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Original Paper

Alkaloid production by somatic embryo cultures *of Corydalis ambigua*

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Abstract Somatic embryos of *Corydalis ambigua* (Papaveraceae), which were cultured in liquid Linsmaier and Skoog medium supplemented with 0.1 μM IAA and 3% sucrose, produced two tetrahydroprotoberberine alkaloids, corydaline (0.03% of dry cell weight) and cavidine (1.09%). Among various plant growth regulators tested, two phenylurea derivatives, thidiazuron and *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea enhanced the alkaloid production. Addition of 1 μM thidiazuron to the medium gave the maximum alkaloid content, 0.12% corydaline and 1.91% cavidine, after 21 days incubation. These results were compared with those from callus cultures and various intact organs. Corydaline and cavidine were accumulated in leaf, tuber and somatic embryos, whereas corybulbine was detected only in tubers. Callus cultures and immature seeds, which lack embryos, contained only trace amounts of these alkaloids, suggesting the necessity for organ differentiation for alkaloid production in *C. ambigua*.

Key words: Papaveraceae, tetrahydroprotoberberine alkaloid.

Corydalis ambigua Cham. et Schltdl. is a perennial herb of the family Papaveraceae and found in the northern areas of the Far East, including Japan. The plant has been used in traditional Chinese medicine or folk medicine as an analgesic, antirheumatic and emmenagogue (Hotta et al. 1989; Chou and Hsü 1992) and contains corybulbine, corydaline and cavidine (Fig. 1) as the major tetrahydroprotoberberine alkaloids in the tuber (Naruto and Kaneko 1973; Yasuda et al. 1988). These tertiary bases are converted to corresponding dehydrogenated quaternary bases, the protoberberine alkaloids (Figure 1), during the drying process of tubers (Yasuda et al. 1987). Dehydrocorydaline is thought to be one of the main effective constituents of the crude drug (Chou and Hsü 1992). Ito et al. (2001) proposed corydaline, dehydrocorydaline and corybulbine to be potential cancer chemopreventive agents. In addition, corydaline has been shown to have anti-thrombic action *in vitro* (Matsuda et al. 1988) and dehydrocorydaline is known for its anti-allergic activity (Matsuda et al. 1997a), anti-inflammatory activity (Kubo et al. 1994b; Matsuda et al. 1997b), inhibitory effects against aldose reductase (Kubo et al. 1994a) and insecticidal activity (Miyazawa et al. 1998). Both cavidine and dehydrocavidine exhibit the spasmolytic activity (Bhakuni and Chaturvedi 1983).

Tissue culture studies on *Corydalis* species have been carried out by several research groups having different interests such as micropropagation (Lee et al. 2001; Sagare et al. 2000; Zhao et al. 1988), alkaloid production (Ikuta et al. 1974; Zhang et al. 1991), biosynthesis (Rueffer et al. 1994) and biotransformation (Iwasa et al. 2003). We previously reported the micropropagation of *C. ambigua* through the direct embryogenesis of tuber sections and the chemical evaluation of regenerants (Hiraoka et al. 2001). A preliminary chemical analysis of the somatic embryos revealed considerable alkaloid accumulation in static cultures. Based on this finding, we attempted to produce alkaloids with *C. ambigua* somatic embryo cultures in liquid medium. In the present study, we investigated medium conditions favorable for alkaloid production, and compared the alkaloid productivity in somatic embryos with that in callus cultures and in various intact plant organs. To our knowledge, this is the first documented report on the production of alkaloids by tissue culture of *C. ambigua*.

Wild plants were collected at Bankei, Sapporo, Japan and maintained at the Herbal Garden of the University of

Abbreviations: BA, 6-benzyladenine; CPPU, *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; LS medium, Linsmaier and Skoog (1965) medium; NAA, α-naphthaleneacetic acid; TDZ, thidiazuron

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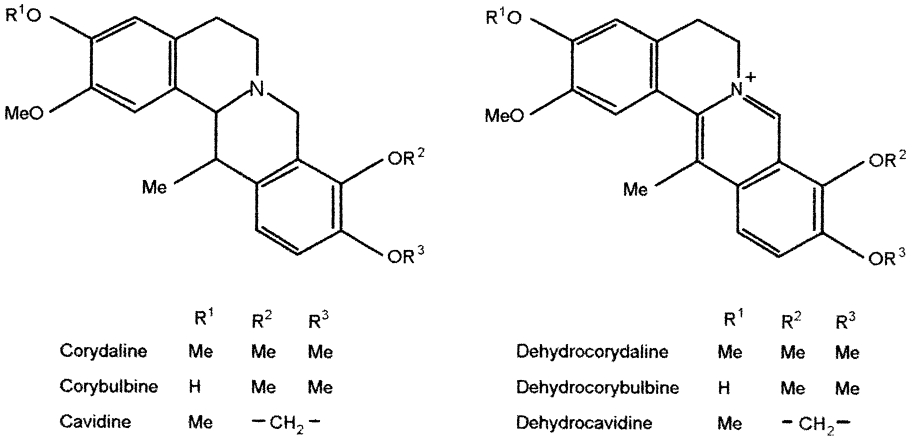

	R^1	R^2	R^3
Corydaline	Me	Me	Me
Corybulbine	H	Me	Me
Cavidine	Me	$-CH_2-$	

	R^1	R^2	R^3
Dehydrocorydaline	Me	Me	Me
Dehydrocorybulbine	H	Me	Me
Dehydrocavidine	Me	$-CH_2-$	

Figure 1. Chemical structure of alkaloids.

Pharmacy and Applied Life Sciences. Leaves and seeds for chemical analysis were harvested in April.

Somatic embryos of *C. ambigua* were developed from a tuber and maintained on half strength LS medium containing 2% sucrose, 0.1 μM IAA and 0.2% Gelrite (Hiraoka et al. 2001). Somatic embryo suspension cultures were established by transferring them into 100 ml of half strength LS liquid medium containing 0.1 μM IAA and 3% sucrose in a 500 ml Erlenmeyer flask. The medium was adjusted to pH 5.6 before autoclaving at 121°C for 20 min. All cultures were grown at 25°C in the dark on a reciprocal shaker (60 rpm, 70 mm stroke) and subcultured every 3 weeks. Among 10 strains established, Cor-17 was selected as the present experimental material based on its better proliferation potential and higher corydaline content. Somatic embryos (3 g) were inoculated in 25 ml of medium in a 100 ml Erlenmeyer flask capped with a silicon sponge plug. All culture experiments were conducted with 3 or 4 replicates and repeated twice.

Following experiments were carried out to optimize the culture medium for alkaloid production. In the first experiment, somatic embryos were cultured in LS medium with different strengths (full, 1/2, 1/4) for standardizing a suitable basal medium concentration. Subsequently embryos were incubated in full strength LS medium supplemented with different sucrose concentrations (1–3%) for the optimization. To determine a suitable culture duration for alkaloid production, somatic embryos were harvested every 3 days up to the culture period of 21 days, which were cultured in LS medium containing 3% sucrose and 0.1 μM IAA. This was used as a standard medium in later experiments. In subsequent experiments, plant growth regulators were added into the culture medium to determine the best combination and concentration for alkaloid production: IAA, IBA, NAA (0.01–1 μM); kinetin, BA, isopentenyladenine (0.01–10 μM); 24-epibrassinolide (10 μM–0.1 M) with or without IAA (0.1–1 μM); CPPU, TDZ (0.01–10 μM) with or without IAA (0.1–1 μM); 1-triacontanol (0.001–0.1 μM) with or without IAA (0.1–1 μM); tiron (0.1 μM–10 mM) with or without IAA (0.1–1 μM). CPPU dissolved in dimethylsufoxide was filter sterilized and added to the medium after autoclaving. Yeast extract (3.75 and 10.0 gl^{-1}) was added into a standard medium 10 days after initiation of the culture and harvested 1, 2, 3, 4, 5 and 21 days after addition of the yeast extract.

Callus tissues were induced from the somatic embryos by transferring them onto LS agar medium supplemented with 10 μM NAA and 10 μM BA, and were subcultured onto the same medium at monthly intervals. Suspension cultures were maintained in liquid medium with the same composition, except for agar, on a reciprocal shaker. After one month of incubation, callus tissues were harvested and used for chemical analysis.

Alkaloids were analyzed using a high-performance liquid chromatographic (HPLC) method previously reported (Hiraoka et al. 2001). The amount of corybulbine, corydaline and cavidine was calculated from calibration curves and expressed as a percentage of the dry weight of the plant material. Alkaloid yields were calculated using the total dry weight of culture multiplied by the percentage of the alkaloid content. The culture medium was extracted twice with ethyl acetate after adjustment to pH 9.0 with 1 M NaOH. The combined ethyl acetate extract was dried with anhydrous Na_2SO_4 and evaporated to dryness at 40°C under reduced pressure. The residue was dissolved in 2 ml of the internal standard solution under sonication. The supernatant of the centrifuged solution was subjected to HPLC analysis of alkaloids. The significance of the difference between the control and treated groups was assessed using the Student's *t*-test.

Since the altered medium strength and sucrose concentration caused no difference in growth and alkaloid production of the somatic embryos (data not shown), the original LS medium containing 3% sucrose

was used as the standard medium in subsequent experiments.

Figure 2 shows growth and alkaloid production of the somatic embryos after the culture period of 21 days in liquid medium. The fresh weight of somatic embryos increased gradually until the end of the incubation period, whereas the dry weight increased only for the first 9 days and remained constant thereafter. The corydaline content increased slightly throughout the culture period and its maximum content and yield reached 0.03% and 0.2 mg per culture, respectively, by day 18. The cavidine content and yield increased constantly until the end of the culture period, reaching 1.05% and 8.4 mg per culture, respectively. Typically, the amount of alkaloids detected in the culture medium was negligible.

Among plant growth regulators tested, only phenylurea derivatives significantly stimulated the growth and alkaloid production of somatic embryos. Two phenylurea derivatives (TDZ and CPPU) gave similar results as shown in Table 1. Both regulators increased the fresh weight of somatic embryos despite a decrease in their dry weight at high concentrations (1–10 μM). Addition of 1 μM TDZ to the standard medium recorded the highest corydaline (0.12%) and cavidine (1.91%) contents in the present study. Such high yields of corydaline (0.7 mg) and cavidine (10.9 mg) were also obtained at the same TDZ concentration (Table 1). The inclusion of BA at different concentrations in combination with 1 μM TDZ in the standard medium enhanced neither alkaloid content nor yield (data not shown). As for the effect of CPPU on alkaloid production, the highest content and yield of corydaline were achieved at 1 μM, whereas those of cavidine were the highest at 10 μM (Table 1). By the treatment with yeast extract, no stimulatory effect on alkaloid production was observed (data not shown).

A comparison of alkaloid contents among intact plant organs (leaf, seed and tuber) and *in vitro* cultures of different state (somatic embryos and calli) are presented in Table 2. Three major compounds, corybulbine, corydaline and cavidine, were present in the tuber as reported previously (Yasuda et al. 1988; Hiraoka et al. 2001). The tuber was the only organ that contained corybulbine at a detectable level in the present plant materials examined. The leaf contained a lower amount of corydaline than found in the tuber but as much cavidine. Seeds contained only a trace amount of corydaline and cavidine. Although corydaline and

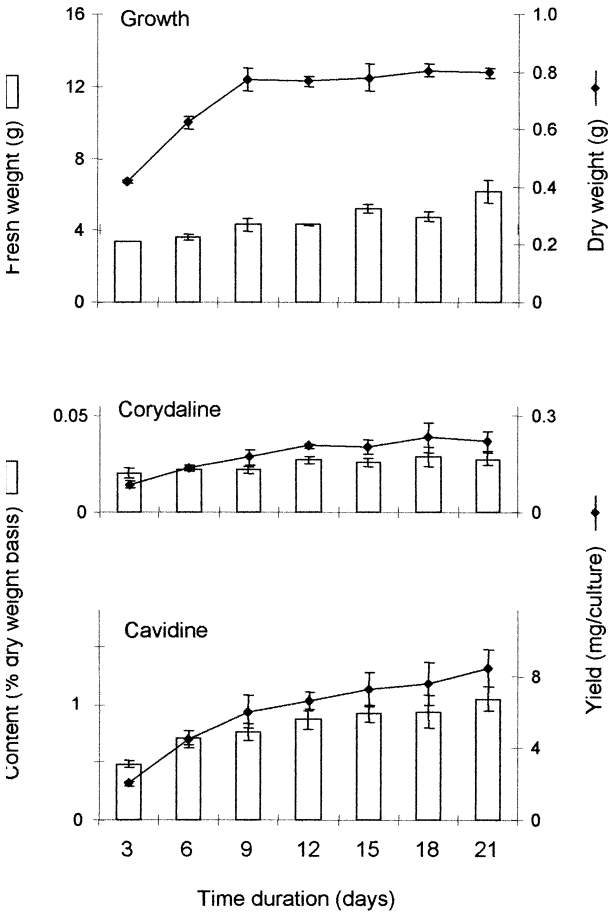

Figure 2. Time course of somatic embryo cultures of *Corydalis ambigua* in LS medium supplemented with 0.1 μM IAA.

Table 1. Effects of TDZ and CPPU on growth and alkaloid production in somatic embryo cultures of *Corydalis ambigua*.

Regulators (μM)		Growth (g)		Alkaloid content (%)		Alkaloid yield (mg/flask)	
		Fresh weight	Dry weight	Corydaline	Cavidine	Corydaline	Cavidine
TDZ	0	5.5±0.4	0.79±0.01	0.03±0.01	0.91±0.20	0.2±0.0	7.2±1.5
	0.01	6.8±0.5**	0.67±0.03**	0.07±0.01***	1.69±0.01***	0.5±0.0***	11.3±0.5*
	0.1	10.4±0.9***	0.59±0.02***	0.08±0.02**	1.60±0.28*	0.45±0.1*	9.4±1.3
	1	11.6±2.3***	0.57±0.04***	0.12±0.04**	1.91±0.22***	0.7±0.2*	10.9±1.5*
	10	11.8±3.3**	0.64±0.05*	0.06±0.01***	1.40±0.19*	0.4±0.01*	9.0±2.0
CPPU	0	6.5±2.0	0.75±0.04	0.03±0.01	0.68±0.06	0.2±0.0	5.1±0.3
	0.01	5.8±0.5	0.77±0.05	0.03±0.01	0.73±0.07	0.2±0.0	5.6±0.4
	0.1	7.5±1.1	0.63±0.08	0.06±0.01***	1.17±0.14**	0.3±0.1*	7.4±1.3*
	1	11.2±2.1*	0.60±0.09*	0.07±0.02*	1.08±0.10***	0.4±0.1***	6.4±0.4*
	10	12.6±4.3*	0.52±0.10*	0.05±0.04	0.70±0.43	0.3±0.2	3.6±2.6

Level of significance when compared to control: * $P<0.05$, ** $P<0.01$, *** $P<0.005$.

Table 2. Alkaloid contents in intact plant organs and *in vitro* cultures of *Corydalis ambigua*.

Plant materials	Alkaloid contents (% of dry weight basis)		
	Corybuline	Corydaline	Cavidine
Intact plant			
Leaf	nd	0.09±0.01	0.07±0.09
Seed	nd	tr	tr
Tuber	0.05±0.01	0.50±0.14	0.08±0.02
In vitro culture			
Somatic embryo (liquid medium)	nd	0.03±0.01	1.05±0.18
Somatic embryo (agar medium)	nd	0.01±0.00	0.23±0.02
Callus (liquid medium)	nd	tr	tr
Callus (agar medium)	nd	tr	tr

Somatic embryos were cultured in LS medium+0.1 μM IAA for 21 days and callus tissues in LS+10μM NAA+10μM BA for 1 month.

nd, not detected; tr, a trace amount.

cavidine accumulated in the somatic embryo cultures in both solid and liquid media, these alkaloids were hardly detectable in the callus cultures. The highest corydaline content was found in the tuber (0.50%), and lower content was also seen in the leaf (0.09%) and somatic embryos in liquid culture (0.03%). Cavidine content was the highest in somatic embryos in liquid culture (1.05%), followed by the tuber (0.08%) and the leaf (0.07%).

Most studies on somatic embryo culture have focused on rapid propagation or fundamental work examining the differentiation and development of a wide variety of plants. In rare cases, this technique has also been applied to the production of useful secondary metabolites such as furanochromone in *Ammi visnaga* (El-Fiky et al. 1989), saponin in *Panax ginseng* (Asaka et al. 1994), naphthoquinone in *Plumbago rosea* (Komaraiah et al. 2004) and others (Payne et al. 1991). Somatic embryos of *A. visnaga* produced 0.57% khellin and 0.72% visnagin on a dry weight basis and were comparable to those of mature fruits. In this study, the cavidine content of *C. ambigua somatic* embryos cultured in the standard medium (1.05%) was 10 times higher than that in the tuber of intact clonal plants of C-17 strain (0.08%), though the corydaline content was lower (Table 2). Static cultures of somatic embryos, with the same medium composition as suspension cultures but with 0.8% agar, produced 0.01% corydaline and 0.23% cavidine, indicating that suspension culture systems are more suitable for the alkaloid production than static systems. Somatic embryos derived from a different strain, Cor-13, had a similar alkaloid composition to Cor-17 (0.01% corydaline and 0.39% cavidine). Zhang et al. (1991) reported that *C. yanhusuo* callus tissues, which possess the ability to redifferentiate tubers and roots, accumulated 0.098% corydaline, 0.124% teterahydropalmatine and 0.834% total alkaloids, which were comparable to the alkaloid contents in the intact tuber.

Numerous research reports exist on the effects of plant growth regulators or elicitors on secondary metabolism of *in vitro* cultures, and in certain cases they enhance the isoquinoline alkaloid production in several plant species, for example, berberine in *Coptis japonica* (Nakagawa et al. 1986; Ikuta and Itokawa 1989), sanguinarine in *Eschscholtzia californica* (Collinge and Brodelius 1989) and *Papaver somniferum* (Tyler et al. 1989). However, most regulators used in this study showed no significant effects on alkaloid accumulation. This is not uncommon in plant tissue culture since plant growth regulators act differently for each plant species. The phenylurea derivatives, synthetic cytokinin analogues, are active in promoting callus growth and organogenesis of plants (Okamoto et al. 1978). They also influence secondary metabolism, for example, Yamamoto et al. (1998) found that among the *Hydrangea callus* tissues subcultured in media supplied with various plant growth regulators, dihydroisocoumarins accumulated only in medium containing a combination of NAA and CPPU. Also, only the phenylureas, TDZ (0.01–10 μM) and CPPU (0.1–1 μM), significantly enhanced alkaloid accumulation in somatic embryos in our study (Table 1). The fresh weight of somatic embryos increased in the medium supplemented with TDZ or CPPU, but the dry weight reduced. This is because embryos started to germinate due to their cytokinin activity, leading to the swelling by the absorption of water.

Despite several studies examining the chemical analysis of *C. ambigua* tubers, no reports to date are available on the alkaloid production in other organs and *in vitro* culture systems. The comparative study on the

alkaloid chemistry of various tissues and organs indicated that corybulbine specifically accumulated in intact tubers and that somatic embryos and leaves contained corydaline and cavidine (Table 2). Somatic embryos also produced a few other unidentified alkaloids other than the above two alkaloids. Despite the high corydaline and cavidine contents in somatic embryos, seeds contained a trace amount of these alkaloids. The reason for this discrepancy is due to the absence of embryos in the seeds collected in April, at which time the plants set seeds. The embryo of this specific species develops gradually from June until January or February of the next year in Hokkaido, as observed by Yasuda et al. (1985). The lack of these alkaloids in undifferentiated callus tissues and immature seeds suggest that alkaloid biosynthesis of *C. ambigua* requires, to some extent, tissue or organ differentiation. The increase in alkaloid accumulation associated with the tuber differentiation was demonstrated also with *C. yanhusuo* callus cultures (Zhao et al. 1988).

In conclusion of this study, we have demonstrated that somatic embryos of *C. ambigua* can produce a considerable amount of corydaline and cavidine under the standard culture condition. The addition of an optimum dosage of TDZ or CPPU enhances the alkaloid production. Considering such a high content (maximum 1.91%) of cavidine in somatic embryos and various important biological activities of corydaline or dehydrocorydaline, the latter two compounds might be suitable, if desired, for further pharmacological exploitation through the production of cavidine using the present or improved culture system followed by its chemical conversion to corydaline (Taguchi and Imaseki 1964).

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