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Enhanced biosynthesis of colchicine and thiocolchicoside contents in cell suspension cultures of *Gloriosa superba* L. exposed to ethylene inhibitor and elicitors

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ABSTRACT

The present investigation reports the influence of ethylene inhibitor and elicitors on enhanced biosynthesis of colchicine and thiocolchicoside contents in cell suspension cultures of *Gloriosa superba* L. Elicitation is one of the approaches used for enhanced commercial production of secondary metabolites from plant cell culture system. *G. superba* is a rich source of colchicine and thiocolchicoside contents that are being used for the treatment of cancer. Callus was obtained from rhizomes of *G. superba* on MS medium containing 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg L^{-1} α-naphthalene acetic acid (NAA) and cell suspension cultures were initiated. The influence of elicitors [salicylic acid (SA), yeast extract (YE), and casein hydrolysate (CH)] as well as ethylene inhibitor [silver nitrate ($AgNO_3$)] on biosynthesis of colchicine and thiocolchicoside contents was studied by exposure of cell suspension cultures with different concentrations for 15 and 30 days period. Among four elicitors tested, CH exhibited the maximum level of colchicine production [8.290 mg g^{-1} dry weight (DW)] at 300 mg L^{-1} concentration over a period of 15 days exposure, compared to the control whereas, SA at 27.624 mg L^{-1} concentration showed the enhanced colchicine production rate (8.149 mg g^{-1} DW) after 30 days exposure time when compared to non-elicited control cultures. Results show that CH and SA treated cells exhibited 8-fold higher level of colchicine biosynthesis over control. The maximum production of thiocolchicoside content in cell suspension culture obtained was 4.550 mg g^{-1} DW and 1.530 mg g^{-1} DW with 200 and 300 mg L^{-1} $AgNO_3$ treatment at 15 and 30 days period, respectively. The colchicine content level was significantly increased when compare to the thiocolchicoside content in cell suspension culture. The accumulation of colchicine and thiocolchicoside contents was influenced by exposure time and doses of elicitors. Results clearly show that the elicitation experiment could enhance the biosynthesis of colchicine and thiocolchicoside contents level in cell suspension cultures of *G. superba* and the present protocol may be used for commercial supply of these important secondary metabolites in the future for therapeutic applications.

1. Introduction

Medicinal plants are the best source of life saving drugs for the majority of the world's population. Plants produce numerous secondary metabolites that are being used for various applications such as pharmaceuticals, bio-pesticides, flavors, fragrances, colors and food additives (Sivanandhan et al., 2014). *In vitro* cell suspension culture was used as additional technique for enhanced production of secondary metabolites (Ahmad et al., 2013). The biosynthesis of valuable secondary metabolites was extracted from cell and organ culture of medicinal plants. More recently, plant cell cultures have been employed to produce foreign gene products (Sukito and Tachibana, 2016). Due to the importance of secondary metabolites, there is an increasing need to obtain more novel secondary metabolites through plant cell suspension culture methods. Different factors such as media composition, elicitors, pH conditions and precursors were influenced for enhanced production of secondary metabolites in the past (Chodisetti et al., 2015; Park et al., 2016; Xia et al., 2016; Cai et al., 2017). Even though, the use of plant cell and organ culture is used to synthesize bioactive compounds with limited commercial success (Xia et al., 2016). Plant cell suspension culture system is being considered as promising alternative source for sustainable production of essential secondary metabolites (Rischer et al., 2013; Yue et al., 2016). Therefore, it is important to establish suitable methods for producing commercially valuable secondary

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metabolites at present.

Cell suspension culture offers a condensed biosynthetic cycle that could be useful for synthesis of highly valuable medicinal bioactive compounds in good quantities (Singh and Chaturvedi, 2012). Elicitation is one of the most effective and important technique to improve *in vitro* production of secondary metabolites. In cell suspension cultures, biotic and abiotic elicitors were used to stimulate the potential secondary metabolites in the recent past (Cai et al., 2017). Cell suspension cultures in liquid medium may allow cells for fast and uniform interaction with nutrition, growth hormones, precursors and elicitors easily (Mustafa et al., 2011). The elicitor is a substance, introduced minor concentration in a cell suspension culture to increase the quantity of specific bioactive compounds rapidly. Among abiotic elicitors, silver nitrate ($AgNO_3$) is used in most of the studies for enhanced production of secondary metabolites in cell suspension culture of various plant species (Xu et al., 2007; Meier et al., 2015; Deepthi and Satheeshkumar, 2016). Earlier studies suggest that the silver nitrate was used as an elicitor on biosynthesis of secondary metabolites in plants, where Ag+ ions is present. Silver nitrate can inhibit the action of ethylene hormone, this may increase the production of secondary plant products, plant regeneration and somatic embryogenesis rate (Mahendran et al., 2017). The positive effect of Ag+ may be attributed to enhance the secondary metabolites production in cell suspension culture (Vildová et al., 2016). Also the effect of yeast extract (YE) and salicylic acid (SA) has been studied and described as elicitors for enhanced secondary metabolite production in various plant species (Sivanandhan et al., 2012a; Shukor et al., 2013; Chodisetti et al., 2015; Park et al., 2016).

Gloriosa superba L. (Liliaceae) is a valuable, important tropical monocot medicinal plant and different plant parts are being used as medicine in indigenous system. Rhizomes are used in several treatments such as paralysis rheumatism, snakebite, insect bites, intermittent fever, wounds, anti-fertility, gonorrhoea, leprosy, piles, dyspepsia, flatulence, haemorrhoids, helminthiasis and inflammations (Pulliah, 2002). It's an important medicinal plant containing crucial compounds, namely colchicine, colchicoside and gloriosine (Sivakumar et Sivakumar and Krishnamurthy, 2004). Colchicine is the major compound derived from rhizomes and seeds of *G. superba* (Ravi et al., 2011). Maximum level of colchicine content noticed was (0.7–0.9%) in rhizomes and seeds (Rajagopal and Kandhasamy, 2009). Colchicine is actively involved in suppression of abnormal cell growth in certain tumours, including animals and its arrest polymerization of tubulin proteins (Andreu et al., 1998). Thiocolchicoside is a semi synthetic derivative of colchicoside which is obtained from *G. superba* and *Colchicum autumnale* (Micheau et al., 2012). Thiocolchicoside is a centrally acting muscle relaxant and used for the treatment of several musculoskeletal disorders. Moreover, their drug exhibited anti-inflammatory and analgesic effects in animal models (Sechi et al., 2003). Although, this drug is already in the use in European countries, in India the first formulation containing thiocolchicoside was approved during the year 2008 (Central Drugs Standard Control Organization, 2008).

Due to the importance of these bioactive molecules, this plant species has potential pharmaceutical value but the quantity of compounds is still low. Also earlier reports indicate the production of low yields of colchicine from callus, adventitious roots and hairy root cultures of *G. superba* (Ghosh et al., 2002; Sivakumar et al., 2004; Ghosh et al., 2006; Pandurangan and Philomina, 2010; Bai and Agastian, 2013). In addition different precursors and nutritional factors were also tested for enhancement of colchicine content in callus cultures (Sivakumar et al., 2004). Though, an attempt was made to increase the colchicine content in cell culture using precursors, no report is available for enhanced production of colchicine and thiocolchicoside till now in *G. superba*. As both colchicine and thiocolchicoside compounds have high pharmaceutical value at present, there is an urgent need to investigate a novel tool for enhanced production of these compounds. In view of the above, the present study is mainly focused to examine four different elicitors at various doses for enhanced biosynthesis of colchicine and thiocolchicoside contents level in cell suspension culture of *G. superba*. In addition, the elicited cells were harvested at different time intervals and colchicine as well as thiocolchicoside contents were determined using HPLC analysis.

2. Materials and methods

2.1. Plant material and culture conditions

Rhizomes of *Gloriosa superba* L. were collected from Thuraiyur, Tiruchirappalli District, Tamil Nadu. The rhizomes were thoroughly washed with running tap water for 20 min and immersion in 2.0% (*w/v*) of commercial Teepol solution for 5 min. Then, the rhizomes were soaked with Bavastin solution for 10 min and washed 3 times with sterilized distilled water. Finally, rhizomes were decontaminated with 0.1% (*w/v*) $HgCl_2$ for 10 min and were washed 5 times with sterilized distilled water and placed on media for initiation of callus culture. The nutrient medium consists of MS (Murashige and Skoog, 1962) basal salts with 3% (*w/v*) sucrose and 0.7% (*w/v*) agar. All the media were adjusted to pH 5.8, before autoclaving at 121 °C for 20 min. For callus initiation, rhizome explants were placed on MS medium supplemented with different concentrations of 2,4-D (1.0–4.0 mg L^{-1}) in combination with 0.5 mg L^{-1} NAA. Cultures were maintained at 25 ± 2 °C and 16:8 h light/dark photoperiod (50 µmol m^{-2} s^{-1}) provided by cool white fluorescent tube lights. After 2 weeks of culture, callus was subcultured on to fresh MS medium containing 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} NAA once in 4 weeks for mass production.

2.2. Establishment of cell suspension cultures

In the present investigation the whitish friable callus was transferred into MS liquid medium to make cell suspension culture. The conical flask (250 mL) containing 100 mL of MS liquid medium containing 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} NAA combination. After sterilization the conical flask containing liquid MS media, were taken to the laminar. White friable callus (1 g) was transferred aseptically into the conical flask with MS liquid medium. The flask was covered with sterile aluminium foil and placed on rotary shaker with agitating at 80 rpm under dark condition.

2.3. Elicitor preparation and cell suspension treatments

In the present study, four elicitors were used and stock solution of salicylic (SA) acid (10 mM) was prepared in 40% ethanol and filtered. Yeast extract (YE), casein hydrolysate (CH) and silver nitrate ($AgNO_3$) were dissolved in sterilized double distilled water for preparation of stock solution (100 mg L^{-1}) and autoclaved all the stock solutions at 121 °C for 15 min (Udomsuk et al., 2011). All the elicitors were used at different concentrations, SA (13.812, 27.624, 41.436, 55.248 and 69.060 mg L^{-1}), YE, CH and $AgNO_3$ (100, 200, 300, 400 and 500 mg L^{-1}) for elicitation experiments and control was maintained without addition of elicitors. After the elicitors treatment (15 and 30 days) cells were harvested for determination of biomass (fresh and dry weight) and quantification of colchicine and thiocolchicoside content.

2.4. Determination of cell suspension biomass

After elicitor's exposure, the cells were collected from the medium for fresh weight measurement. Then the cells were placed on a sterile Whatman filter paper to remove the water drops and measured the fresh weight. Further, cells were placed on a Petri dish and dried in a hot air oven at 60 °C for 2 days and dry weight was measured. Each treatment was repeated twice.