Figure 5: Oxytmatine content of different tissue sample



Figure 6: Inflorescence (a) and Fruit (b) of *sophora alopecuroides*

Cells initiated its multiplication after a short lag phase was further moved to the exponential phase of 3-6 days, attained maximum biomass from the 9th day of incubation when it was supplemented with 1.0mg/L Kin, 1 mg/L 2,4-D. Similar results were recorded by Xu *et al* (2009).

Different elicitors *viz.*, salicylates, jasmonates, yeast extract, cork tissue, and nitric oxide were tested and found to play an important role in the production of secondary metabolites [15,16,7]. Similarly, Jasmonic acid influences the plant defense responses and could be a potential inducer for the biosynthesis of secondary biosynthesis [17,18,19].

Sodium nitroprusside is responsible to generate NO in solutions [20]. In the plant system, nitric oxide plays a role in root growth, stimulation of seed germination, defense gene activation, and induction of plant defense response [21,22]. In accordance with Xu. (2009), the effect of matrine production of *S. flavescens* cells increased by three times when it has treated with jasmonic acids and nitric oxide. Moreover, Nitric oxide enhances the jasmonic acid level and in turn, it activates the endogenous jasmonic acid biosynthesis of *S. flavescens* cells. In our study, we reported that the accumulation of oxytmatine increased by three times when comparing to non-elicited one. Also, it is about 8.3 µg/g higher when comparing with the non-elicited cell culture. From the earlier study, Xu *et al.*, 2009 reported that jasmonic acid and nitric oxide influence the

synergistic stage of matrine accumulation but the loss of cell viability and increased oxymatrine production depends on the defense response of the cells by the elicitors.

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