Original Paper

Utilization of Nitrate for Early Androgenic Embryogenesis
of *Brassica napus* L.

Yasunobu OHKAWA*, Hiroyuki FUKUOKA[1], Taiichi OGAWA and Harufumi MINAMI[2][3]

National Institute of Agrobiological Resources, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan
[1] *Chugoku National Agricultural Experiment Station, 6-12-1 Nishifukatsu, Fukuyama, Hiroshima
721-8514, Japan*
[2] *Tokyo Metropolitan Industrial Technology Institute, 2-11-1 Fukazawa, Setagaya, Tokyo
158-0082, Japan*
[3]*Present address: Tokyo Metropolitan Agricultural Experiment Station, 273-1 Koshimizu, Motomachi,
Ohshimamati, Tokyo 100-0101, Japan*
Corresponding author E–mail address: ohkawa@abr.affrc.go.jp

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Abstract

The utilization of nitrate in microspore culture of *Brassica napus* was examined. The microspores developed into embryos in a medium containing glutamine as a sole source of nitrogen (medium A) as well as in a medium containing nitrate and glutamine (medium C). On the other hand, no embryo could develop from the microspores in a medium with nitrate as a sole source of nitrogen (medium B). Transfer experiments from one medium to another during various periods of culture indicated that the microspores cultured in medium A stopped growing as soon as they were transferred to medium B. On the other hand, the microspores cultured in medium B did not show any change even after transfer to medium A on the 4th day of culture. Although the amount of nitrate in medium B increased within the first 14 days, the increase was less pronounced in medium C. The activity of nitrate reductase *in vivo* was not detected in the developing androgenic embryos before the 8th day of culture, whereas 15 days–old embryos showed a higher activity of nitrate reductase than leaf. From these results it was concluded that nitrate was not used for early androgenic embryogenesis in *B. napus* before 15 days of culture because of the lack of nitrate reductase activity. Negative effect of the presence of nitrate on androgenic embryogenesis was not observed, either. On the contrary, the presence of nitrate promoted the growth of heart–shaped or more developed embryos after rapid and complete consumption of glutamine in the culture medium C. Nitrate in the medium for microspore culture of *B. napus* was considered to be an ideal source of nitrogen at the later stages of androgenic embryogenesis.

Introduction

Androgenic haploids obtained by anther or isolated microspore culture were produced in many plants after the first report of Guha and Maheshwari (1964). Some of them were directly derived from microspores without callus formation, i.e., by androgenic embryogenesis (Kiyosue *et al.*, 1993; Sangwan and Sangwan–Norreel, 1987). The microspores induced embryogenesis from pollen maturation with procedures such as low temperature pre–treatment, sugar starvation, elevated temperature and so on (Sangwan–Norreel, 1977; Aruga and Nakajima, 1985; Lichter, 1982; Keller and Armstrong, 1979). Sangwan & Sangwan–Norreel (1987) pointed out that a trigger was essential for embryogenic pollen

to start embryogenesis. On the other hand, culture media and conditions did not affect the earliest stages of androgenic development, especially the initiation of androgenesis. However, when microspores were cultured in unsuitable media, no embryos in general could develop even after the initiation of embryogenesis by the triggers. The use of a suitable culture medium is considered to be essential for microspores to continue the embryogenesis.

Up to the present, many studies have been carried out to develop suitable culture media for each plant and organ. Most of the culture media were developed from several major media with modification, especially addition of components, by the method of trial and error (White, 1951). Then some components of the culture media were found to be un-

necessary or detrimental for some species. In rice culture, Ogawa et al. (1999) showed that a high concentration of nitrate, which was suitable for many varieties, inhibited the multiplication of calli in certain varieties. In these varieties a high activity of nitrate reductase was revealed while nitrite reductase showed a low activity leading to the accumulation of toxic nitrite which induced cell death. In the microspore culture of B. rapa, Burnett et al. (1992) reported that the presence of iron in the culture medium was toxic during the initial phase of androgenic embryogenesis. It is important to study the role of each component of the medium for the culture of plant materials for developing simple and suitable culture media.

Androgenic embryos were reported to be similar to zygotic embryos in terms of morphology (Crouch and Sussex, 1981; Ilic-Grubor et al., 1998a), accumulation of seed proteins (Crouch, 1982), and of fatty acids (Taylor et al., 1990). Furthermore, the culture medium for androgenic embryogenesis should be equivalent to the embryosac fluid for zygotic embryo development. The cultured microspores receive nutrients from the surrounding culture medium, while zygotic embryos received them from source tissues by translocation via the embryosac fluid or endosperm (Bhojwani and Bhatnagar, 1992; Peoples et al., 1985). Therefore, nutritional studies on zygotic embryo development may contribute to the promotion of studies on androgenic embryogenesis in terms of composition of culture media and development of appropriate culture media. Peoples et al. (1985) reported that amino acids were a common source of nitrogen nutrition for the development of pea fruit. In wheat, proline was the main chemical of the phloem (Fisher and Macnicol, 1986) and a high concentration of glutamine was detected in the embryosac fluid of immature seeds of Brassica napus L. (Ohkawa and Maeda, 1992).

Since androgenic embryogenesis was reported in B. napus (Lichter, 1982), similar culture media have been used for producing embryos from isolated microspores in other Brassica species (Sato et al., 1989; Lichter, 1989; Chuong and Beversdorf, 1985). The media contained a high concentration (0.38M) of sucrose and glutamine which were known to stimulate androgenesis in anther culture (Keller et al., 1975) and potassium nitrate as a presumed source of nitrogen. On the other hand, the exudate of cowpea fruits from phloem origin contained 0.4 - 0.8 M of sucrose (Pate et al., 1984). Recently, Ilic-Grubor et al. (1998b) have shown that the microspores developed into mature embryos in culture media containing 3 mM sucrose, 0.44 M

mannitol and 25 % of PEG, suggesting that a high concentration of sucrose was not necessary as a carbon source but was important for keeping the osmotic pressure as high as that of the embryosac fluid (650 - 680 mOsmol which was nearly equal to 0.6 M of sucrose) (Ohkawa and Maeda, 1992). Meanwhile, the role of nitrate in the medium in microspore culture whose content was low in the embryosac fluid had not been elucidated. In this paper, the utilization of nitrate in the androgenic embryogenesis of B. napus was investigated.

Materials and Methods

Plant Materials and Isolation of Microspores

Seeds of rapeseed (Brassica napus L. cv. Lisandra), given by Dr. Z. Fan, Agriculture Canada, were sown in pots 0.02 m^2 in size with commercial pre-mixed soil (Ibaraki Iseki Co.,Ltd., Ibaraki, Japan) and cultivated at 15 °C (day)/10 °C (night) under a 16 hrs day-length (Keller and Stringam, 1978). At the flowering stage, buds 3-4 mm long which were assumed to contain microspores at the late uninucleate stage were collected, surface-sterilized with 1 % of sodium hypochlorite for 15 min and washed three times with sterilized water. Microspores were isolated from the buds, according to the method of Keller et al. (1987). Released microspores from anthers by squashing of buds with a syringe in B5-13 medium (Keller et al., 1987) containing 13 % of sucrose without hormones were washed three times by centrifugation with B5-13 medium. After the wash, the microspores were resuspended in the following culture media.

Microspore Culture

Microspore culture was conducted by the method of Keller et al. (1987) with modifications. The isolated microspores were cultured at 32.5 °C for the first four days to induce androgenesis then cultured at 25 °C. All the cultures were kept in the dark. Following three media were used for microspore culture: glutamine medium (medium A), nitrate medium (medium B) and medium containing nitrate and glutamine (medium C: reported as NLN medium by Keller et al., 1987)(Table 1). The microspores cultured in these media were collected by centrifugation (1,000 rpm, 5 min.) on the 4th, 6th and 8th days of culture. The pellets were washed once by replacing media and were resuspended with the medium at the density of 5 x 10^4 cells/ml. The suspended microspores were plated in petri dishes (6 cm in diameter) and cultured again. Continuous culture of microspores in medium C with different concentrations of glutamine (0.05, 0.1, 0.55, 2.74,

Table 1. Composition of the medium for isolated microspore culture

Component	Concentration (mM)		
	medium A	medium B	medium C
KNO₃	–	1.24	1.24
Ca(NO₃)₂ • 4H₂O	–	2.12	2.12
L–Glutamine	8.21	–	5.47

Other components were the same as those in NLN– 13 medium (Keller *et al.*, 1987).

5.47 and 10.9 mM) was also performed. In all the culture experiments, the embryos derived from microspores were counted on the 14th day of culture.

In vivo Nitrate Reductase Activity of Androgenic Embryos

The developing androgenic embryos in medium C were collected by centrifugation (1,000 rpm, 5 min.) on the 4th, 8th and 15th days of culture. The precipitated embryos weighing 100–400 mg were washed twice with phosphate buffer (pH 7.5) and resuspended in 10 m*l* of the reaction mixture containing 100 mM KH_2PO_4 (pH 7.5), 25 mM KNO_3 and 5 % n–propanol. The suspension was incubated at 30 °C for 60 min in the dark. The reaction was stopped by soaking the reaction vessel into boiling water for 2 min. The reaction mixture was centrifuged at 1,000 × g for 10 min and the concentration of NO_2^- in the supernatant was determined by a colorimetric method (Hageman and Hucklesby, 1971; Hageman and Reed, 1980). The activity in the leaves of 30–day–old seedlings was also measured for confirming the validity of the measuring methods. The *in vivo* nitrate reductase (NR) activity of the embryos at different developmental stages was measured twice and two independently cultured embryos were used.

Determination of Nitrate and Ammonium Ions and Glutamine in Culture Media

Microspores were cultured in medium C. The concentrations of nitrate and ammonium ions in medium C on day 0 and day 14 of culture were determined by ion–chromatography (Ion–chromatograph QIX, Dionex Co.,Ltd., San Jose, USA). The concentration of glutamine of the media on day 0, 5, 11, 14 and 20 of culture was also determined using HPLC (L8500, Hitachi Koki Co.,Ltd., Tokyo, Japan) with an Ion–exchange column PH#2622SC.

Results

Androgenic Embryogenesis in Culture Media with Different Nitrogen Sources

In medium C, the general medium for the microspore culture in *Brassica* which contained nitrate and glutamine, a large number of androgenic embryos were obtained within 14 days of culture. Embryos were characterized by various sizes and developmental stages (**Fig. 1**). After treatment with elevated temperature as a trigger, some of the microspores rapidly grew into more developed embryos, i.e., heart–shaped, torpedo and early cotyledonal embryos larger than 250 μm long. On the other hand, some of them still remained at the multi –cellular stage (smaller than 50 μm). The microspores did not develop into embryos synchronously. The early cotyledonal embryos grew up to normal haploid plants when they were transferred to B5 medium (Gamborg *et al.*, 1968) without hormones under light conditions (**Fig. 2**).

The microspores in medium A as well as in medium C became embryos after 14 days of culture (**Fig. 1** and **3**). The embryos were also at various developmental stages. About one–third of the embryos was larger than 250 μm including torpedo and heart–shaped embryos. Globular embryos with sizes in the range of 100 to 250 μm and smaller embryos under 100 μm accounted for the other two –thirds. On the other hand, no embryos were developed in medium B (**Fig. 1**). Medium C contained both glutamine and nitrate whereas medium A contained glutamine but not nitrate and medium B contained nitrate but not glutamine (**Table 1**). Thus, these results indicated that androgenic embryos could be obtained in the presence of glutamine regardless of the presence of nitrate. Although microspores developed to embryos in medium A as well as in medium C and there was no significant difference in the number of embryos smaller than
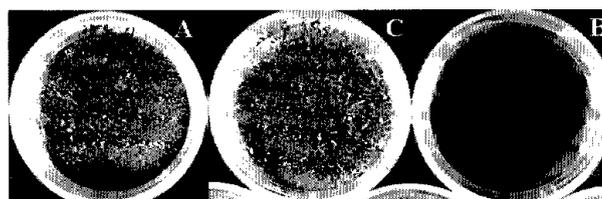

Fig. 1. Embryos from microspores cultured in different media after 14 days of culture.

   A: cultured in medium A containing glutamine as a sole nitrogen source

   B: cultured in medium B containing nitrate

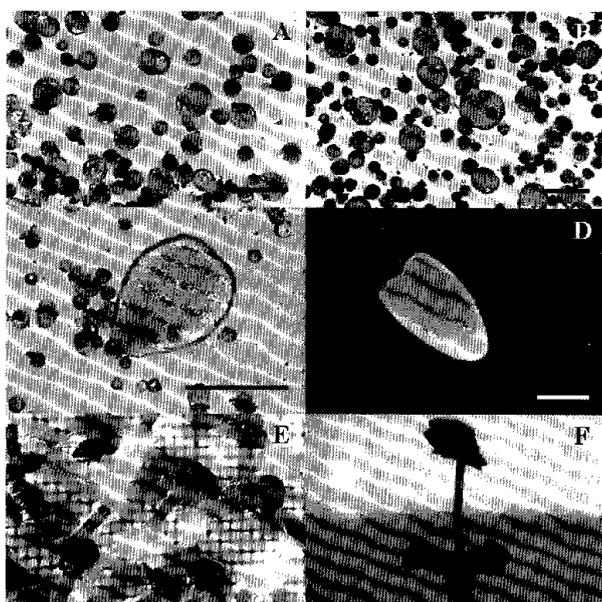   C: cultured in medium C containing glutamine and nitrate

Fig. 2. Androgenic embryo development and regeneration of haploid plant

A: fourth day of culture (bar = 100 μm),

B: globular stage (bar = 100 μm),

C: heart-shaped stage (bar=250 μm),

D: torpedo stage (bar = 1 mm),
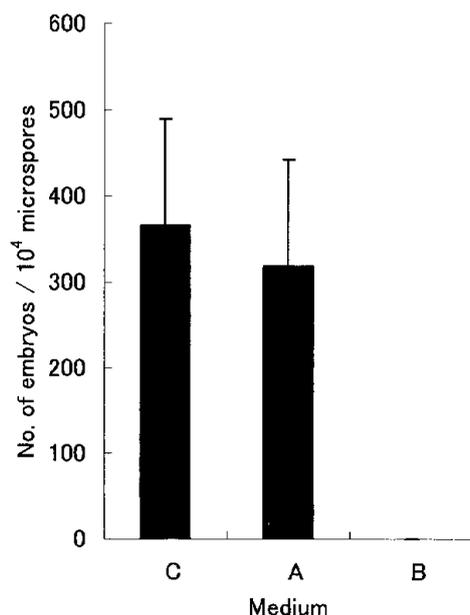
E, F: regenerated haploid plant

Fig. 3. Number of embryos larger than 50 μm derived from microspores cultured in different media.

250 μm in the media, the larger embryos (>250 μm) were significantly (P<0.01) fewer in medium A than in medium C (**Table 2**). Absence of nitrate tended to slightly reduce the number of well-developed androgenic embryos.

The microspores cultured in medium B, containing nitrate as a sole nitrogen source, did not show any change and no androgenic embryos were

Table 2. Number of embryos developed in different media

Medium	Embryo Size	
	50-250 μm	>250 μm
C	218 ± 102[1]	137 ± 29[2]
A	204 ± 103	107 ± 25[2]
B	0	0

[1] Number of embryos/1 × 10^4 microspores ± s.e.

[2] Significant difference was detected between them at 1% level by t-test.

produced (**Fig. 1, 3**). No embryos developed when the microspores were cultured in medium B for the first four days or more. Even after transfer from medium B to medium A, no cell masses were observed (**Fig. 4** BAAA, BBAA, BBBA and BBBB). In contrast, divided cells were observed even in medium B when the microspores were cultured in medium A for the first four or more days (**Fig. 4** ABBB, AABB, AAAB and AAAA). On the 14th day of culture, only small-size (50-100 μm) cell masses were detected when the microspores were transferred from medium A to B on the 4th or 6th day of culture. More cell masses were counted in the 6th-day-transfer culture than in the 4th one. When the cultured microspores were transferred to medium B on the 8th day of culture, many more cell masses and early globular embryos (100-250 μm) were observed on the 14th day of culture. A large number of microspores developed to heart-shaped or torpedo embryos (over 250 μm) on the 14th day of continuous culture in medium A. The number of early globular embryos was also much higher than that in the 8th-day-transfer culture. There was no significant difference in the number of cell masses (<50 μm) between the 8th-day-transfer culture and continuous culture. The earlier the transfer to medium B, the younger the embryos on day 14. These results suggested that the development of the embryos from microspores cultured in medium A seemed to stop when they were transferred to medium B.

When the microspores were cultured in medium C with different concentrations of glutamine, the total number of embryos (larger than 50 μm) ranged from 0.1 to 241.0 embryos/1 x 10^4 microspores (**Fig. 5**). There were few embryos in the media with 0.05, 0.1, 0.55 mM of glutamine. In the medium with 2.74 mM of glutamine, 164.1 ± 4.3 embryos grew from 1 x 10^4 microspores. The frequency of embryo formation in the medium with 10.9 mM of glutamine (232.7 ± 23.1) was as high as that with 5.47 mM
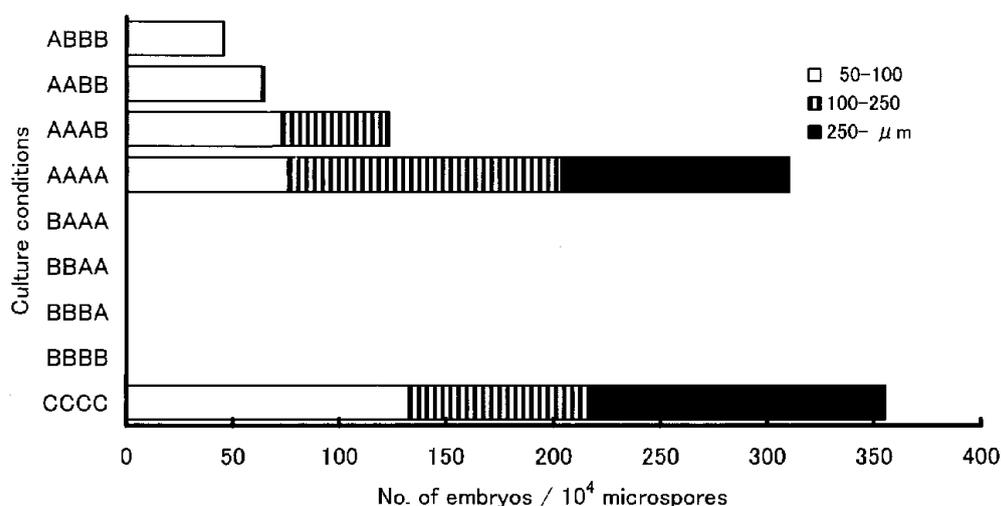
Fig. 4. Effect of changes of media at different developmental stages of androgenic embryogenesis.

ABBB or BAAA: microspores cultured in medium A or B for the first 4 days were transferred to medium B or A, respectively.

AABB or BBAA: microspores cultured in medium A or B for the first 6 days were transferred to medium B or A, respectively.

AAAB or BBBA: microspores cultured in medium A or B for the first 8 days were transferred to medium B or A, respectively.

AAAA, BBBB or CCCC: microspores were continuously cultured in medium A, B or C, respectively.
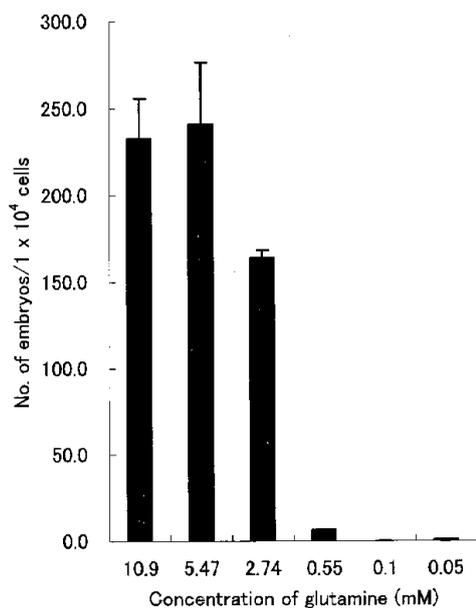

Fig. 5. Effect of glutamine concentration in medium C on embryo formation from microspores.

(241.0 ± 35.5). A concentration above 5.47 mM of glutamine was optimum for microspore culture. On the other hand, the numbers of embryos larger than 250 μm were 74.0 ± 3.0, 81.9 ± 10.0 and 137.1 ± 46.0 in the medium C with 2.74, 5.47 and 10.9 mM of glutamine, respectively. There was no significant difference in the amount of embryos larger than 250 μm among them.

In Vivo Nitrate Reductase Activity of Androgenic Embryos

On day 4 in the culture, most of the microspores were at the first cell division stage in medium C and on day 8, there were cell masses and early globular embryos (<250 μm) (**Fig. 2**). A very low activity of nitrate reductase was detected in these young embryos on the 4th and 8th days of culture (**Fig. 6**). On day 15, the embryos were at various developmental stages and most of them that were larger than 250 μm were heart-shaped or torpedo embryos. The large embryos collected showed a high activity of nitrate reductase (1,457 μmoles NO_2^- synthesis/gfw/hr). In the heart-shaped and more developed embryos, the activity was about 50 times higher than in the globular and less developed embryos. The activity was also higher than that in a young leaf (860 μmoles NO_2^- synthesis/gfw/hr).

Changes in the Concentrations of Nitrate and Ammonium Ions in Culture Media during Androgenic Embryogenesis

Microspores cultured in medium C or medium A developed to the heart-shaped or torpedo stage by day 14. However, the concentration of NO_3^- ion in the culture medium C after 14 days of culture was as high as that on day 0 (**Table 3**). On the contrary, the concentration of NO_3^- ion increased in medium B after 14 days of culture. On day 14, the micro-
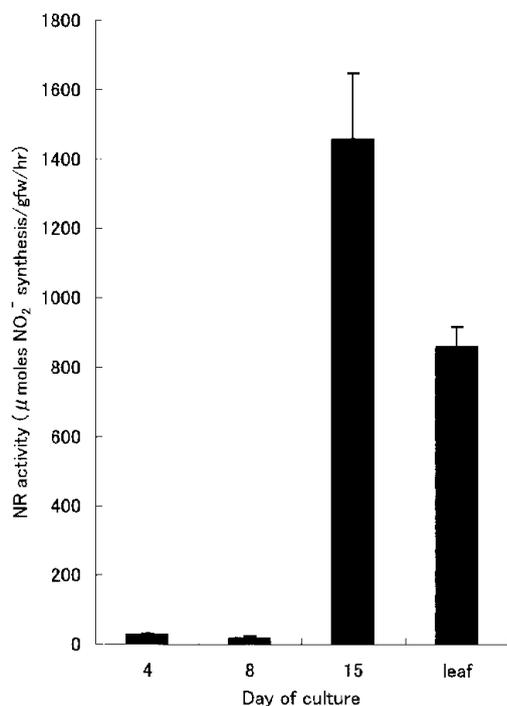
Fig. 6. Nitrate reductase activity of androgenic embryos at different developmental stages.

Fig. 7. Changes of glutamine content of medium C through microspore culture.

Table 3. Concentration of nitrate and ammonium ions in the media

Day of culture	Medium C		Medium A		Medium B	
	0	14	0	14	0	14
NO_3^-	7.62[1]	7.73	N.D.	N.D.	7.79	8.90
NH_4^+	N.D.[2]	2.92	N.D.	3.86	N.D.	N.D.

[1] mM

[2] not detected

spores did not display any change in medium B and a large number of undeveloped microspores (embryogenic frequency was about 2-3 %) was observed in medium C. Media, A, B and C, did not contain NH_4^+ ions at first, but after 14 days of culture, 3-4 mM of NH_4^+ ions were detected in medium A and C in which androgenic embryos developed. In medium B, androgenic embryogenesis was not observed and NH_4^+ ions were not detected both on day 0 and day 14. Then, a small amount of NH_4^+ ions was produced when androgenic embryos developed.

Changes in the Concentration of Glutamine in Medium C during Androgenic Embryogenesis

The concentration of glutamine in medium C gradually decreased during the first 11 days of culture and rapidly decreased thereafter (**Fig. 7**). On the 8th day of culture, there were few large embryos while the size of smaller embryos rapidly increased
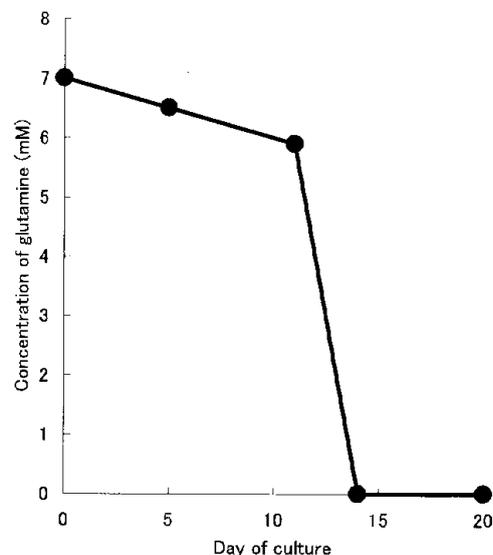
subsequently. On day 14, glutamine was completely consumed.

Discussion

Tissue culture is an important technique for genetic engineering and for studying the physiology of plants. A complete plant can regenerate from a part of plant tissue or a single cell via callus or direct embryogenesis because of totipotency. Although a green plant is autotroph and can be hydroponically cultured with inorganic compounds, some organic compounds, like sugars, are necessary for culturing tissues or cells separated from the plant body (White, 1951). Typical tissue culture media contain nitrate, ammonium, sucrose, vitamins and so on. On the other hand, a tissue, a part of an individual plant, receives some compounds from other parts of the plant by the vascular system (Heldt, 1997). Nitrate and ammonium absorbed by roots are generally transferred to other parts of the plant, mainly leaves, and converted into glutamine in chloroplasts. Nitrate ion is also converted into amide in leucoplasts of root cells (Heldt, 1997). Then, most parts of the plant are considered to display a nitrate reductase activity and are able to be cultured in a medium with nitrate and ammonium as nitrogen sources (White, 1951).

A fertilized egg, which is different from the mother plant, parasitically lives on the mother plant. The fertilized egg develops into an embryo by getting nutritious substances from the mother plant via the embryosac fluid or endosperm (Bhojwani and Bhatnagar, 1992). The embryosac fluid of *B. napus*, mainly contained glutamine as a nitrogen compound (Ohkawa and Maeda, 1992). In wheat,

proline was the main chemical of phloem (Fisher and Macnicol, 1986). For the fruit development of *Vigna unguiculata*, amino acids were the main source of nitrogen nutrition (Peoples *et al.*, 1985). Pate *et al.* (1984) concluded from a comparison between the root breeding xylem sap and the fruit phloem sap of *V. unguiculata* that the stem utilized NO_3^- nitrogen to synthesize amino acids prior to phloem transfer of nitrogen to the fruit. Then, amino acids were considered to be used mainly for zygotic embryo development. Raghavan (1966) reported the requirements of nutritional components for the development of extracted immature embryos from the developing ovule of *Capsella bursa-pastoris*. He pointed out that the globular and younger embryos developed heterotrophically while the heart-shaped and older embryos tended to develop more autotrophically. The late globular embryos grew in the culture medium containing inorganic salts, vitamins, sucrose and growth regulators but lacking amino acids. Nitrate was good enough for the growth of the late globular embryos (60–80 μm). For the early globular embryos (<40 μm), there was no information about the necessary components for development.

In the formation of androgenic embryos in *B. napus*, glutamine was mainly consumed and no embryo formation was detected in the medium with nitrate as a sole source of nitrogen. Even globular embryos (100–250 μm) did not continue to develop when they were transferred from the medium containing glutamine to the nitrate medium. The nitrate medium contained the same amounts of vitamins, sucrose and growth regulators as those in medium C with glutamine and nitrate (Lichter, 1982). The androgenic globular embryos in medium C did not display a nitrate reductase activity which is generally promoted by the presence of nitrate (Dougall, 1977). The enzyme mediates the first reaction of nitrogen assimilation whereby nitrate ion is converted to nitrite ion. Fukuoka *et al.* (1996) revealed the expression of the nitrate reductase gene at the heart-shaped stage of androgenic embryos but not at the globular stage. During the zygotic embryo development, the gene also started to be expressed at the heart-shaped stage. These results suggested that the development of globular or younger embryos was not stimulated by nitrate because of the lack of nitrate reductase activity.

In other species, *Nicotiana* and *Oryza sativa*, the culture media for isolated microspores without any pre-treatment of anther or bud, also contained 3–5 mM of glutamine (Imamura *et al.*, 1982; Kyo and Harada, 1985; Ogawa *et al.*, 1995). In the case of anther culture of *N. tabacum*, Aruga and Nakajima

(1985) showed the presence of glutamine and asparagine inside the anther which was cultured in a medium without amino acids and concluded that these amino acids produced by the anther wall were necessary for the initiation of androgenic embryogenesis. On the other hand, another type of embryogenesis, somatic embryogenesis, has been reported in many plants (Kiyosue *et al.*, 1993). Nomura and Komamine (1985) observed the formation of somatic embryos with a high frequency from isolated single cells of carrot. The culture medium for the formation of somatic embryos from isolated single cells contained nitrate and ammonium but not glutamine or other amino acids (Fujimura and Komamine, 1979). Smith and Krikorian (1990) reported that proembryos at the preglobular stage in carrot suspension culture could be maintained well on a medium containing ammonium ion or ammonium and nitrate ions but that the growth was poor with nitrate ion alone. Based on these findings, young embryos before the globular stage were not considered to use nitrate as a nitrogen source, irrespective of the origin of the cells, zygotes, microspores and somatic cells.

There were no significant differences in the total amount of embryos and also in that of larger embryos (>250 μm) between medium C with 5.47 and 10.9 mM of glutamine. Medium A contained 8.21 mM of glutamine and there was no significant difference in the total amount of embryos between medium A and medium C (with 5.47 mM of glutamine), too. But in medium A, there were fewer larger embryos than in medium C. The total amount of nitrogen in both media was the same. These results suggested that nitrate promoted embryo development more than glutamine at later stages. Although the amount of nitrate in medium B increased within the first 14 days, the increase was less pronounced in medium C. On day 14, the microspores did not display any change in medium B and a large number of undeveloped microspores (embryogenic frequency was about 2–3 %) was observed in medium C. It thus, appeared that a certain amount of nitrate in medium C was used by a small number of developed embryos after the heart-shaped stage. The microspores in medium A and medium C were considered to grow at the same speed before the heart-shaped stage and to be subjected to the expression of nitrate reductase at the heart-shaped stage even under the presence of glutamine and absence of nitrate (Fukuoka *et al.*, 1996). However the embryos in medium C were supposed to use nitrate and grow rapidly, while those in medium A used glutamine and grew slowly. Therefore, a larger number of embryos were pro-

duced in medium C than in medium A.

Nitrate did not inhibit androgenic embryogenesis at the early stage unlike in rice tissue culture (Ogawa *et al.*, 1999) and promoted embryo development after the heart-shaped stage. Nitrate seemed to remain in the culture medium and not to affect the androgenic embryogenesis until the heart-shaped embryo development. To cope with the change in the nutritional requirements of further developing embryos, preparation of nitrate in the culture medium at first seemed to be suitable.

Acknowledgments

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