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Isoquinoline alkaloid production by transformed cultures of *Papaver somniferum*

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Three clones of transformed cultures of opium poppy (*Papaver somniferum* L.) were established by infection with *Agrobacterium rhizogenes* MAFF 03-01724. MAFF clone 1 being capable of forming somatic embryos was selected and its growth and isoquinoline alkaloid production was investigated. The illumination, temperature and nutrient medium composition greatly affected growth, cell morphology and alkaloid accumulation. The MAFF clone 1 cultured in Root Culture medium in the dark at 22℃ accumulated a high quantity of sanguinarine (652 μg/g dry weight) though the growth was poor (4.4 fold as fresh weight basis after 2 months of culture). The MAFF clone 1 cultured in a quarter macro salt strength Woody Plant medium under 14 h/day light at 22℃ developed into plantlets and accumulated significant quantity of codeine (648 μg /g dry wt) together with papaverine, noscapine, and sanguinarine. This clone was applied to a rotating drum fermenter (2 L working volume), and *ca.* 0.3 mg codeine and 0.06 mg sanguinarine were obtained after 4 weeks of culture. One quarter of the codeine produced was found in the culture medium.

Key Words: *Papaver somniferum*, *Agrobacterium rhizogenes*, transformation, somatic embryo, isoquinoline alkaloid

Introduction

Opium poppy (*Papaver somniferum* L., Papaveraceae) is one of the most well known medicinal plants since ancient times. It is a plant which causes narcosis; however, its alkaloids, such as morphine, codeine, papaverine and noscapine are therapeutically valuable[1]. Many researches have so far been conducted to produce these alkaloids *in vitro*, and most cultured cells including transformed ones readily produce sanguinarine and its analogues[2-6]. There are some examples of production of morphinan alkaloids in differentiated cells such as shoots, plantlets and somatic embryos[2-5].

We have previously reported the induction of transformed cultures of *P. somniferum* by infection with *A. rhizogenes* MAFF 03-01724[7,8]. In that study, we found that one of the three transformed clones capable of forming somatic embryo contained considerable quantities of morphinan alkaloids as indicated by enzyme-linked immunosorbent assay (ELISA). The present study describes isoquinoline alkaloid production by transformed cultures under different cultural conditions.

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Materials and methods

Plant material

Transformed and non-transformed cultures of *P. somniferum* L. var. *Ikkanshu* were established as described previously[7,8]. They were maintained on phytohormone-free (HF) Murashige and Skoog (MS)[9] solid medium at 22℃ in the dark with subculturing at 4 weeks intervals.

General culture conditions

All the media used for the experiments were HF liquid medium supplemented with 30 g/L sucrose and adjusted to pH 5.7 before autoclaving at 121℃ for 15 min. The MAFF clone 1 grown on the MS solid medium was used as experimental materials except Table 4. In the case of Table 4, each DT grown in the Table 3 experiment was subcultured in the same liquid medium. Other culture conditions employed are indicated in the tables.

Extraction and high pressure liquid chromatography (HPLC) analysis for isoquinoline alkaloids

Alkaloid extraction and HPLC analysis were carried out as described previously[7,8]. When a rotating drum fermenter (RDF) was used, 50 mL of acetic acid was added to *ca.* one L liquid medium (pH 2 - 3) and solution was extracted twice with 100 ml of chloroform. The aqueous layer was made alkaline (pH 8 - 9) with 28% ammonium hydroxide and then extracted three times with 100 mL of a mixture of chloroform and isopropanol (3:1). A

combined extract was evaporated *in vacuo* and residue was dissolved in an appropriate volume of methanol for HPLC analysis. The data from these extracts except RDF are shown as the mean of two replicates.

Results and discussion

Three transformed clones (MAFF clone 1, 2 and 3) were established on MS solid medium by infection with *A. rhizogenes* MAFF 03-01724[7,8]. Alkaloids in these clones grown on the MS solid medium in the dark at 22℃ were analyzed by HPLC (Table 1). Codeine and sanguinarine were detected in MAFF clone 1, which appeared to be most embryogenic and contained the highest level of morphinan alkaloids by ELISA (1.76 μg morphine equivalents/g fresh weight)[7,8].

Table 1. Morphinan alkaloid in the calli of *Papaver somniferum* cultured on MS solid medium at 22℃ in the dark for 4 weeks

Cultures	Embryogenesis	HPLC (μg/g dry weight)	
		Codeine	Sanguinarine
non-transformant	+++	nd*	nd
MAFF clone 1	+++	trace	29
MAFF clone 2	-	nd	nd
MAFF clone 3	+	nd	nd

+++: vigorous, ++: moderate, +: poor, - : none

*Not detected

As far as we know, there is another example of transformation of *P. somniferum* with *A. rhizogenes* by Williams and Ellis[6], who reported that transformed tissue led to the production of disorganized cell cultures rather than hairy root cultures. This tendency may be attributed less to the insertion and/or position effects of the T-DNA of Ri plasmid than to intrinsic properties of *P. somniferum*, such as peculiar response to the endo/exo-genous phytohormones. In our study, transformed cells tended to form embryogenic cells though two of the three clones lost their embryogenic capability after the long repeated subculture time. Even the non-transformed cultures, any parts of axenic *P. somniferum* plants could not generate ordinary root cultures by the addition of auxins but embryogenic cells instead[7,8].

MAFF clone 1 was selected for further experiments because it has maintained stable embryogenic capability for years and accumulated alkaloids.

Influences of temperature and basal medium in the dark

MAFF clone 1 grown on MS solid medium was transferred into a half macro salt strength MS (1/2 MS), MS, Gamborg B5 (B5)[10], Woody Plant (WP)[11] and Root Culture (RC)[12] liquid media and cultured for 2 months at either 22℃ or 25℃ in the dark (Table 2). Although the mixture of undifferentiated cells (UC) and differenciated tisssues (shoots and embryos) (DT) were used as inoculum, UC predominantly proliferated in the liquid medium. The cells grew well in all liquid media except RC medium, however, DT production was restricted to B5 and WP media at 22℃. In this experiment, sanguinarine could be detected by HPLC with showing highest levels in the cells grown in the RC medium.

Table 2. Influences of temperature and basal media on the growth and alkaloid contents in MAFF clone 1

Temperature	Medium	Morphology	Growth index*	Sanguinarine (μg/g dry weight)
22 ℃	1/2 MS	UC[b]	14.4	11
	MS	UC	8.5	5
	B5	UC and DT[c]	12.3	2
	WP	UC and DT	13.2	136
	RC	UC	4.4	652
25 ℃	1/2 MS	UC	14.3	25
	MS	UC	13.1	nd[d]
	B5	UC	15.1	14
	WP	UC	7.6	3
	RC	UC	4.6	503

Mixture of UC and DT was cultured in liquid medium (20 mL/100 mL flask) in the dark on a rotary shaker (30 rpm) for 2 months.

*Final fresh weight / initial fresh weight (50-100 mg)

[b]Undifferentiated cells (UC)

[c]Differentiated tissues (DT)

[d]Not detected

Effects of WP macro salts concentration

Since both UC and DT proliferated well and contained a considerable amount of sanguinarine when grown in WP liquid medium at 22℃, the effect of WP macro salts concentration on growth and alkaloid production was investigated by using either UC or DT as inoculum. Both of the growth and alkaloid concentration of the cultures under 14 h/day light were superior to those in the dark, though their growth pattern and morphology were similar. When UC were inoculated and cultured in liquid medium, only UC was formed and no DT formation was observed (Fig. 1A). The UC growth in full, double, and three-times strength macro salts WP (WP, 2WP and 3 WP) media were better than those in a quarter and a half strength macro salts WP (1/4 WP and 1/2 WP) media, but alkaloids were negligible in all the UC cultures (data not shown). Both UC and DT proliferated in the liquid medium when DT were used as inocula (Table 3). The UC to DT ratio increased along with the WP macro salts concentration (Fig. 1B). DT growth without UC proliferation was observed when the DT was cultured in the 1/4 WP (in the light and dark) and the 1/2 WP

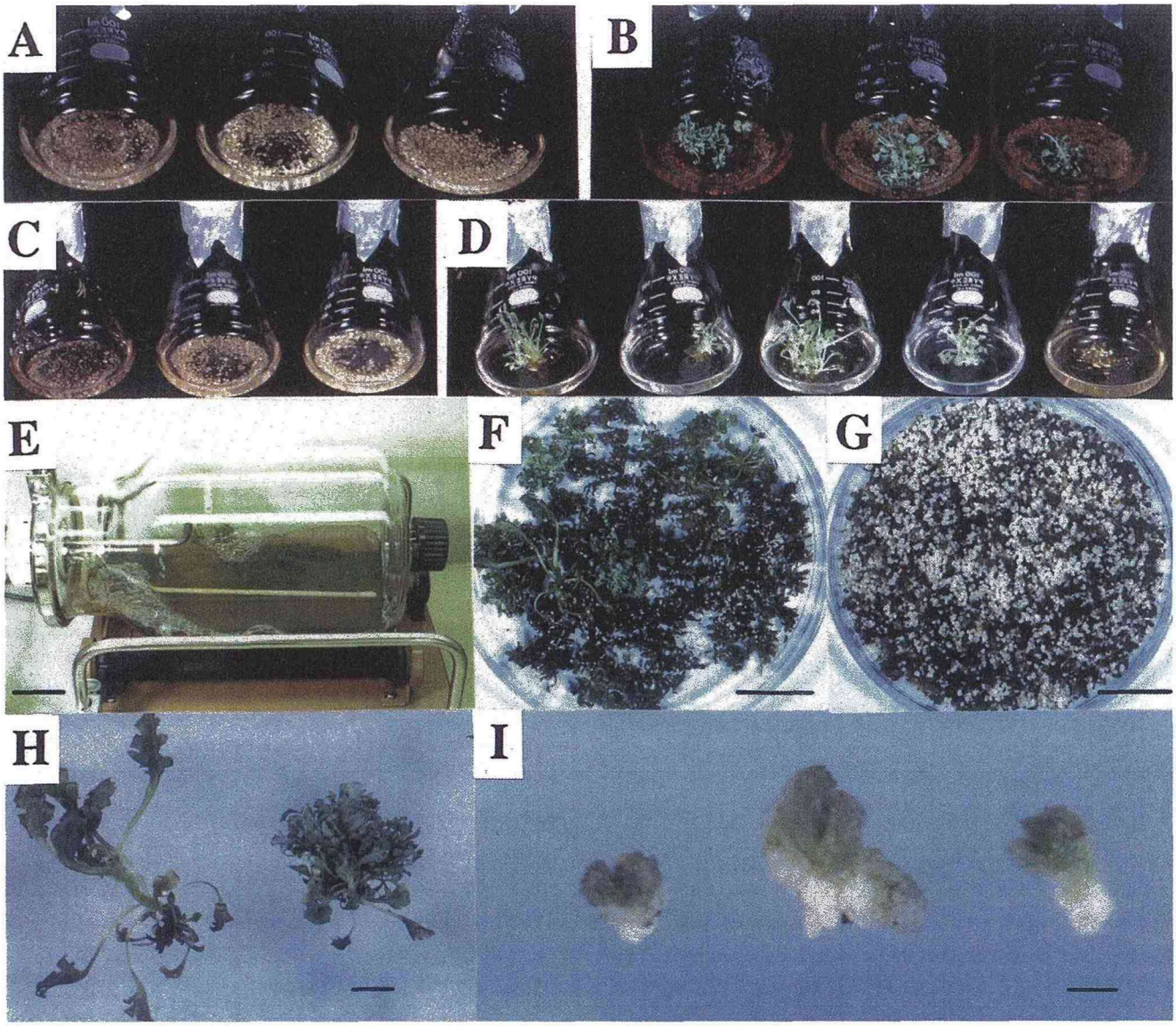

Fig. 1. *Papaver somniferum* cultures

A, MAFF clone 1 (undifferentiated cells, UC) cultured in WP, 2WP and 3WP (left to right) liquid medium (22℃, 14 h light, 1 month); B, MAFF clone 1 (differentitated tissues, DT) cultured in WP, 2WP and 3WP (left to right) liquid medium (22℃, 14 h light, 1 month)(see table 3); C, MAFF clone 1 (UC) subcultured in WP, 2WP and 3WP (left to right) liquid medium (22℃, 14 h light, 1 month); D, MAFF clone 1 (DT) subcultured in 1/4 WP, 1/2 WP, WP, 2WP and 3WP (left to right) liquid medium (22℃, 14 h light, 1 month)(see table 4). E, MAFF clone 1 culturing in a rotating drum fermenter (RDF); F, MAFF clone 1 (DT) proliferated in a RDF; G, MAFF clone 1 (UC) produced in a RDF; H, MAFF clone 1 shoots grwon in a RDF; I, MAFF clone 1 embryos produced in a RDF (see Table 5). Bars represent 5 cm (E), 3 cm (F and G), 1 cm (H) and 1 mm (I), respectively.

(in the light) medium and the highest concentration of alkaloids was detected in the DT grown in 1/4 WP medium under 14 hr/day light (Table 3).

Then the cultures in the light were subcultured under the same conditions. When UC were subcultured, no alkaloid was detected in any of the UC cultures, though almost the same UC proliferation was observed (Fig. 1A and C). When DT were subcultured, the best plantlet formation (rooting and further development of the shoots, Fig. 1D) was observed and the highest concentration of codeine (648 μg /g dry weight) together with other alkaloids (papaverine, noscapine, and sanguinarine) was detected in the 1/4 WP medium though the DT did not proliferate (growth index: 12.0, Table 4). In the first passage (Table 3), high sanguinarine concentration (300 μg /g dry weigt) was obtained in 1/4 WP medium, however, it decreased drastically in the second passage (9 μg /g dry weight). As shown in Table 2, much more sanguinarine was produced when the MAFF clone 1 was cultured in more starved cultural condition (in the RC medium, much lower inor-

Table 3. Effect of WP macro salts concentration on the gorwth and alkaloid production on MAFF clone 1 using differentiated tissues (DT) as inoculum (first passage)

Macro salts conc.	Light condition	Morphology	Growth index[a]	Alkaloid μg/g dry weight	
				Codeine	Sanguinarine
1/4	dark	DT[b]	11.7 ± 0.2	11 ± 9	110 ± 115
	light	DT	15.9 ± 1.1	67 ± 0	300 ± 32
1/2	dark	DT and UC[c]	6.0 ± 1.4	nd[d]	9 ± 0
	light	DT	30.3 ± 1.9	27 ± 0	60 ± 33
1	dark	DT and UC	12.5 ± 2.1	nd	18 ± 15
	light	DT and UC	36.8 ± 1.6	29 ± 30	11 ± 4
2	dark	DT and UC	39.6 ± 15.9	6 ± 1	7 ± 7
	light	DT and UC	45.7 ± 10.1	26 ± 0	2 ± 1
3	dark	DT and UC	25.9 ± 1.1	nd	8 ± 7
	light	DT and UC	53.3 ± 0.1	5 ± 0	1 ± 1

DT was cultured in liquid medium (20 mL/100 mL flask) at 22 °C in the dark or under 14 hr/day light (80μ $Em^{-2}S^{-1}$) on a rotary shaker (50 rpm) for 1 month.

Data were shown as mean ± standard deviation.

[a]Final fresh weight / initial fresh weight (50-100 mg)

[b]Undifferentiated cells (UC)

[c]Differentiated tissues (DT)

[d]Not detected

ganic salts concentration than MS). Therefore, high concentration of sanguinarine in 1/4 WP medium at the first passage might be attributed to the drastic change of culture medium (high to low concentration of inorganic salts). At the second passage, as the cultures were adapted in their culture conditions, effects of culture media on the growth and alkaloid production might be expected. Moderate shoot development and rooting were observed in 1/2 WP medium (Fig. 1D) with showing the highest concentrations of noscapine and sanguinarine among the cultures. On the other hand, both shoot development and proliferation were completely suppressed in 3 WP medium (Fig. 1D), resulting in a low concentration of alkaloids (Table 4).

In WP medium, the best DT proliferation (growth index: 24.5 in Table 4) and shoot development (Fig. 1D) were observed and the third highest concentration of codeine (158 μg /g dry weight) was obtained. Papaverine and noscapine were detected only in plantlets (shoots with root system). These results imply that alkaloid concentration and composition in MAFF clone 1 are greatly influenced by their morphology and the degree of plant development. For the accumulation of alkaloids, to some extent, establishment of the root system appears to be important. Morphine was not detectable level in any of the cultures as reported previously[7,8].

Table 4. Effect of WP macro salts concentration on the gorwth and alkaloid production on MAFF clone 1 using differentiated tissues (DT) as inoculum cultured under 14 h/day light (second passage)

Macro salts conc.	Shoot development	Rooting	Growth index[a]	Alkaloid μg/g dry weight			
				Codeine	Papaverine	Noscapine	Sanguinarine
1/4	+++	+++	12.0 ± 7.0	648 ± 289	29 ± 21	48 ± 16	9 ± 0
1/2	++	++	6.4 ± 0.0	60 ± 52	nd[b]	59 ± 0	15 ± 19
1	+++	+	24.5 ± 14.2	158 ± 11	11 ± 0	3 ± 1	1 ± 0
2	+++	+	11.4 ± 3.3	179 ± 117	8 ± 2	10 ± 5	nd
3	+	-	7.6 ± 5.2	6 ± 3	nd	nd	1 ± 0

DT was subcultured in the same liquid medium (20 mL/100 mL flask) at 22 °C under 14 hr/day light (80μ $Em^{-2}S^{-1}$) on a rotary shaker (50 rpm) for 1 month.

Data were shown as mean ± standard deviation.

+++: vigorous, ++: moderate, +: poor, - : none

[a]Final fresh weight / initial fresh weight (50-100 mg)

[b]Not detected

Table 5. Growth and alkaloid production of MAFF clone 1 cultured in a RDF

Parts	fresh weight(g)	dry weight(g)	Alkaloid μg/g dry weight		Alkaloid μg	
			Codeine	Sanguinarine	Codeine	Sanguinarine
DT[a]	24.9	2.40	81	3	194.4	7.2
UC[b]	27.0	3.65	15	4	54.8	14.6
medium					79.3	36.8
Total	51.9	6.05			328.5	58.6

DT was cultured in WP liquid medium (2.1 L, rotated at 3 rpm) at 23 °C under 14 hr/day light

(80μ $Em^{-2}S^{-1}$) at 0.2 vvm (vol. of air, vol. of medium per min) for 4 weeks.

[a]Differentiated tissues (DT)

[b]Undifferentiated cells (UC)

Culture of differentiated tissues (DT) in a rotating drum fermenter (RDF)

In the preliminary experiment for MAFF clone 1 in liquid culture using a 100 ml flask, the ratio of UC to DT tended to increase along with the increase of rotation rates. In the case of *Cephaelis ipecacuanha*, the frequency of adventitious shoot formation on internodal segments was 83.7% when cultured in a RDF, whereas it below 30 % when cultured in an air lift type fermenter[13]. The inhibition of shoot formation might be attributed to the hydrodynamic stress produced by aeration and/or rotation. Therefore a RDF (Fig. 1E) was chosen for *P. somniferum* cultures because the production of DT seemed to be associated to the codeine formation. Since moderate growth and alkaloid accumulation and proliferation of DT were observed in WP medium, DT were cultured using WP liquid medium (Table 5). Both DT and UC proliferated (Fig. 1F-I) as observed in the liquid culture in a 100 ml flask. After 4 weeks of culture, 24.9g fresh weight of DT and 27.0g fresh weight of UC were obtained (initial fresh weight of DT: 3.8 g). Codeine and sanguinarine were detected in these cultures and

the culture medium with showing the better codeine productivity. After 4 weeks of culture, *ca.* 0.3 mg codeine and 0.06 mg sanguinarine were obtained.

Conclusion

It was reported that the transformed cultures established by Williams and Ellis accumulated sanguinarine and its analogues in high quantities but almost no morphinans[6]. In our transformed cultures, only one (MAFF clone 1) of the three clones maintained stable embryogenic capability for 8 years and accumulated morphinan alkaloids. Therefore the lack of accumulation of morphinans in their cultures may be due to the genetic variation among the clones which were inevitably induced by Ri plasmid.

In the present study, nutrient medium composition, temperature and illumination crucially affected cell morphology and alkaloid composition and accumulation. Lower temperature (22℃), lower inorganic salts concentrations (a quarter to full strength macro salts WP) and illumination were required for the stable production of DT and alkaloids. Higher inorganic salts concentrations of WP promoted the formation and propagation of UC that caused diminishing alkaloids, especially codeine.

We demonstrated the production of codeine by transformed cultures of *P. somniferum* using a RDF. The yields of alkaloids were not economically feasible at present. However, the data suggest possibilities for the continuous production of codeine because one quarter of it was detected in the culture medium. In addition, productivity of alkaloids might be improved by further optimization including the selection of appropriate transformed clones and culture apparatus and conditions.

There are two possible biosynthetic pathways for the conversion of thebaine to morphine[14]. The one, which is well established, is described in the literature[4,5], *i.e.* morphine biosynthesis from thebaine via codeinone and codeine. Another pathway, first demonstrated by Brochmann-Hanssen in 1984[15], occurs via oripavine and morphinone. In the present study, the transformed clone could synthesize codeine but lacked morphine, though the non-transformed clone obtained from the same plant material accumulated morphine at the latter developmental stage[7,8]. Therefore genetic transformation of *P. somniferum* by *Agrobacterium* may offer the biochemical variations which enables us to unravel the complicated terminal steps in the biosynthesis of morphine.

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