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Enhancement of Podophyllotoxin Production by Biogenetic Precursors and Elicitors in Cell Suspension Cultures of *Juniperus chinensis*

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Abstract: *Juniperus chinensis* suspension cultures were conducted in Schenk and Hildebrandt medium. On addition of phenylalanine and coniferyl alcohol to the cultures, podophyllotoxin production was enhanced 2.6 and 6-folds compared with the control, respectively. Chito-oligosaccharides, chitopentaose and methyl jasmonate stimulated cells to produce podophyllotoxin at 5, 5.2 and 1.5 times the control level, respectively. The combination of two elicitors, chitopentaose and methyl jasmonate, markedly increased 15-folds relative to the control.

Key words: Cell suspension cultures, podophyllotoxin, biogenetic precursor, elicitor

Introduction

Juniperus chinensis, belongs to the Cupressaceae family and is an ornamental plant typically growing in Japan, Korea, China and the Himalayas (Uehara, 1969; Hora, 1981; Bodkin, 1986). In recent years, it has attracted considerable attention due to the isolation of podophyllotoxin (Miyata *et al.*, 1998). The podophyllotoxin aryltetralin lignin, serves as a commercial precursor of semi-synthetic etoposide and teniposide, important drugs in the treatment of testicular tumors, small-cell lung cancer and acute leukemia (Williams *et al.*, 1987; Ayres and Loike, 1990; Canel *et al.*, 2000). Total chemical synthesis of both compounds is possible, but not an economic proposition (Broomhead and Dewick, 1990). Although podophyllotoxin has been isolated from whole plant of *J. chinensis*, commercial-scale production of podophyllotoxin is not yet realistic.

Rapid developments in plant biotechnology in recent years have provoked much discussion about the overall potential of commercial applications of the technology. Cell and tissue cultures have been widely used as processing vehicles for the synthesis of natural products (Fowler, 1998). Cultured plant cells are in principle totipotent and, therefore, any product present in the parent should also be synthesized in culture under the appropriate incubation conditions (Funk *et al.*, 1987). Previously, we described podophyllotoxin production in *J. chinensis* callus cultures on addition of phenylalanine, coniferyl alcohol and chito-oligosaccharides (COS), as well as the influence of the composition of the cell medium on production (Premjet *et al.*, 2002). The production was significantly increased by adding COS, an elicitor, to the callus cultures (Muranaka *et al.*, 1998; Premjet *et al.*, 2002). It was proved that the elicitation is effective in stimulating the production of podophyllotoxin in *J. chinensis* callus cultures. In present study, we therefore attempted to improve the productivity of podophyllotoxin in cell suspension cultures based on results with established callus cultures of *J. chinensis* (Premjet *et al.*, 2001, 2002; Muranaka *et al.*, 1998). Phenylalanine and coniferyl alcohol were added as biogenetic precursors. Two kinds of β-1,4-linked glucosamine oligosaccharides derived from chitosan, chito-oligosaccharides and chitopentaose, were used as elicitors. The combination of oligosaccharides and methyl jasmonate has been employed to induce phytoalexin in rice systems (Nojiri *et al.*, 1996). Production of palitaxel in *Taxus canadensis* cell suspension cultures was enhanced when the cultures were treated with a combination of N-acetylchito-hexaose and methyl jasmonate (Linden *et al.*, 2000). In this study, methyl jasmonate, a lipid-derived elicitor, was also applied as an elicitor in combination with chitopentaose to *J. chinensis* cell suspension cultures. The effects of each individual compound or a combination of the elicitors on podophyllotoxin production in *J. chinensis* cell suspension cultures were investigated.

Materials and Methods

Study area: The experiment was carried out from 1997 to 2001 in the Faculty of Agriculture, Ehime University, Matsuyama, Japan.

Plant material: *Juniperus chinensis* was collected from Ibuki shrine in Uwajima City, Ehime Prefecture, Japan between 1997 and 2001.

Induction and proliferation of callus: Callus cultures of *J. chinensis* were developed from stem and maintained in SH medium as previously described (Premjet *et al.*, 2002).

Cell suspension cultures: SH medium modified by addition of casamino acid, half- strength ammonium salt and SH standard medium were used to establish cell suspension cultures. The SH medium was found to be suitable for podophyllotoxin production in *J. chinensis* cell suspension cultures, while the medium containing casamino acid and half-strength ammonium salt depressed podophyllotoxin production. Cell suspension cultures were established as previously described (Premjet *et al.*, 2001) at 25°C under 120 rpm in the dark.

Addition of biogenetic precursors and elicitors: The podophyllotoxin arises from phenylpropanoid metabolism. Phenylalanine is an important precursor for auxin-type plant hormones and various secondary compounds including phenylpropanoids. The coupling of two units of phenylpropanoid, coniferyl alcohol, affords pinoresinol, which then undergoes sequential enantiospecific reduction to give larciresinol, secoisolariciresinol and matairesinol in downstream metabolism. Two kinds of precursors, phenylalanine and coniferyl alcohol, involve in the biosynthesis of podophyllotoxin were selected to improve production in cell suspension cultures of *J. chinensis*. In order to determine the optimum concentrations of the precursors for maximum podophyllotoxin production, phenylalanine and coniferyl alcohol in amounts of 0, 5, 10, 15, 25 and 50 mg l^{-1} were added to cell suspension cultures on day 7 before cells had entered a log phase of growth. For induction experiments, cell suspensions from stock solutions were pooled then filtered and transferred (1 g of fresh cells) to 10 ml of fresh SH medium in a 25-ml flask. Phenylalanine and coniferyl alcohol as a biogenetic precursor at concentrations of 0, 5, 15, 25 and 50 mg l^{-1}, respectively, were added to 7-days-old cell suspension cultures. Chito-oligosaccharides were synthesized by the method of Kikkawara *et al.* (1990). Aqueous solutions (5 mg l^{-1}) of chito-oligosaccharides, chitopentaose and methyl jasmonate (10 mg l^{-1}) as elicitors were added, after filter sterilization, to cell suspension cultures on day 7. Culture conditions were maintained as above. Cell viability, cell fresh weight, cell dry weight, packed cell volume (PCV) and podophyllotoxin contents were monitored every seven days over a period of four weeks. The experiments were performed in three replicates. Each feeding experiment was performed separately with its own control group.

Cell fresh weight, cell dry weight and cell viability: Cell fresh weights were measured directly, while dry weights were measured after the freeze-drying of tissue. Cell viability was assessed by fluorescein diacetate (FDA) staining (Jeffrey and John, 1990).

Podophyllotoxin analyses: Cell suspension cultures were filtered under suction and the cell and culture filtrates were collected to measure podophyllotoxin content. The freeze-dried cells and media were powdered in a mortar with a pestle. Samples (50-100 mg) were extracted with 50% methanol (10 ml). Then the mixture was filtered, the residue was washed with a further 50% methanol and the combined crude extracts were evaporated to give a final volume (5 ml) and partitioned between water and dichloromethane (1:1 v/v). Dichloromethane solubles were evaporated to dryness and analyzed by HPLC performed on a reversed-phase column (Hitachi Gel No. 3056, 0.4 x 30 cm) in a Shimadzu LC-10A liquid chromatograph with SPD-10A UV (Ultraviolet-visible light detector wavelength: 290 nm) by isocratic elution with water: acetonitrile (2:1 v/v) as the mobile phase. The flow rate was 0.8 ml/min and all chromatograms were plotted at the absorption maximum of podophyllotoxin, 290 nm. The podophyllotoxin in the dichloromethane soluble was identified by comparing its retention time with that of authentic podophyllotoxin.

Results

Characteristics and Growth of cell suspension cultures: Cell suspension cultures of *J. chinensis* were normally light brown. Cell diameter was found to vary in the range of 100-180 microns (Fig. 1). Cells were spherical in shape and tended to present as aggregates in cell suspension cultures. The growth of cell suspension cultures of the control and on addition of precursors and elicitors was assessed by measuring the cell dry weight of each cell suspension culture every seven days over a period of four weeks. Growth patterns of control and treated cultures were similar (Fig. 2a, b, c). The 'lag' phase was from day 0 to 7 during which little growth was detected. The 'log' phase, a dramatic increase in cell growth, occurred during day 8 to 21. After the log phase, the cell growth decreased. At the beginning of the cell suspension cultures, cell viability was 89-60%, but it decreased after addition of the compounds and in the fourth week had dropped to 8%. The loss of viability may be related to a lack of nutrients and accumulation of compounds toxic to cells during cultivation (Table 1). Non-treated cell suspensions produced a dry mass of approximately 147 mg/flask within 21 days of cultivation (average mean of three experiments, (Fig. 2a,b,c). Phenylalanine at all concentrations did not promote any cell growth (Fig. 2a). Addition of coniferyl alcohol at low concentrations (5-15 mg l^{-1}) increased cell dry weight, while higher concentrations gave a low dry mass (Fig. 2 b). Chitopentaose (5 mg l^{-1}) and chito-oligosaccharides (5 mg l^{-1}) gave the maximum dry weight, 180-mg/flask (Fig. 2 c). A methyl jasmonate concentration over 10 mg l^{-1} was toxic to cells.

Podophyllotoxin in cell suspension cultures of *J. chinensis*: Analyses for podophyllotoxin were carried out on cells and culture filtrate. Podophyllotoxin was not excreted into the medium. The determination of the podophyllotoxin content of harvested cells showed that cell suspension cultures of *J. chinensis* were still capable of producing podophyllotoxin, but the amount was five to ten times lower than that produced in parent callus cultures (data not shown). Variability was observed in the amount of podophyllotoxin in non-treated cell suspension cultures among experiments depending on the cell line used to establish the cultures.

Effects of precursors on podophyllotoxin production: The accumulation of podophyllotoxin in cell suspension cultures of *J. chinensis*, usually paralleled growth up to three weeks and then decreased. The increase in the phenylalanine concentration resulted in less accumulation of podophyllotoxin (Fig. 3). Podophyllotoxin production on addition of phenylalanine (5-10 mg l^{-1}) to cell suspension cultures increased 1.6-2.6 folds relative to the control. Addition of coniferyl alcohol at all concentrations stimulated podophyllotoxin accumulation in cell suspension cultures (Fig. 4). Podophyllotoxin production was enhanced 7- fold when (25 mg l^{-1}) coniferyl alcohol was added to the cultures.

Effects of elicitors on podophyllotoxin production: Chitopentaose was found to be the compound most effective at stimulating the podophyllotoxin production in *J. chinensis* callus cultures in a previous paper (Premjet *et al.*, 2002). The optimum concentration of both chito-oligosaccharides and chitopentaose used in this study was based on the previous data (Premjet *et al.*, 2001). Elicitation of *J. chinensis* cell suspension cultures with chito-oligosaccharides and chitopentaose resulted in obviously greater podophyllotoxin production when compared with control. The production stimulation by chito-oligosaccharides was 5 times that of the control. Chitopentaose produced a slightly higher yield than chito-oligosaccharides at 14 and 28 days (Fig. 5). Addition of a combination of elicitors, methyl jasmonate and chitopentaose, to the cultures enhanced the podophyllotoxin production 15-folds relative to the control, 1.5-folds compared cultures elicited with chitopentaose and 6-folds relative to the culture elicited with methyl jasmonate alone (Fig. 6).

Discussion

Phenylalanine, coniferyl alcohol, chito-oligosaccharides, chitopentaose and methyl jasmonate could enhance the intracellular content of podophyllotoxin in *J. chinensis* cell suspension cultures. The addition of precursors and elicitors caused cell stress leading to browning. After seven days, the suspension cultures overcome the stressful conditions and cell growth accelerates. From the observations of *J. chinensis* cell suspension cultures in several experiments through three years, browning cells produced more podophyllotoxin than normal. Treatments with phenylalanine in cell suspension cultures also resulted in an increase in pH from 5.8 to 8; other phenolic compounds, flavanoids or alkaloids along with podophyllotoxin might be simultaneously produced (Moreno *et al.*, 1995; Fukuda *et al.*, 1997). Ayes (1969) reported the incorporation of phenylalanine to podophyllotoxin by *Podophyllum hexandrum* Royle and later on addition of phenylalanine to culture media of several plants have been carried out. Phenylalanine has affected stimulation of the production of several secondary metabolites, rosmaric acid in *Salvia officialis* and taxol in *Taxus cuspidata* and *T. baccata* cell cultures (Ellis *et al.*, 1970; Fett-Neto *et al.*, 1994; Rosa *et al.*, 1999). An exogenous supply of a biosynthetic precursor to the culture medium sometimes stimulates secondary metabolite production where the productivity is limited by lack of that particular precursor (Moreno *et al.*, 1993; Misawa, 1994). The *J. chinensis* cell suspension cultures utilized the exogenous phenylalanine and coniferyl alcohol to produce podophyllotoxin. Chito-oligosaccharides are a common elicitor used in our laboratory to elicit the production of secondary metabolites in plant cell cultures. Chito-oligosaccharides have strong effects on elicitation in cell cultures not only of *J. chinensis* but also of *Taxus cuspidata* var nana (Yoshida *et al.*, 2002). We found that chitopentaose was effective in stimulating the podophyllotoxin production both in callus cultures and in cell suspension cultures of *J. chinensis*. Methyl jasmonate stimulated enzymes of the phenylpropanoid pathway and plays an important role in plant defense response (Farmer and Ryan, 1992). Our results demonstrated that methyl jasmonate stimulated cells to produce podophyllotoxin. However, podophyllotoxin production was the most enhanced in cell suspension cultures treated with a combination of methyl jasmonate and chitopentaose. The mechanism behind the elicitation by those elicitors in *J. chinensis* cells is still not clear. The maximum level of podophyllotoxin was detected when cells reached the highest dry mass. These results

Table 1: Cell viability of *J. chinensis* cell suspension cultures on addition of precursors and elicitors

Added compounds (ml l^{-1})	% Cell viability at days			
	7	14	21	28
Phenylalanine 0	84	71	68	65
Phenylalanine 5	84	65	50	42
Phenylalanine 10	84	59	47	38
Phenylalanine 15	84	66	42	35
Phenylalanine 25	84	65	53	49
Phenylalanine 50	84	60	49	30
Coniferyl alcohol 0	57	46	42	12
Coniferyl alcohol 5	57	52	35	18
Coniferyl alcohol 10	57	52	19	10
Coniferyl alcohol 15	57	50	44	10
Coniferyl alcohol 25	57	51	11	9
Coniferyl alcohol 50	57	33	26	8
Chito-oligosaccharide 0	83	70	56	35
Chito-oligosaccharide 5	83	74	49	31
Chito-oligosaccharide 10	83	76	43	30
Chito-oligosaccharide 15	83	72	53	35
Chito-oligosaccharide 25	83	70	50	33
Chito-oligosaccharide 50	83	76	52	30
Metyhyl jasmonate 0	89	80	61	45
Metyhyl jasmonate 10	89	63	40	24
Chitopentaose (5) + methyl jasmonate (10)	89	65	57	38
Chitopentaose 5	89	77	61	40

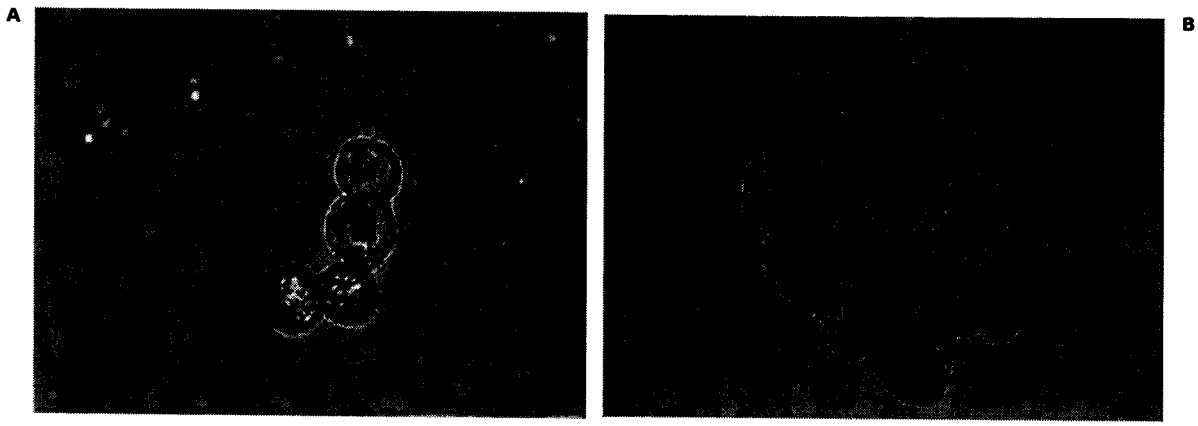

Fig. 1: Micrographs of *J. chinensis* cells. (A), spherical single cells in cell suspension cultures. (B), cell aggregates. The cell diameter is 100-180 micron. The aggregate diameter is 3 mm

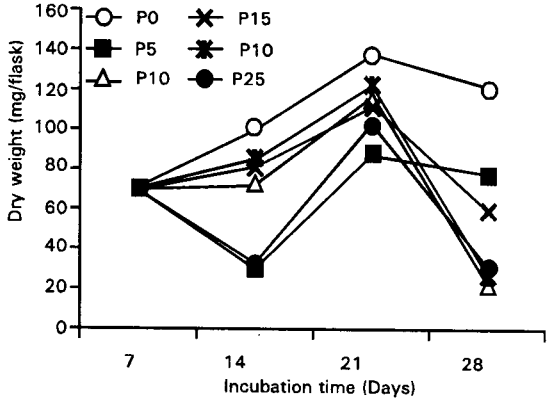

Fig. 2a: Growth of *J. chinensis* cell suspension cultures on addition of various phenylalanine (P) concentrations (0, 5, 10, 15, 25 and 50 ml l^{-1})

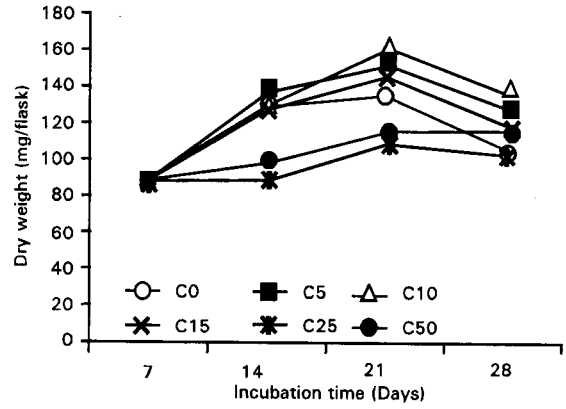

Fig. 2b: Growth of *J. chinensis* cell suspension cultures on addition of various coniferyl alcohol (C) concentrations (0, 5, 10, 15, 25 and 50 ml l^{-1})

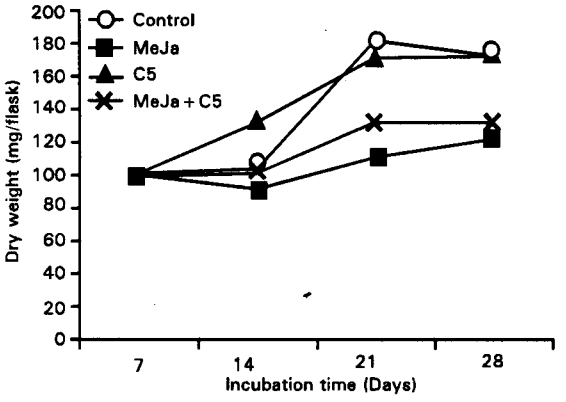

Fig. 2c: Growth of *J. chinensis* cell suspension cultures on addition of chitopentaose (C5, 5 mg l^{-1}) methyl jasmonate (Meja, 10 ml l^{-1} and concentrations of the two.

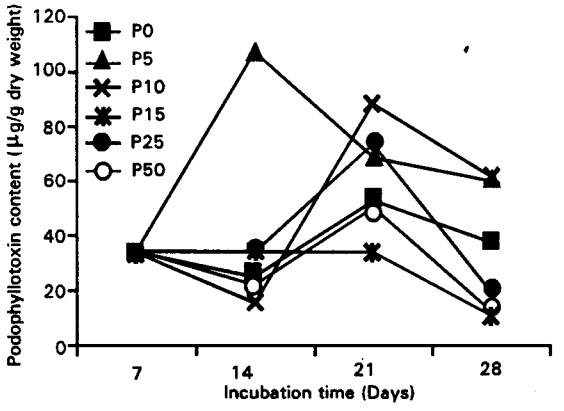

Fig. 3: Effect of phenylalanine on podophyllotoxin production in *J. chinensis* cell suspension cultures.
Phenylalanine 0 mg l^{-1} (PO), phenylalanine 5 mg l^{-1} (P5), phenylalanine 10 mg l^{-1} (P10),
Phenylalanine 15 mg l^{-1} (P15), phenylalanine 25 mg l^{-1} (P25), phenylalanine 50 mg l^{-1} (P50),

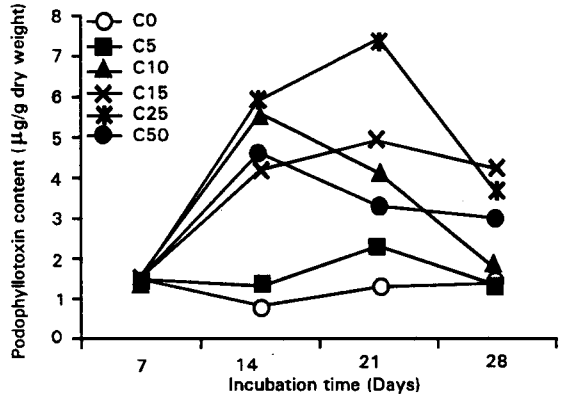

Fig. 4: Effect of coniferyl alcohol [e] on podophyllotoxin production in *J. chinensis* cell suspension cultures.
Coniferyl alcohol 0 mg L^{-1} (C0), coniferyl alcohol 5 mg L^{-1} (C5), coniferyl alcohol 10 mg L^{-1} (C10), coniferyl alcohol 15 mg L^{-1} (C15), coniferyl alcohol 25 mg L^{-1} (C25), coniferyl alcohol 50 mg L^{-1} (C50)

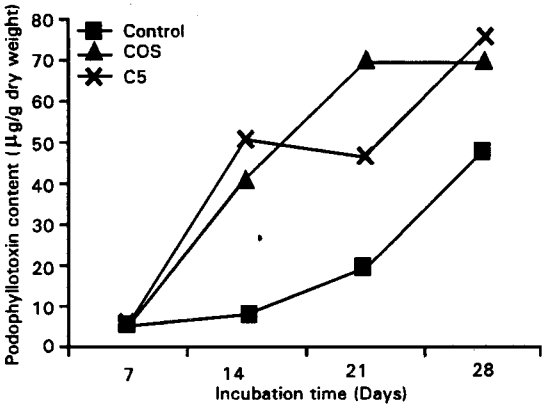

Fig. 5: Effect of chito-oligosaccharides (COS) and chitopentaose (C5) on podophyllotoxin in *J. chinensis* cell suspension cultures

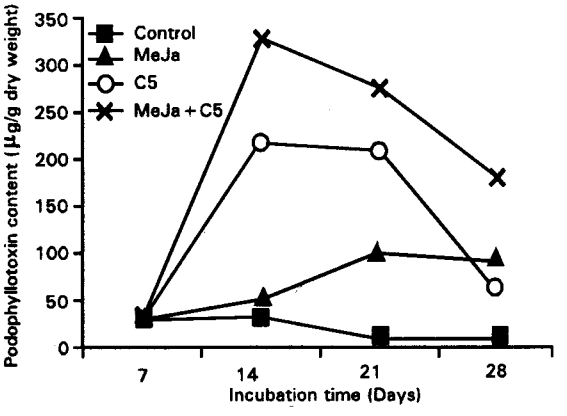

Fig. 6: Effect of methy jasmonate (MeJa), chitopentaose C5) and combination of the two on podophyllotoxin production in *J. chinensis* cell suspension cultures.

indicated that the accumulation of podophyllotoxin in *J. chinensis* cell suspension cultures was dependent on both the concentration of added compounds (precursors, elicitors) and cell dry mass and incubation period. The results obtained here showed that the *J. chinensis* cell suspension cultures were an alternative source of podophyllotoxin production. Addition of biogenetic precursors and elicitors into the culture media is a promise method for yield improvement since the compounds used were inexpensive. More effort should be paid to developing other biotechnological techniques to maintain cell suspension cultures using podophyllotoxin production in the long run. Optimization of the cell cultivation regime might lead to a renewable source of cytotoxic lignans for medical use. We are now conducting podophyllotoxin production by immobilized cell cultures of *J. chinensis*.

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