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Optimization of Aconitine Production in Suspension Cell Cultures of *Aconitum napellus* L.

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ABSTRACT : Aconitine alkaloids produced from cell suspension cultures of *Aconitum napellus* for the first time. The effects of various culture conditions on cell biomass and aconitine accumulation in cell suspension cultures were investigated. Suspension cell cultures of *A. napellus* were established by transferring callus tissues from leaf explants onto liquid MS medium supplemented with 1 ㎎/ℓ NAA and 0.1 ㎎/ℓ kinetin. Among the culture media tested, MS medium had a pronounced effect on cell growth and aconitine accumulation. The maximum dry cell weight was obtained at inoculum size of 3 g (FCW) per flask and in MS medium supplemented with 5% sucrose after 8 weeks. The addition of salicylic acid (SA) and yeast extract (YE) in the MS medium enhanced aconitine accumulation. Using a proper combination of culture condition and supplements, aconitine content could reach 0.043% (dry weight basis), that was 2.5~3 fold higher that detected in control cultures.

Key words : *Aconitum napellus*, suspension cell cultures, aconitine

INTRODUCTION

Aconite, also known as *Aconitum,* is a genus of plants belonging to the natural order Ranunculaceae, the buttercup family, commonly known as aconite, monkshood, or wolfsbane, and embracing about 60 species, chiefly natives of the mountainous parts of the northern hemisphere. The crude drug "bushi", the roots of certain species of *Aconitum* plants, has been utilized as an important material for clinical therapy in Oriental medicine. The plants' alkaloids have been identified as pharmacologically active compounds. For some of the alkaloids, such as aconitine, hypoconitine, mesaconitine and pyro-type alkaloids, the anti-inflamatory action has been verified experimentally in carrageenin-induced hind paw oedema (Hikino *et al.*, 1980; Murayama *et al.*, 1991). The antinociceptive properties of Aconitum alkaloids have been proven for aconitine, hypaconitine, 3-acetylaconitine and lappaconitine in the writhing assay and the tail-flick test (Hikino *et al.*, 1979; Dang *et al.*, 1986; Liu *et al.*, 1987).

Plant cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic transformation and hence is able to produce the range of chemicals found in the parent plant. Plant cell culture technology is a promising alternative for producing a large variety of useful secondary metabolites that are widely used as pharmaceuticals, food additives, and dairy products(Verpoorte *et al.*, 1999). However, there are still problems in the production of metabolites by cell cultures resulting from the instability of cell lines, low yields, slow growth and scale-up problems (Ravishankar & Venkataraman, 1993). Development of an efficient cell culture system for commercial production of aconitum alkaloids requires an integrated approach combining the effects of various enhancement strategies. Several approaches have therefore been made in order to increase the accumulation of useful

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compounds in the cultures.

In this communication, we report for the first time the optimal conditions for cell growth and aconitine production of suspension cell cultures, and compared the contents of aconitine with those of the cultivated plants.

MATERIALS AND METHODS

Callus cultures

The seeds of *A. napellus* were surface sterilized by dipping into 70% ethanol for 2 min followed by 5 min in a 2% sodium hypochlorite, and rinsed with ditilled water. Subsequently, the seeds were germinated on half strength MS basal medium at 25±1℃ with 16-h photoperiod under fluorescent light at 20 μmol m^{-2} s^{-1}. After 6 weeks culture, the expended first true leaves of seedlings were cut into 1 ㎠ segments and cultured in petri-dishes containing MS medium containing 3% sucrose, 7% agar, and supplemented with 1 ㎎/ℓ NAA, and 0.1 ㎎/ℓ kinetin. These cultures were incubated in growth chamber at 25±1℃ in the darkness, subcultured them in the same medium and conditions every 2 weeks.

Cell suspension cultures

To establish suspension cultures, calli were first transferred into the liquid MS medium containing 1 ㎎/ℓ NAA and 0.1 ㎎/ℓ kinetin and cultured for 3-4 passages, from which fine cell clumps harvested with stainless steel mesh (pore size 50 ㎛) during exponential growth phase were used as inoculum. Fast-growing cells were resuspended in 50 ㎖ liquid medium in 100 Erlenmyer flasks and cultivated on a gyratory shaker (120 rpm) at 25±1℃ with a 16 h photoperiod.

The effect of basal media

To investigate the effects of basal medium on cell growth and productivity, the cells were cultured in MS (Murashige & Skoog, 1962), 1/2MS, WPM (Lloyd & McCown 1980), B_5 (Gamborg *et al.*, 1968), and SH (Schenk & Hildrbrandt, 1972) medium under the conditions described above, respectively. After 8 weeks of growth, fresh cells were harvested to compare cell yield.

The effect of sucrose

The effect of carbon source on the cell growth and productivity was investigated by using initial sucrose concentrations of 3, 5, 7 and 10% in MS basal medium.

The effect of inoculum size

The optimum inoculum size of the suspension culture was determined by change the weight of starting fresh cells. Initial inoculum are fed from 1 to 7 g fresh cell weight (FCW) per 50 ㎖ culture in liquid MS medium.

Elicitations

Elicitation was carried out with salicylic acid (SA) and yeast extract (YE). Stock solutions were prepared by dissolving each elicitor in ditilled water and adjusting the pH 5.7. The solutions were sterilized by autoclaving at 121℃ for 15 min. The elicitors were added to 2-weeks-old cultures and these were incubated according to the conditions described above.

Determination of cell growth

Fresh cell weight (FCW) was measured after the cell aggregates had been separated from the medium on Whatmann No.1 filter paper under vaccum and washed three times with distilled water. Dry cell weight (DCW) was determined by freeze-drying biomass until constant weight. Data shown are mean of three replicates and ±S.D. Values are presented as error bars.

Analytical method

Aconitine extraction and determination was carried out as previously described (Mori *et al.*,1998). The aconitine content in independent samples was determined by HPLC analysis using TSK-GELODS-80TM (4.6 X 150) column with a detection wave length 231 ㎚. The mobile phase was 0.05 M phosphate buffer (pH2.7) - tetrahydrofuran (183:17) and the peak area was caculated by comparing with an authentic sample of aconitine from the Sigma Chem. Co., USA.

RESULTS AND DISCUSSION

Callus could be initiated from seedling leaf explants on MS medium containing 1 ㎎/ℓ NAA and 0.1 ㎎/ℓ

kinetin. Well-developed callus was used as starting material for establishing the suspension cell cultures which grown on various culture media with supplememts. We compared cell growth and aconitine accumulation by suspension cell cultures in five basal media; MS, 1/2MS, WPM, B_5, and SH medium. The MS medium was best for cell biomass (4.1 g DCW/flask) and aconitine content (0.014%) (Fig. 1 and 2).

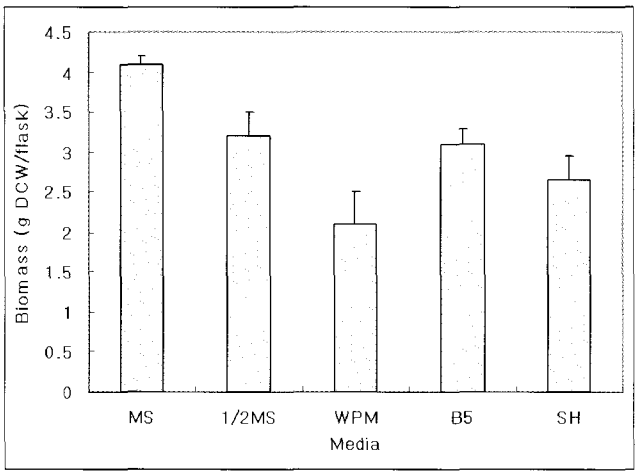

Fig. 1. Effects of culture media on cell growth in *A. napellus* cell suspension culture.

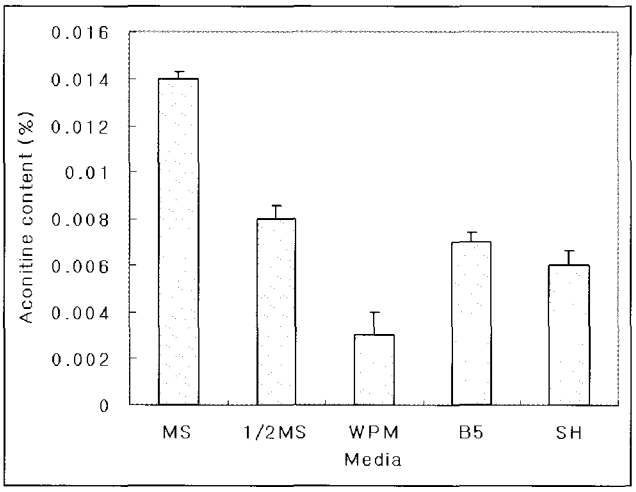

Fig. 2. Effects of culture media on aconitine production in *A. napellus* cell suspension culture.

Suspension cell cultures are usually grown heterotrophically using simple sugars as carbon source and inorganic supply of other nutrients. The carbon source was effect not only growth and differentiation of suspension cultured cells but also secondary metabolism. We examined the effect of initial sucrose concentration on cell biomass and aconitine production. The result given in Fig. 3 shows that, with the increase in sucrose concentration in the basal medium, the dry cell mass increased and reached 3.8 g DCW/flask at 3% and 4.1 g DCW/flask at 5% sucrose. The aconitine content also increased with the increase in the concentration of sucrose in the basal medium. Quantities of 0.008% (dry weight basis) for cultivation in a medium with 3% sucrose and 0.014% (dry weight basis) for cultivation in a medium with 7% sucrose were found (Fig. 4). Misawa (1985) reported that

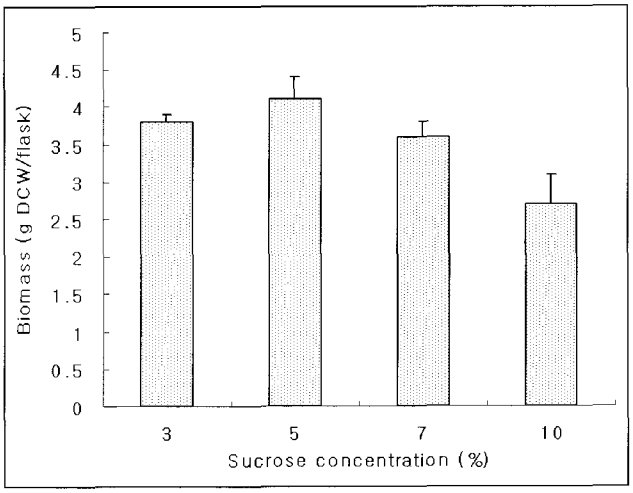

Fig. 3. Effects of initial sucrose concentration on cell growth in *A. napellus* cell suspension culture.

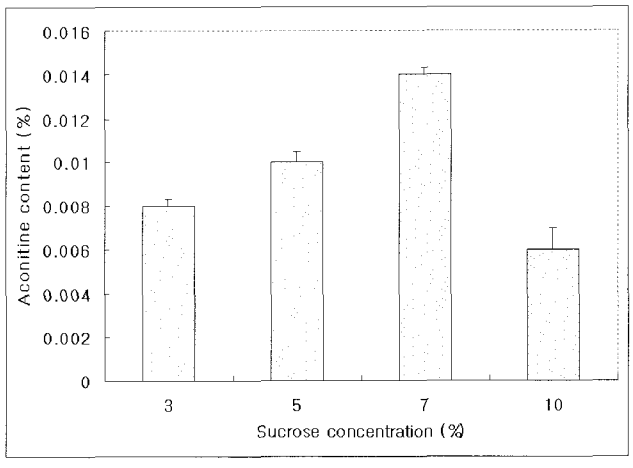

Fig. 4. Effects of initial sucrose concentration on aconitine production in *A. napellus* cell suspension culture.

initial sucrose concentration of 2.5% and 7.5% in *Coleus blumei* cell culture media brought about rosmarinic acid yields of 0.8 and 3.3 g/ℓ, respectively. For indole alkaloid accumulation in cell suspension culture of *Catharanthus roseus*, 8% sucrose was found to be optimal in the tested concentration range of 4–12% (Knobloch & Berlin, 1980). Our results are contrast to those of Sakamo *et al.* (1993), who found that higher concentrations of sucrose at 5% reduced the anthocyanin production in cell suspension cultures of *Aralia cordata*, where 3% favoured the anthocyanin accumulation. Such disparities may be attributed to the differences in cell strains, osmotic stress response, biosynthetic pathway of the metabolites, and culture factors such as medium compositions.

Inoculum size is also an important factor in plant cell suspension cultures. In general, cells fail to grow below a critical minimum inoculum size (van Gulik *et al.*, 1994). There exists the most suitable inoculum density as reported for the cell lines of *Perilla frutescens* (Zhong & Yoshida, 1995) and *Anchusa officinalis* (Su & Lei, 1993). Fig. 5 shows the effect of inoculum size on cell suspension cultures of *A. napellus* in shake flask. A maximum biomass yield of 4.1 g (DCW) per flask was obtained with 3 g (FCW) inoculum size after 8 weeks. The decrease in the growth rate with inoculum size was due perhaps to the increasing limitation of key nutrients, including oxygen, for cells at higher densities. A similar effect was obseved previously in a *Catharanthus roseus* cell cultures (Contin *et al.*, 1998).

For enhancement of aconitine alkaloid in the *A. napellus* cell suspension cultures, SA and YE added to the basal MS medium. Fig. 4 shows the effect of elicitors on aconitine production in *A. napellus* cell suspension cultures. The addition of 0.1 mM SA or 0.5 mg/mℓ YE to MS medium stimulated aconitine production, although YE was less effective than SA (Fig. 6 and 7). SA is one of the most widely studied stress-signaling molecules; its role in influencing plant resistance to pathogens and other

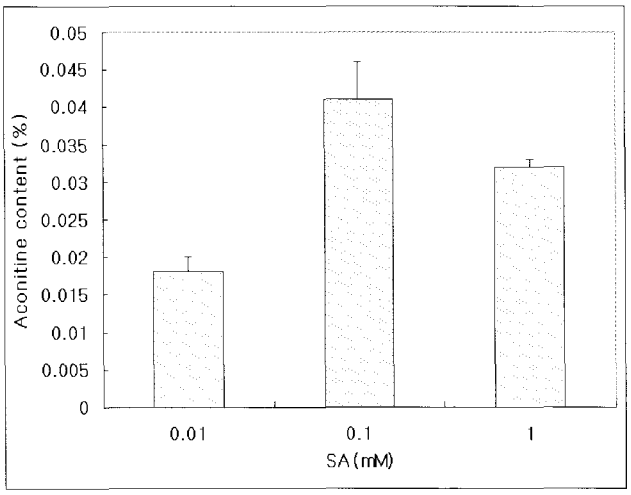

Fig. 6. Effects of salicylic acid (SA) on aconitine production in *A. napellus* cell suspension culture.

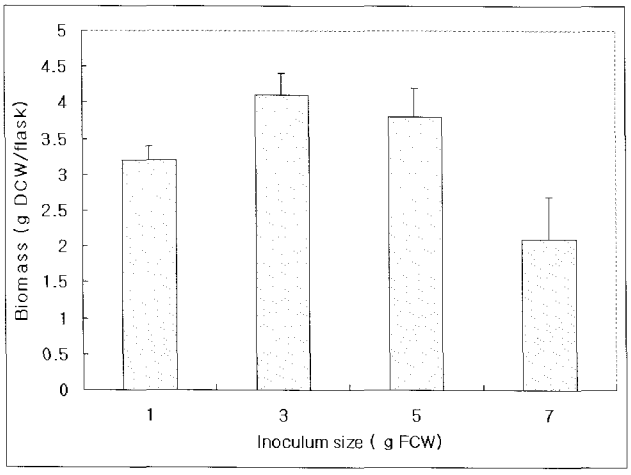

Fig. 5. Effects of initial inoculum size on cell growth in *A. napellus* cell suspension culture.

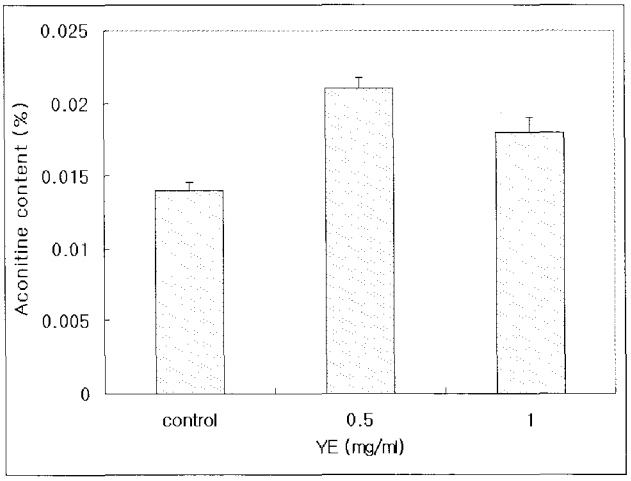

Fig. 7. Effects of yeast extract (YE) on aconitine production in *A. napellus* cell suspension culture.

stress factors is well documented (Draper, 1997). SA and its derivative has been reported to enhance the productivity of some secondary metabolites in cell and tissue cultures. Mehmetoglu & Curtis (1997) reported that 40 μM SA was the optimal concentration for elicitation in suspension cell cultures of *Hyoscyamus muticus* and increased the lubimin production up to 50% of the control. Furthermore, an increase in total alkaloid production of 505% was achieved by adding 1 mM ASA to tumor lines of *Catharanthus roseus* cultured *in vitro* (Gergorio & Victor, 1997). The results of the experiment in this study show that biosynthesis of aconitine alkaloids can be enhanced by the addition of SA. Therefore, addition of SA or SA derivatives could be a useful tool for the enhancement of aconitine alkaloids production in biotechnological processes.

The capability of suspension cell cultures for producing aconitine alkaloid was shown to be much higher than that of *in vitro* cultivated plantlets in which the aconitine content in the root was 0.018% of the dry weight (Fig. 8). This high biosynthetic activity in suspension cell cultures might be partly due to an escape from negative feedback or other physiological disturbance. However, the production of aconitine in suspension cell cultures was also stimulated by the addition of elicitors.

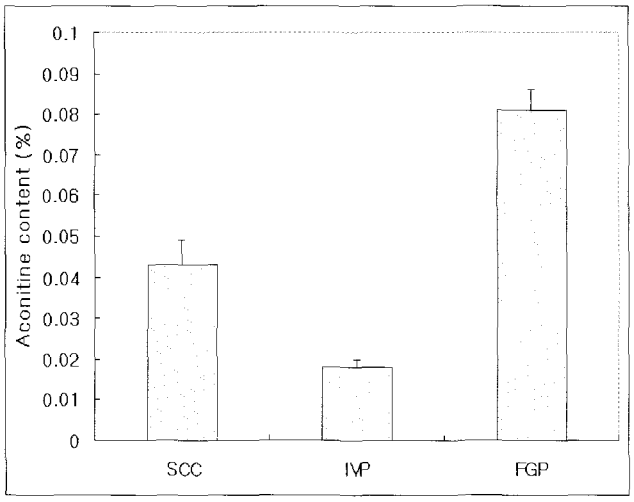

Fig. 8. Contents of aconitine in suspension cell cultures (SCC), *in vitro* cultivated plantlets (IVP), and field-grown plant roots (FGP) of *A. napellus*.

ACKNOWLEDGEMENTS

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