In Vitro Cell. Dev. Biol.—Plant 37:268–273, March–April 2001
DOI: 10.1079/IVP2000146

1054-5476/01 $10.00+0.00

THE EFFECTS OF GLUTAMINE ON THE MAINTENANCE OF EMBRYOGENIC CULTURES OF *CRYPTOMERIA JAPONICA*

SHINJIRO OGITA[1]*, HAMAKO SASAMOTO[1], EDWARD C. YEUNG[2], AND TREVOR A. THORPE[2]†

[1]*Cell Manipulation Laboratory, Bio-Resources Technology Division, Forestry and Forest Products Research Institute, Ibaraki, 305-8687 Japan*
[2]*Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4*

(Received 14 August 2000; accepted 31 October 2000; editor D. W. Altman)

Summary

Embryogenic tissues of sugi (*Cryptomeria japonica*) were induced on a modified Campbell and Durzan (CD) medium containing 1 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) and 600 mg l^{-1} glutamine, and subcultured in the medium of the same composition for over 1 yr. This resulted in a mixed culture of embryogenic and non-embryogenic cells. When embryogenic cells were isolated and cultured independently, their capacity to form embryogenic aggregates was lost. Thus, the non-embryogenic cells present within a mixed culture system were essential to the formation of embryogenic aggregates. When embryogenic tissues were isolated and cultured independently on a high glutamine-containing (2400 mg l^{-1}) medium, dry weights and endogenous levels of glutamine increased, and the tissue could generate a large number of embryogenic aggregates. Amino acid analysis of embryogenic and non-embryogenic cells from the maintenance culture indicated a higher level of glutamine was present in the latter. The high endogenous level of glutamine in the non-embryogenic portion of mixed cell masses may be the supplier of glutamine for maintaining the embryogenic property of the tissues.

Key words: amino acid; *Cryptomeria japonica*; embryogenic tissue; glutamine, HPLC; somatic embryogenesis.

Introduction

The quality of embryogenic tissues from conifer species can be judged by the presence of well-organized embryonal heads and elongated suspensors (Ogita et al., 1997, 1999a; Egertsdotter and von Arnold, 1998). Upon prolonged culture, it is generally known that the quality of embryogenic tissues of conifers can deteriorate and lose their embryo-forming ability, e.g. *Pinus banksiana* (Park et al., 1999). In order to maintain such a culture, usually subculture at short intervals into fresh medium, improvement of medium composition, and cryopreservation are needed (Dunstan et al., 1995).

Embryogenic cell lines of sugi (*Cryptomeria japonica*) were induced on a modified CD medium (Campbell and Durzan, 1975) and subcultured in medium of the same composition at 2–3-wk intervals for over 1 yr (Ogita et al., 1999a). At that time, the culture consisted of a mixture of yellow to yellow-green non-embryogenic cells and the white embryogenic cell masses. Non-embryogenic cells were comprised of vacuolated spherical or elliptic cells of similar sizes containing chloroplasts, while the embryogenic cell aggregates were characterized by the presence of well-organized embryonal heads and elongated suspensors. When we tried to optimize the embryogenic cell line by isolating and subculturing just the embryogenic portion of the culture, the opposite effect was noted, i.e. the embryogenic tissue deteriorated rapidly and lost its embryogenic potential (Ogita and Sasamoto, unpublished).

Glutamine is a common organic nitrogen source used in higher plant tissue culture media (Franklin and Dixon, 1994). In conifer somatic embryogenesis, glutamine has been shown to be an effective compound which assists in the induction, maintenance, and maturation of somatic embryos (Guevin and Kirby, 1996; Barrett et al., 1997). Furthermore, glutamine also plays an important role in conifer zygotic embryo development (Feirer, 1995). The purpose of this study was to investigate whether the addition of glutamine could maintain and/or improve the quality of embryogenic tissues of sugi after a long subculturing period. The possible interactions between non-embryogenic and embryogenic cells were also examined.

Materials and Methods

Experimental materials. An embryogenic cell line of *Cryptomeria japonica* D. Don, which originated from a single individual zygotic embryo collected in 1997, was induced and maintained as described by Ogita et al. (1999a). The maintenance medium was the modified Campbell and Durzan medium (CD; Campbell and Durzan, 1975), containing 1 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D), 600 mg l^{-1} glutamine (Gln), 30 g l^{-1} sucrose, and 0.2 g l^{-1} gellan gum at pH 5.6. Five portions of the embryogenic tissues were put on the medium in a 9-cm Petri dish and were subcultured at 2–3-wk intervals. The cultures were maintained at 25°C, with a 16-h photoperiod under fluorescent illumination at a photon flux density of 60 μE m^{-2} s^{-1}.

Over 1 yr of subculture, the maintenance cell line became a

*Present address: Research Association for Biotechnology, Technology Department, 8916-5, Takayama, Ikoma, Nara, 630-0101, Japan.

†Author to whom correspondence should be addressed: Email tthorpe@ucalgary.ca

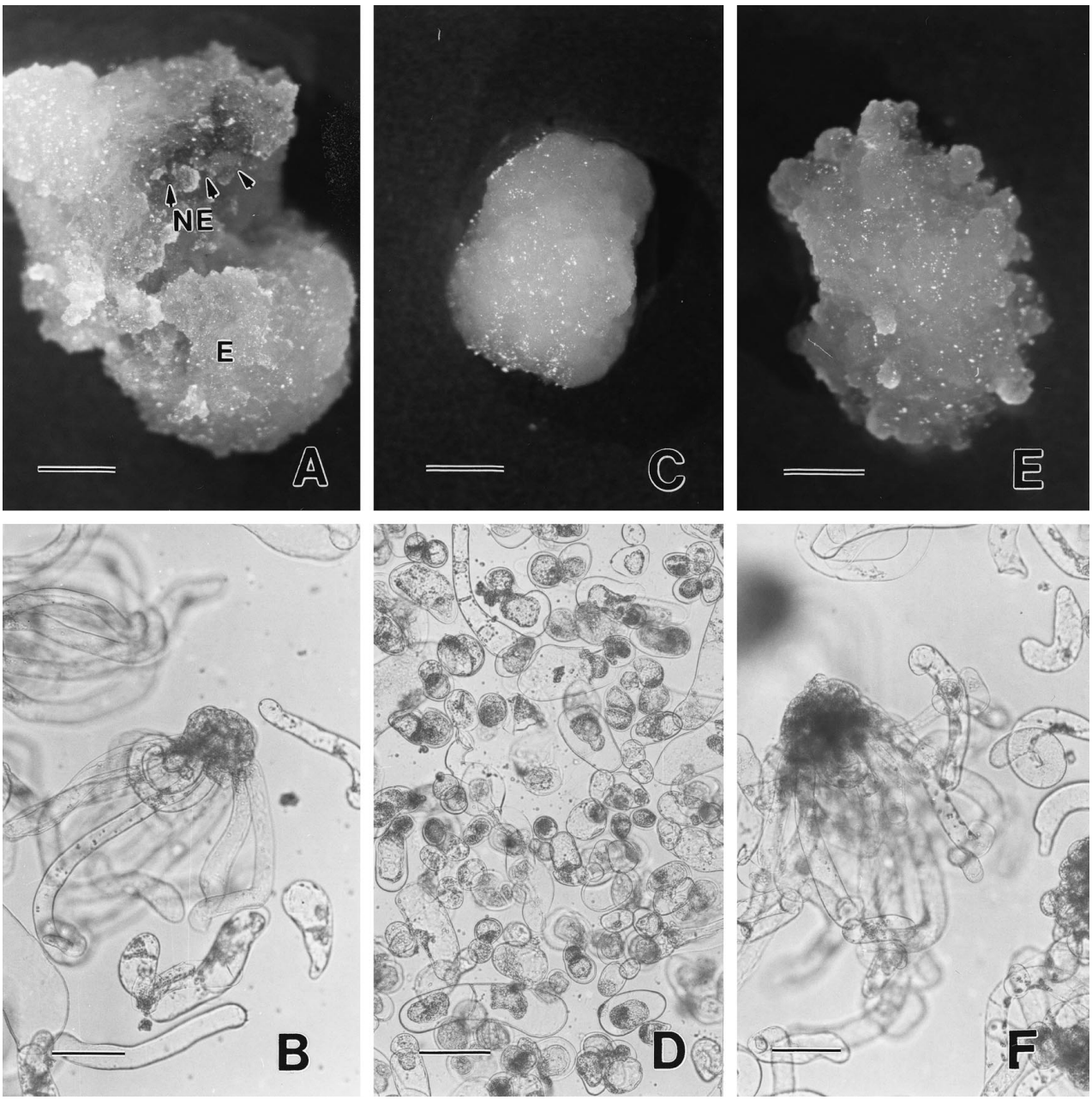


FIG. 1. Morphology of embryogenic and non-embryogenic cell masses of *C. japonica*. A, A mixed-culture showing the embryogenic (E) and non-embryogenic portion (NE) of the cell mass cultured on the mCD(600) medium. B, Embryogenic aggregates with well-defined embryonal head and suspensor can be found in the embryogenic portion of the cell mass (Control-E). C, D Independent embryogenic tissues cultured on the mCD(600) medium (IET-1). D, Cells tend to become spherical and separate from one another under the IET-1 culture condition. E, F, Independent embryogenic tissues cultured on the mCD(2400) medium (IET-2). F, Embryogenic aggregate with well-defined embryonal head and suspensor can be found when cultured under the IET-2 condition. A, C, E, *bar* = 2 mm. B, D, F, *bar* = 100 μm.

heterogeneous mixture of cell masses that had both embryogenic portions and non-embryogenic portions. The mixed cell masses were maintained on the same conditions for another year of subculture (more than 2 yr, from 1997 to the present). These cell masses were used as experimental materials.

Glutamine treatment and culture conditions. Three modified CD media with 600 (mCD600), 1200 (mCD1200), and 2400 (mCD2400) mg l^{-1} of Gln were used for investigating the effect of glutamine on maintenance subcultures. Other nutrients in these three media remained the same as described above. The mixed cell masses which consisted of both embryogenic and non-embryogenic cell masses, or an independent embryogenic tissue which was isolated from the mixed cell masses, were cultured on these three media.

Determination of the quality of the cultured cell masses. The cultured cell masses were collected at days 0, 10, 20, and 30. Morphological characteristics of each of the cell masses were examined using a stereomicroscope and/or a fluorescence microscope under UV-excitation,

TABLE 1

QUALITY OF EMBRYOGENIC AND NON-EMBRYOGENIC CELL MASSES OF SUGI (*C. JAPONICA*)

Experimental conditions	Time in culture (d)	DW (mg DW g FW^{-1})	No. of embryogenic cells (aggregates g FW^{-1})
Control-E: the embryogenic portion of the mixture cell masses cultured on the mCD(600) medium	0	48.6[a] (2.8)	4447[a] (369)
	10	42.3[a] (2.7)	n.d.
	20	44.9[a] (1.4)	n.d.
	30	43.9[a] (2.7)	4121[a] (496)
Control-NE: the non-embryogenic portion of the mixture cell masses cultured on the mCD(600) medium	0	64.8[bc] (4.0)	0[b]
	10	53.8[ab] (3.2)	n.d.
	20	54.6[ab] (5.4)	n.d.
	30	55.6[ab] (2.5)	0[b]
IET-1: the independent embryogenic tissues cultured on the mCD(600) medium	10	43.2[a] (1.5)	n.d.
	20	49.3[a] (3.4)	n.d.
	30	59.1[b] (2.1)	0[b]
IET-2: the independent embryogenic tissues cultured on the mCD(2400) medium	10	42.8[a] (1.7)	n.d.
	20	74.6[c] (3.4)	n.d.
	30	86.1[c] (10.2)	12139[c] (1074)

Dry weight (DW) was calculated from five measurements (mean with standard deviation in parentheses). The values were obtained from at least three independent measurements. n.d.: not determined. Means with the same letter are not significantly different in DW ($P < 0.05$), in number of embryogenic cell aggregates per g fresh weight (FW) ($P < 0.01$).

after 4,6-diamidino-2-phenylindole (DAPI) staining (Ogita et al., 1999a), and the number of embryogenic cell aggregates within each cultured cell mass was counted under the microscope.

Fresh (FW) and dry (DW) weights of each cell mass were measured. The cell masses were dried for 20 h at 80°C to measure DW. Each value was obtained from at least three independent measurements. The data were judged as significant at $P < 0.01$ for the number of embryogenic cell aggregates and at $P < 0.05$ for the DW employing the Student's *t*-test (Zar, 1974).

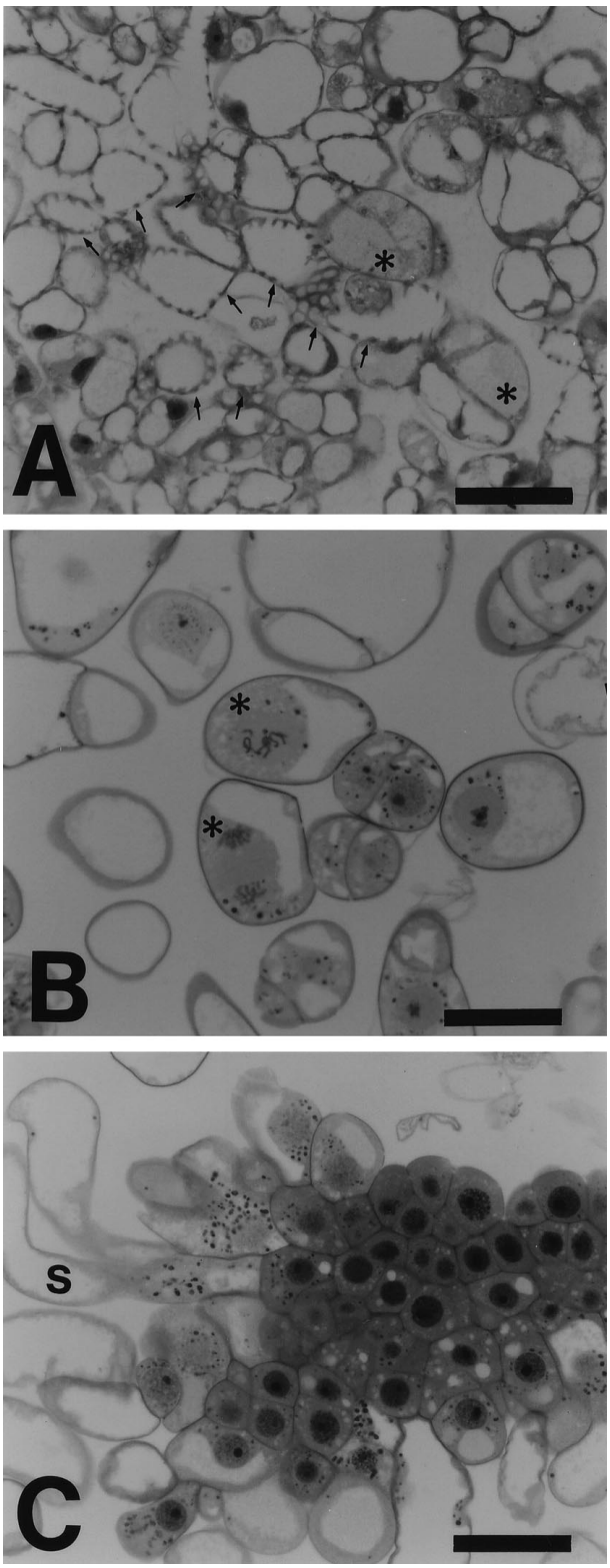
Extraction and analysis of free glutamine level. The cultured cell masses (20–30 mg FW) were measured and collected at each culture period in a 2-ml micro-tube (assist tube, Assist, Tokyo, Japan), frozen in liquid nitrogen, and stored at −85°C. Free amino acids were extracted from the samples by boiling them three times for 10 min each at 60°C with a total of 1.2 ml of 80% (v/v) ethanol. The extracts were evaporated to dryness using a centrifugal vaporizer (CVE-100, EYELA, Tokyo, Japan) with a uni-trap (UT-50, EYELA, Tokyo, Japan). The residues, which were dissolved in 50 m*M* borate buffer (pH 8.0, with 0.05 m*M* EDTA) and filtered by 0.22 μm mesh filter (Millex 4 mm GV, Millipore), were used as the free amino acid fractions. Automatic amino acid analysis utilizing 4-fluoro-7-nitrobenzo-*s*-oxa-1,3-diazole (NBD-F, Dojindo Labs., Kumamoto, Japan) was based on the report by Kotaniguchi and Kawakatsu (1987). The amino acid fractions were diluted with the borate buffer at three to five different concentrations of amino acids (approximately 20–300 pmol levels) per independent sample. Two independent samples per culture period were measured by the NBD-F method. The free amino acid fractions and a known amount of norleucine, as an internal standard mixed (50 μl:50 μl), were automatically labeled with 40 m*M* NBD-F (25 μl). Subsequently, they were incubated for 1 min at 60°C and separated on a YMC-Pack ODS-A reversed-phase column (150 mm × 4.6 mm i.d., YMC Co., Ltd., Kyoto, Japan; temperature 30°C) using a gradient high-performance liquid chromatography (HPLC) system (Gilson Medical Electronics, France) with a fluorescent detector (FP-920, JASCO, Tokyo, Japan; excitation 470 nm, emission 540 nm). Free glutamine was identified by co-chromatography with a standard.

Histological preparations. Tissues were fixed in 1.6% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 50 m*M* phosphate buffer, pH 6.8 for 24 h at 4°C. After fixation, the tissues were dehydrated in methyl cellosolve, followed by two changes of absolute ethanol, and embedded in Technovit 7100 (Kulzer & Co., Wehrheim, Germany; Yeung, 1999). Serial 3-μm sections were cut with glass knives on a Reichert-Jung 2040 Autocut rotary microtome. These sections were stained with Periodic acid–Schiff's (PAS) reaction for total carbohydrates and counterstained with amido black 10B for proteins or toluidine blue O for general histological organization (Yeung, 1984).

Results and Discussion

The induction of embryogenic tissues from developing embryos and their morphological characteristics were previously described by Ogita et al. (1999a). As we continued to maintain these cell lines using the mCD(600) medium, the tissues became more heterogeneous with a mixture of cell masses that had both embryogenic portions and non-embryogenic portions. In order to improve the performance of the culture, embryogenic tissues were separated from the non-embryogenic portion of the tissue and cultured independently on mCD(600) medium. Unexpectedly, the ability to form embryogenic cell aggregates deteriorated after a few weeks of culture. Therefore, it appears that a division of labor may have taken place within the maintenance culture, and the growth of the embryogenic tissue depended on the non-embryogenic portion of the tissue. Based on this observation, further experiments were carried out to elucidate the interactions between the two portions of the tissue.

Preliminary amino acid analyses indicated that Gln was one of the major components in embryogenic cell lines of *C. japonica* (Ogita et al., 2000). High concentrations of Gln have also been observed in embryogenic cultures of carrot (Kamada and Harada, 1984; Joy et al., 1996) and white spruce (Joy et al., 1997). Thus, the effects of increased levels of glutamine on the embryogenic portion of the tissue were tested. For these studies three mCD media with 600, 1200, and 2400 mg l^{-1} of Gln were used. Independent embryogenic tissue also was isolated from the mixed cell masses and cultured on these three media. The initial experiments established that a higher concentration of glutamine, both 1200 and 2400 mg l^{-1}, but particularly 2400 mg l^{-1} Gln, enhanced proliferation of the embryogenic tissue (data not shown). Based on these preliminary observations, the following combinations were studied in greater detail: (1) Control: the mixed cell masses which consisted of both embryogenic (Control-E) and non-embryogenic (Control-NE) cell masses cultured on the mCD(600) medium; (2) independent embryogenic tissue 1 (IET-1): the independent



embryogenic tissues cultured on the mCD(600) medium; and (3) independent embryogenic tissue 2 (IET-2): the independent embryogenic tissues cultured on the mCD(2400) medium. In order to observe the cell morphology and quantify the number of embryogenic aggregates, a small amount of the tissues was collected and stained with DAPI solution. DW (mg DW g FW^{-1}) was also measured as an index of the quality of the embryogenic tissue.

As shown in Fig. 1A, B, well-defined embryogenic aggregates were readily detected in the embryogenic portion of the mixed cell masses. The embryonal heads were cytoplasmic-rich, and they were subtended by elongated suspensor cells (Fig. 1B). Approximately 4000 aggregates per g FW were present (Table 1; Control-E). Over a 30-d culture period, there was no significant difference ($P < 0.01$) in the number of embryogenic cell aggregates and in the DW ($P < 0.05$) of the embryogenic portion of the tissue. Thus, under the maintenance condition, an equilibrium was reached between the embryogenic and non-embryogenic cells in terms of embryogenic aggregate production on a FW basis. The non-embryogenic cells were highly vacuolated (Fig. 2A). Phenolic compounds could be detected within the vacuole, as it stained intensively blue with toluidine blue O. Lignified tracheary cells could also be found among the non-embryogenic cells. Both the phenolic compounds and the presence of tracheary cells could account for the higher DW observed in the non-embryogenic portion of the tissue mass (Table 1; Control-NE, Fig. 1A (NE) and Fig. 2A).

However, when the embryogenic portion of the tissue was isolated and cultured independently on the mCD(600) medium, i.e. the 'IET-1' condition, the cell mass gradually loosened, i.e. became more friable, and the surface of the tissue became 'smooth' (Fig. 1C). Furthermore, embryogenic aggregates could not be detected after 30 d of culture. Although cytoplasmic-rich cells were present, a majority of the cells appeared as single cells forming a loose aggregate (Table 1, Fig. 1D). The failure to form embryogenic aggregates clearly indicates that the culture medium was not optimal and some factor(s) were missing in the absence of non-embryogenic cells. Judging from the presence of mitotic figures, the cytoplasmic-rich cells still continued to increase in number. The slight but significant ($P < 0.05$) increase in the DW of the IET-1 culture at the end of the culture period indicates this was likely due to the accumulation of single cytoplasmic-rich cells (Fig. 2B).

In the 'IET-2' condition, a higher concentration of glutamine (2400 mg l^{-1}) clearly stimulated the production of embryogenic aggregates and maintained the embryogenic property of the tissue (Table 1). The embryogenic cell masses had an 'irregular' surface (Fig. 1E) and produced significantly ($P < 0.01$) more embryogenic aggregates as compared to the Control (Table 1). The high glutamine treatment might have increased the synthesis of certain macromolecules or metabolites that are essential to the maintenance of embryogenic properties of cells. In addition, there was a significant difference ($P < 0.05$) in DW accumulation compared to

FIG. 2. Histology of embryogenic and non-embryogenic cell masses of *C. japonica*. A, The non-embryogenic cells in the 'Control-NE' are highly vacuolated. Phenolic compounds (*) can be detected within vacuoles as the phenolics stain intensively blue with toluidine blue O. Lignified tracheary cells (arrows) can also be found among the non-embryogenic cells. *Bar* = 50 μm. B, Embryogenic aggregates are not present when the embryogenic tissue is cultured under the 'IET-1' condition after 30 d of culture. Although cytoplasmic cells with mitotic figures (*) are present, embryonal heads and suspensors fail to develop. *Bar* = 50 μm. C, Embryogenic aggregate with well-defined embryonal head and suspensor (S) can be found in the 'IET-2' cultures. The head cells have a strong affinity for the protein stain. *Bar* = 50 μm. Sections (A) and (B) were stained with Periodic acid–Schiff (PAS) reaction and counterstained with toluidine blue O. Section (C) was stained with PAS reaction and counterstained with amido black 10B.

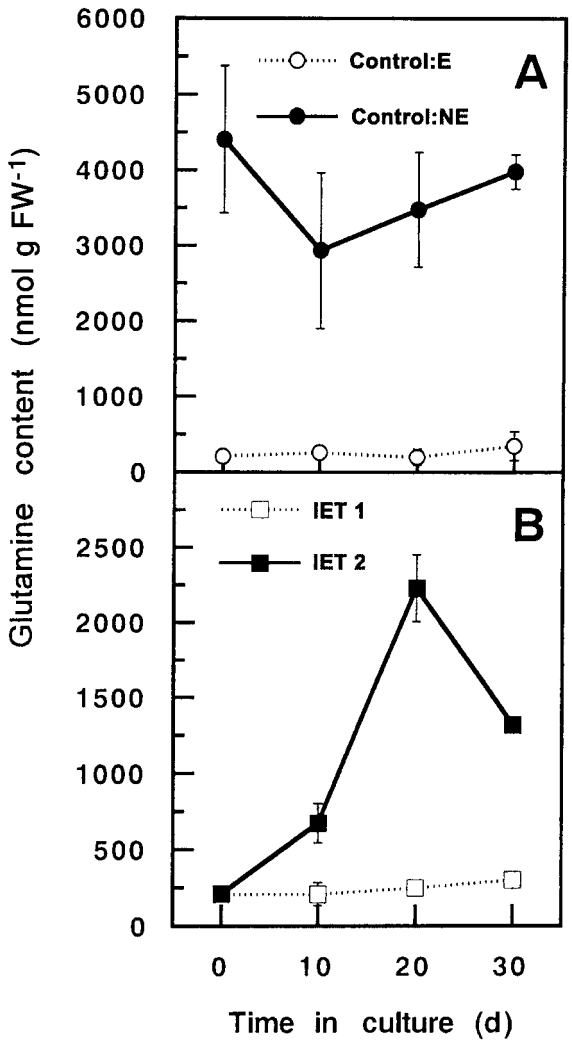


FIG. 3. The free glutamine content in embryogenic and non-embryogenic cell masses of *C. japonica*. A, The free glutamine content from the embryogenic portion (Control-E) and the non-embryogenic portion (Control-NE) of the tissue. B, The free glutamine content from tissues cultured under the 'IET-1' and the 'IET-2' conditions. Error bars show the standard deviation for each sample.

all other treatments. The increase in DW accumulation is most likely due to an increase in the number of embryogenic aggregates (Figs 1F and 2C). As well, the head cells had a strong affinity for the protein stain, which could also account for the increase in the DW of the culture. These results clearly established the importance of glutamine in the maintenance of embryogenic cells.

Kamada and Harada (1984) analyzed the intracellular levels of free and protein-bound amino acids during carrot somatic embryo formation. The authors demonstrated that glutamine and glutamic acid are in a group of amino acids reaching the highest concentrations during somatic embryo formation of carrot. Additionally, it was suggested that glutamine might play a central role in somatic embryo development as a nitrogen source and/or the main transport molecule in amino acid metabolism (Kamada and Harada, 1984). During white spruce somatic embryo development, glutamine was the first amino acid labeled from $^{15}NH_4^+$, and it continued to increase along with glutamate and arginine during development (Joy et al., 1997). Feirer (1995) also suggested the importance of glutamine in conifer embryo development based on an analysis of free amino acid content of developing *Pinus strobus* ovules and seeds. In order to ascertain the role of glutamine in somatic embryogenesis of sugi, the endogenous levels of glutamine were determined. As shown in Fig. 3A, in the maintenance medium, i.e. mCD(600), the non-embryogenic portion of the tissue had a high glutamine level (approximately more than 3000 nmol g FW^{-1}), and this level was maintained over a 30-d culture period. In the embryogenic portion, the endogenous glutamine level remained low (approximately 200–300 nmol g FW^{-1}). It is also of interest that when different fractions of carrot cell suspensions were fed ^{14}C-[U]-sucrose, non-embryogenic cells accumulated more glutamine than embryogenic cell masses, globular or torpedo-stage embryos (Joy et al., 1996). When isolated embryogenic cell masses were cultured under the IET-1 condition, the endogenous level of glutamine also remained low (Fig. 3B). On the other hand, when the isolated embryogenic tissues were cultured with a high glutamine level (2400 mg l^{-1}), the endogenous glutamine level continued to increase up to about 10 times from the initial level (Fig. 3B).

It is therefore interesting to note that glutamine appears to play an important role related to proliferation and/or differentiation of somatic embryos. In *C. japonica*, the results clearly demonstrated the importance of glutamine in maintaining the embryogenic property of the tissues. Since a low concentration of exogenous glutamine could not maintain the embryogenic potential of the tissue, it appears that under normal maintenance conditions (with mixed cell culture), the non-embryogenic cells, which contained high levels of free glutamine, are responsible for the continuous supply of glutamine to the embryogenic tissues. The non-embryogenic cells may function as nurse tissue in a mixed culture condition.

A high level of exogenous glutamine (IET-2) greatly stimulates the production of embryogenic aggregates and maintains the embryogenic tissue potential through an increase in endogenous glutamine levels. This indicates that embryogenic cells might have a high metabolic requirement for glutamine for synthesis of other metabolites and proteins. In both carrot and white spruce somatic embryogenic tissues the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway is the route of inorganic nitrogen assimilation (Joy et al., 1996, 1997). Thus glutamate becomes available to play a central role in the synthesis of other amino acids and other biomolecules. Therefore, without an adequate supply of glutamine/glutamate, embryogenic tissues will deteriorate and lose their embryogenic characteristics.

ACKNOWLEDGMENTS

The work was supported in part by grants from the Bilateral International Joint Research – Special Coordination Funds for Promoting Science and Technology (Japan).

REFERENCES

Barrett, J. D.; Park, Y. S.; Bonga, J. M. The effectiveness of various nitrogen sources in white spruce (*Picea glauca* (Moench) Voss) somatic embryogenesis. Plant Cell Rep. 16:411–415; 1997.

Campbell, R. A.; Durzan, D. J. Induction of multiple buds and needles in tissue cultures of *Picea glauca*. Can. J. Bot. 53:1652–1657; 1975.

Dunstan, D. I.; Tautorus, T. E.; Thorpe, T. A. Somatic embryogenesis in woody plants. In: Thorpe, T. A., ed. *In vitro* embryogenesis in plants. Dordrecht: Kluwer Academic Publishers; 1995:471–538.

Egertsdotter, U.; von Arnold, S. Development of somatic embryos in Norway spruce. J. Exp. Bot. 49:55–162; 1998.

Feirer, R. P. The biochemistry of conifer embryo development: amino acids, polyamines and storage proteins. In: Jain, S. et al., eds. Somatic embryogenesis in woody plants, vol. 1. Dordrecht: Kluwer Academic Publishers; 1995:317–336.

Franklin, C. I.; Dixon, R. A. Initiation and maintenance of callus and cell suspension cultures. In: Dixon, R. A.; Gonzales, R. A., eds. Plant cell culture – a practical approach. New York: Oxford University Press; 1994:1–25.

Guevin, T. G.; Kirby, E. G. Effects of glutamine and osmoticum on somatic embryo maturation in norway spruce (*Picea abies* L.) Karst. In: Ahuja, M. R.; Boerjan, W.; Neale, D. B., eds. Somatic cell genetics and molecular genetics of trees. Dordrecht: Kluwer Academic Publishers; 1996:11–16.

Joy, R. W., IV; McIntyre, D. D.; Vogel, H. J.; Thorpe, T. A. Stage-specific nitrogen metabolism in developing carrot somatic embryos. Physiol. Plant. 97:149–159; 1996.

Joy, R. W., IV; Vogel, H. J.; Thorpe, T. A. Inorganic nitrogen metabolism in embryogenic white spruce cultures: a nitrogen 14/15 NMR study. J. Plant Physiol. 151:306–315; 1997.

Kamada, H.; Harada, H. Changes in endogenous amino acids compositions during somatic embryogenesis in *Daucus carota*. Plant Cell Physiol. 25:27–38; 1984.

Kotaniguchi, H.; Kawakatsu, M. Automatic amino acid analysis utilizing 4-fluoro-7-nitrobenzo-*s*-oxa-1,3-diazole. J. Chromat. 420:141–145; 1987.

Ogita, S.; Ishikawa, H.; Sasamoto, H.; Kubo, T. Somatic embryogenesis from immature and mature zygotic embryos of *Cryptomeria japonica*: embryogenic cell induction and its morphological characteristics. J. Wood Sci. 45:87–91; 1999a.

Ogita, S.; Kubo, T.; Fushitani, M. Anatomical characteristics in early embryogenesis from immature embryo of *Larix leptolepis*. For. Res. Environ. 35:45–51; 1997.

Ogita, S.; Sasamoto, H.; Ohta, A.; Kubo, T. Changes in levels of amino acids during growth and development of embryogenic cells of *Cryptomeria japonica*. 50th Mtg. Abstracts of the Jap. Wood. Res. Soc.: 38; 2000 (in Japanese).

Park, Y. S.; Bonga, J. M.; Cameron, S. I.; Barrett, J. D.; DeVerno, L. L.; Klimaszewska, K Somatic embryogenesis in Jack pine (*Pinus banksiana* Lamb). In: Jain, S. M. et al., eds. Somatic embryogenesis in woody plants, vol. 4. Dordrecht: Kluwer Academic Publishers; 1999:491–504.

Yeung, E. C. Histological and histochemical staining procedures. In: Vasil, I. K., ed. Cell culture and somatic genetics of plants, vol. 1. Laboratory procedures and their applications. Orlando: Academic Press; 1984:689–697.

Yeung, E. C. The use of histology in the study of plant tissue culture systems - some practical comments. In Vitro Cell. Dev. Biol. Plant 35:137–143; 1999.

Zar, J. H. Biostatistical analysis. Englewood Cliffs, NJ: Prentice-Hall; 1974.