J Plant Growth Regul (1999) 18:89–92

Journal of
Plant
Growth
Regulation

Effect of Brassinolide on Growth and Shikonin Formation in Cultured *Onosma paniculatum* Cells

Y.-H. Yang,* H. Zhang, and R.-Q. Cao

Pharmaceutical Biotechnology Key Laboratory, College of Life Science, Nanjing University, Nanjing 210093, P.R. China

Received November 2, 1998; accepted August 25, 1999

Abstract. The effect of brassinolide (BR) on cell growth and shikonin and its derivative formation in *Onosma paniculatum* cell culture was studied. BR addition with IAA and BAP (+BR/+IAA/+BAP) in B_5 medium slightly increased the cell growth at 0.01–0.1 ppb concentration compared with a growth control (−BR/+IAA/+BAP). Only BR addition (+BR/−IAA/−BAP) at 0.001–100 ppb in B_5 medium significantly increased the cell fresh weight compared with a growth control (−BR/−IAA/−BAP). The same concentration of BR tested at 0–1000 ppb increased the cell fresh weight of +IAA/+BAP significantly more than that of −IAA/−BAP. BR at 0.001–0.1 ppb with IAA and BAP added (+BR/+IAA/+BAP) in M_9 medium increased shikonin and its derivative content markedly by 31–87%, compared with its control (−BR/+IAA/+BAP). BR at 0.001–1000 ppb without IAA and BAP added to M_9 medium (+BR/−IAA/−BAP) also increased shikonin and its derivative content compared with its control (−BR/−IAA/−BAP). However, the amount of shikonin and derivative formed of +IAA/+BAP was greater than that of −IAA/−BAP only at the same concentration of BR at 0–1 ppb. These combined results show that BR at 0.01 ppb with IAA and BAP added was the best for cell growth and shikonin formation. Formation of shikonin and its derivative by adding BR at 0.01 ppb with IAA and BAP (+BR/+IAA/+BAP) in M_9 medium was significantly enhanced 4 days after BR addition compared with a production control (−BR/+IAA/+BAP). In contrast, +BR/−IAA/−BAP vs. −BR/−IAA/−BAP was not as effective as +BR/+IAA/+BAP vs. −BR/+IAA/+BAP for the shikonin formation. The time course study for shikonin formation also showed that +BR/+IAA/+BAP and −BP/+IAA/+BAP only slightly increased cell growth in M_9 medium. Similarly, soluble protein content in the cells treated by BR at 0.01 ppb with IAA and BAP (+BR/+IAA/+BAP) exceeded that of the control (−BR/+IAA/+BAP) 4 days after BR addition. And +BR/−IAA/−BAP only slightly increased the soluble protein content over that of −BR/−IAA/−BAP.

Key Words. Brassinolide—*Onosma paniculatum*—Plant secondary metabolites—Shikonin—Plant cell culture

Brassinosteroids refer to a group of steroidal lactones with high plant physiological activity. Brassinolide (BR) is one member of this group with an extremely high activity. Brassinolides were first isolated from rape (*Brassica nupus* L.) pollen by Mitchell et al. (1970), at which time they were called brassins. The chemical structure of brassinosteroids was described by Grove et al. (1979) and a close structural similarity between BR and animal steroid hormones was found. Subsequently, brassinosteroids were isolated from a variety of other plants and are now known to be ubiquitous in the plant kingdom. BR was synthesized by Thompson et al. (1979).

The special effect of BR is that it can stimulate cell elongation and division when applied to a plant at nanomolar levels. The effects of BR are tissue specific. It can promote elongation of epicotyls, hypocotyls, mesocotyls, and coleoptiles. This effect was found to be accompanied by a stimulation of proton extrusion from the cell (Cerana et al. 1983), which is similar to that produced by auxin, but BR-induced elongation shows a longer latent time than auxin (Clouse et al. 1992). The elongation of bean epicotyls was developed as the standard assay method of BR. When applied to a plant, BR can enhance

Abbreviations: BR, brassinolide; IAA, indoleacetic acid; BAP, 6-benzylaminopurine; CIRFW, cell increase ratio for fresh weight; BSA, bovine serum albumin; FW, fresh weight; DW, dry weight.

*Author for correspondence.

expression of the BRU1 gene whereas auxin cannot (Zurek and Clouse 1994). The genetic sequence of BRU1 showed homology to cell wall–loosening enzymes. BR's unique chemical structure, high biological activity at very low concentration, and wide distribution in the plant kingdom make BR worthy of consideration as a new kind of phytohormone.

Although numerous experiments have been done on BR, they primarily focused on intact plants or cuttings. Few reports exist about its effects on cultured cells, especially for the formation of secondary metabolites. This article will cover this topic with callus of *Onosma paniculatum* as our experimental material. These cells are capable of accumulating shikonin and its derivatives, red naphthoquinone pigments, when grown in M_9 medium (Fujita et al. 1981).

Materials and Methods

Material Preparation and Culture Method

The callus was derived from young shoots of *O. paniculatum.* We adopted a two-stage culture system (a growth stage for cell proliferation and a production stage for shikonin and its derivatives), used a small cell aggregate selection method (Ning and Cao 1995), and obtained a somatic line YN12 to produce shikonin and its derivatives. This cell line was maintained in a growth medium (B_5) (Gamborg et al. 1968) with IAA and BAP added as described by Ning and Cao (1995) at 25°C in light (4000 lux 8 h/day). The subculture was carried out every 16–18 days (Ning and Cao 1995). To produce shikonin, 2.5 g of callus was inoculated in a 250-mL Erlenmayer flask containing a 50-mL production medium (M_9 liquid) (Fujita et al. 1981) and cultured, in the dark, on a rotary shaker at a speed of 120 rpm. The calli were harvested after 20–24 days.

BR Reagent

BR was kindly supplied by Professor Luo Bingshan, Department of Agronomy, Huazhong Agricultural University. It was dissolved in absolute ethanol to yield a 3 ppm (i.e., μg/mL) stock solution. The ethanol in this solution showed no effect on the treated cells.

Measurement of Cell Growth

The cells growing in the media were weighed at the beginning and end of the culture. The cell increase ratio for fresh weight (CIRFW) was defined as the difference of these two weights divided by the beginning weight.

Analysis of Shikonin and its Derivative Content

The shikonin and its derivative in the cells were determined as described by Heide and Tabata (1987). The amount of shikonin and its derivatives was reported as mg/g DW cells.

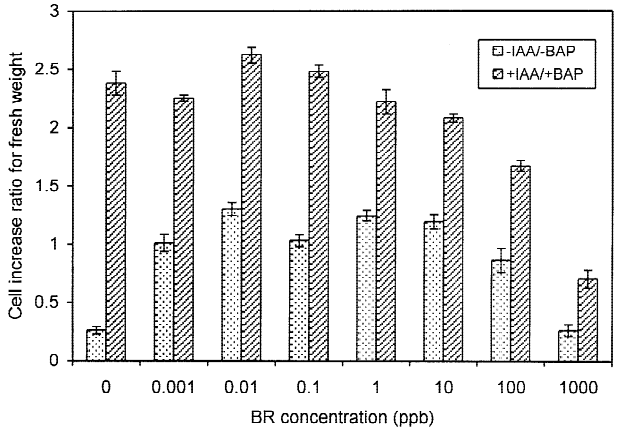

Fig. 1. Effect of BR addition on cell increase ratio for fresh weight of cultured *O. paniculatum* cells in B_5 medium with or without IAA and BAP added. Vertical bars represent SD of the means with three replications.

Soluble Protein Analysis

The fresh cells (0.25 g) were homogenized in a 2.5 mL 0.1 M potassium phosphate buffer (pH 6.5), then centrifuged at 10,000 × g for 20 min. The supernatant was used to determine soluble protein content according to the method of reference (Ning et al. 1996), using bovine serum albumin (BSA) as standard protein. Protein content was reported as mg/g FW cells.

Experimental Design

All treatments were replicated three times in a randomized complete block design.

Results

Effect of BR on cell growth in the growth stage

BR addition only at 0.01–0.1 ppb in the growth medium (B_5) with IAA and BAP added (+BR/+IAA/+BAP) as described by Ning and Cao (1995) slightly increased the cell increase ratio for fresh weight (CIRFW) compared with a growth control without BR added (−BR/+IAA/+BAP) (Fig. 1). The cell weight was increased by 248–262% for +BR/+IAA/+BAP at 0.01–0.1 ppb BR addition, and by 238% for −BR/+IAA/+BAP. When BR at 0.001–100 ppb was added to the B_5 medium without IAA and BAP added (+BR/−IAA/−BAP), CIRFW was significantly more than that of a growth control (−BR/−IAA/−BAP). In contrast, the cell weight was increased by 86–130% for +BR/−IAA/−BAP at 0.001–100 ppb BR, and by 26% for −BR/−IAA/−BAP. The CIRFW increase of +IAA/+BAP was significantly more than that of −IAA/−BAP, when the same concentration of BR was used at 0–1000 ppb (Fig. 1).

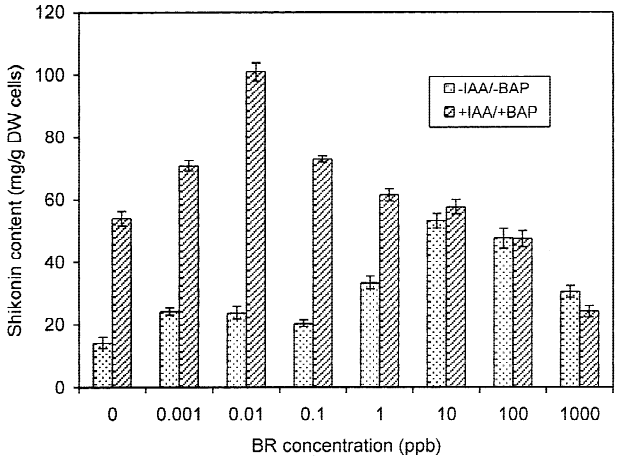

Fig. 2. Effect of BR addition on shikonin formation of cultured *O. paniculatum* cells in M_9 medium with or without IAA and BAP added. Vertical bars represent SD of the means with three replications.

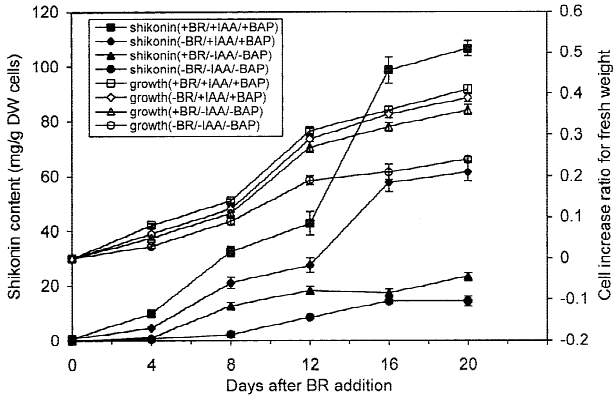

Fig. 3. Kinetic curve of cell growth and shikonin formation of cultured *O. paniculatum* cells by adding BR at optimum concentration (0.01 ppb) in M_9 medium with or without IAA and BAP added. Vertical bars represent SD of the means with three replications.

Effect of BR on Shikonin Formation in the Production Stage

BR addition at 0.001–1,000 ppb in the production medium (liquid M_9), with other regulators added as previously (+BR/+IAA/+BAP), showed that BR had an increasing effect on shikonin and its derivative formation at lower concentrations (less than 10 ppb) compared with a production control (−BR/+IAA/+BAP) as described by Ning and Cao (1995) (Fig. 2). BR at 0.001, 0.01, and 0.1 ppb increased shikonin derivative content by 31%, 87%, and 35%, respectively. BR at 0.001–1000 ppb in the production medium without other regulators (+BR/−IAA/−BAP) consistently raised shikonin derivative content compared with a production control (−BR/−IAA/−BAP) by 43–173%. However, when concentrations of BR less than 10 ppb were used, more shikonin derivatives were formed with IAA and BAP added (+IAA/+BAP) in the medium than those without IAA and BAP (−IAA/−BAP) (Fig. 2). BR at 0.01 ppb with IAA and BAP added (+BR/+IAA/+BAP) was optimal for cell growth and shikonin formation among all treatments tested at 0–1000 ppb BR (Figs. 1 and 2).

Kinetic Curve of Shikonin Formation During the Production Stage

The time course study of shikonin formation at 0.01 ppb BR with IAA and BAP added in the medium (+BR/+IAA/+BAP) showed that shikonin content of the treated cells exceeded that of no BR added (−BR/+IAA/+BAP) 4 days after BR addition (Fig. 3). BR alone (+BR/−IAA/−BAP) also increased shikonin content more than no growth regulator addition (−BR/−IAA/−BAP), but the increase was much less than that of +BR/+IAA/+BAP vs. −BR/+IAA/+BAP 12 days after BR addition. During the production stage, cell growth was not as effective as in the growth stage. Cell weight increased over that of the initial inoculum by 39–41% for +BR/+IAA/+BAP and −BR/+IAA/+BAP at the end of culture. When IAA and BAP were not used, cell weight increased by 24–36% for +BR/−IAA/−BAP and −BR/−IAA/−BAP (Fig. 3).

Change in Soluble Protein During the Production Period

BR addition at 0.01 ppb in the production medium with IAA and BAP added as above (+BR/+IAA/+BAP) showed that soluble protein content in the cells was markedly higher than that of −BR/+IAA/+BAP during the production stage 4 days after BR addition (Fig. 4). Soluble protein content only increased during the first 8 days after the addition and decreased 12 days after the addition. In contrast, only BR addition (+BR/−IAA/−BAP) slightly increased the content compared with −BR/−IAA/−BAP. No regulator addition (−BR/−IAA/−BAP) did not significantly changed the content during the whole production period (Fig. 4).

Discussion

The best known characteristic of BR is its effect on growth: it has been shown to stimulate cell elongation and division, in studies on intact plants or cuttings. In cultured cells, BR, especially at higher concentrations, did not show significant enhanced growth effects. Conversely, in a tobacco callus culture, BR showed a broader

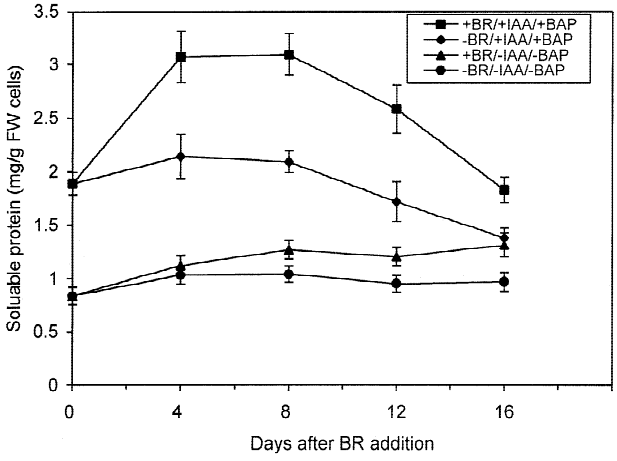

Fig. 4. Kinetic curve of soluble protein content in cultured *O. paniculatum* cells by adding BR at optimum concentration (0.01 ppb) in M_9 medium with or without IAA and BAP added. Vertical bars represent SD of the means with three replications.

inhibitory concentration (Roth et al. 1989). In our experiment, BR, with IAA and BAP also added as described by Ning and Cao (1995), inhibited cell growth at concentrations higher than 0.1 ppb.

Although no documentation of BR's effect on secondary metabolite formation exists, our experiment showed that BR can stimulate shikonin formation at concentrations lower than 10 ppb. This is perhaps partly due to BR's effect on IAA activity. Many experiments have shown an enhancement of IAA activity in BR-treated tissue, and two hypotheses have been advanced to explain it: either (a) BR is involved in sensitizing the tissue to auxin by increasing the activity of auxin receptors, or (b) BR may increase synthesis, transport, or deconjugation of auxin. The second hypothesis seems to be discredited (Cohen and Meudt 1983). IAA can decrease the Ca^{2+} level in cells (De Guzman and Dela Fuente 1984), and a low intracellular Ca^{2+} level is suitable for shikonin formation (Ning et al. 1996). Because BR can stimulate the content of soluble protein in the cells, these proteins might include some enzymes that are involved in initiating cell wall loosening and secondary metabolite formation. Therefore, effects of BR addition on key enzymes involved in shikonin and its derivative formation remain to be studied further.

References

Cerana R, Bonetti A, Marre MT, Romani G, Lado P, Marre E (1983) Effects of a brassinosteroid on growth and electrogenic proton extrusion in Azuky bean epicotyls. Physiol Plant 59:23–27

Clouse SD, Zurek DM, McMorris TC, Baker ME (1992) Effect of brassinolide on gene expression in elongating soybean epicotyls. Plant Physiol 100:1377–1383

Cohen JD, Meudt WJ (1983) Investigations on the mechanism of the brassinosteroid response, I. Indole-3-acetic acid metabolism and transport. Plant Physiol 72:691–694

De Guzman CC, Dela Fuente RK (1984) Polar calcium flux in sunflower hypocotyl segments. Plant Physiol 76:347–352

Fujita Y, Hara Y, Suga C, Morimoto T (1981) Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*, II. A new medium for the production of shikonin derivatives. Plant Cell Rep 1:61–63

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspension culture of soybean root cells. Exp Cell Res 50:151–158

Grove MD, Spencer GF, Rohwedder WK, Mandava NB, Worley JF, Warthen JD Jr, Steffens GL, Flippen-Anderson JL, Cook JC Jr (1979) Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* L. pollen. Nature 281:216–217

Heide L, Tabata M (1987) Enzyme activities in cell-free extracts of shikonin-producing *Lithospermum erythrorhizon* cell suspension cultures. Phytochem 26:1645–1650

Mitchell JM, Mandava NB, Worley JF, Plimmer JR, Smith MV (1970) Brassins—a new family of plant hormones from rape pollen. Nature 225:1065–1066

Ning W, Cao RQ (1995) *Onosma paniculatum:* in vitro culture and the production of purple-red pigment. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry 37: Medicinal and aromatic plants IX. Springer-Verlag, Berlin Heidelberg New York, pp 226–241

Ning W, Yu TT, Cao RQ (1996) Effect of a shikonin elicitor produced by *Aspergillus oryzae* on the metabolism of *Onosma paniculatum* cells. Acta Phytophysiologica Sinica 22:74–80 (in Chinese)

Roth PS, Bach TJ, Thompson MJ (1989) Brassinosteroids: potent inhibitors of growth of transformed tobacco callus cultures. Plant Sci 69:63–70

Thompson MJ, Mandava NB, Flippen-Anderson JL, Worley JF, Dutky SR, Robbins WE, Lusby WE (1979) Synthesis of brassinosteroids: new plant-growth-promoting steroids. J Org Chem 44: 5002–5004

Zurek DM, Clouse SD (1994) Molecular cloning and characterization of a brassinosteroid-regulated gene from elongating soybean (*Glycine max* L.) epicotyls. Plant Physiol 104:161–170.