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Full Length Research Paper

Microspore culture of *Zantedeschia aethiopica*: The role of monosaccharides in sporophytic development

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Various factors affecting microspore culture were studied in *Zantedeschia aethiopica*. Starting microspores at late-unicellular stage, NLN as culture medium and treatment of culture at 32°C for 2 days prior to incubation at 25°C, gave the best early sporophytic response. A comparison of different carbohydrates applied in culture medium showed that glucose and fructose favored more occurrences of early sporophytic divisions than sucrose and maltose. However, multicellular structures stopped growing in continues cultivation with each carbohydrate. The development of multicellular structures was sustained and calli were formed by culturing microspores in the medium containing glucose or fructose for initial 7 days, subsequently changing to the medium containing sucrose. Thus, this study revealed an essential and novel role of monosaccharides in sporophytic development of *Z. aethiopica* microspores, whereas, it is known that monosaccharides are less effective for many other crops. In total, 24 calli were obtained, of which, 18 produced plants. Chromosome number counting of 18 plants indicated that 8 were haploid, 6 were diploid and the rest were other numeric chromosomal constitutions.

Key words: Calla lily, haploid plant production, isolated microspore culture, glucose, fructose.

INTRODUCTION

Calla lily (*Zantedeschia* spp.), a genus of the Araceae family, is an important ornament grown worldwide (Kuehny, 2000; Snijder et al., 2004; Wright et al., 2005; Ni et al., 2009, 2010). The genus is native to southern Africa and consists of many valuable species. Diploid plants in all *Zantedeschia* spp. contain 32 chromosomes (Yao et al., 1994). Various modern cultivars of calla lily are developed by crosses from different species and propagated vegetatively, therefore, have a high degree of heterozygosity. The juvenile phase of calla lily is quite long, lasting for 1 to 2 years. These characterizations hamper genetic analysis of important traits and efficient breeding of the ornamental. Breeding efficiency could be improved by inbreeding programs, but it is time consuming to obtain pure lines by numerous cycles of self pollination.

Compared with conventional inbreeding, haploid technology allows obtaining of homozygous doubled haploid plants (DHs) in a single generation; thus, tremendously facilitating breeding programs (Ferrie et al., 1995; Forster and Thomas, 2005). Haploid plants are also of benefit in genetic transformation and mutation studies (Ferrie et al., 2008). However, data on haploid plant production are rare in the Araceae family. Ko et al. (1996) earlier reported the obtaining of haploid plant using anther culture in classical white calla lily, *Zantedeschia aethiopica*. Later, Eeckhaut et al. (2001) reported on ovule culture in *Spathiphyllum wallisii* genotypes. But efficiency of these reports is low. Only one or two haploid

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origin plants were obtained from hundreds of anthers or ovules. On the other hand, these methods also give rise of diploids that could be somatic origin. In order to confirm that they are valuable DHs, laborious genetic analyses are required. Alternatively, haploid plants can be produced through isolated microspore culture. This method offers an advantage of using unique haploid cells as source of regenerants and has potential to increase yield. Nevertheless, there are a number of factors that influence the success of microspore culture, including microspore developmental stage, stress treatment of culture, culture medium and carbohydrate source provided in medium. However, no data has been published on isolated microspore culture in the Araceae family. In this study, effect of various factors on isolated microspore culture was investigated and a successful microspore culture procedure was reported in *Z. aethiopica*. Moreover, this study revealed an essential and novel role of monosaccharides in the microspore culture of this ornamental.

MATERIALS AND METHODS

Plant material

Z. aethiopica cultivar 'Hong Gan' was used in this study. Donor plants were grown in open field in Kunming, China. Water was applied through irrigation tubes at a depth of 1 to 5 cm and N: P: K (16%:16%:16%) granule fertilizer was applied every two weeks (10 g/m^2). Newly sprouting inflorescences were harvested during 2007 and 2008 in winter (November to January). During this period, the temperature ranged from 15 to 19°C during day and from 2 to 10°C during night; average photoperiod was 10 h day/14 h night. The spadices were taken, disinfected for 10 min in 1% NaOCl with 0.05% (v/v) Tween-20 and then rinsed three times in sterile tap water.

Microspore isolation

For each isolation, one spadix was taken from a plant. About 500 anthers were excised from middle part of a spadix, transferred to a sterile Petri dish (60 × 15 mm) and 2 ml of culture medium were added. Microspores were released by pressing anthers with a turning movement using injection piston, followed by addition of 8 ml culture medium. The suspension obtained was filtered through a nylon filter (mesh width 150 µm). Microspores were collected by centrifugation for 3 min at 100 *g* and washed twice by re-suspending the microspores in 10 ml fresh culture medium and centrifugation as earlier mentioned. The total number of microspores was determined with a hemacytometer. Microspore suspensions were adjusted to a density of 4×10^4 microspores/ml using culture medium. Usually, one spadix yielded up to 12 ml culture. Developmental stage of freshly isolated microspores was determined on the basis of the nucleus position after 4',6-diamidino-2-phenylindole (DAPI) staining. Experimental design for studying various factors affecting the microspore culture is described further.

Microspore culture

Initially, the method described for *Brassica napus* microspore culture (Custers, 2003; Lichter, 1982) was used as a provisional protocol. Briefly, (1) microspores at late-unicellular stage were isolated and cultured in NLN medium (Lichter, 1982), supplemented with 13% sucrose (w/v), pH 5.8; (2) cultures were stressed at 32°C for 2 days in the beginning of the culture and then transferred to 25°C in darkness. With the provisional protocol, we studied the effect of various factors in more detail.

Developmental stage

Microspore populations at mid, late-unicellular and early-bicellular stages were cultured. Inflorescences containing microspores at appropriate developmental stages were classified as follows: (1) inflorescences in 3 to 5 cm length, enclosed inside leaf sheath, contained anthers with about 90% mid-unicellular microspores; (2) inflorescences in 5 to 7 cm length, emerged for about 2 cm out of leaf sheath, contained anthers with about 90% late-unicellular microspores (Figure 1a); (3) inflorescences in 7 to 9 cm length, emerged for about 4 cm out of leaf sheath, contained anthers with about 90% early-bicellular microspores. These classes of inflorescences were used for the isolation of microspores at mid, late-unicellular and early-bicellular stages.

Temperature stress

Microspore cultures were kept at different temperatures (4, 25 or 32°C) for the first 2 days and then maintained at 25°C.

Culture medium

Four culture media, NLN, B5 (Gamborg et al., 1968), CHU (N6) (Chu et al., 1975), Nitsch (Nitsch and Nitsch, 1969), were compared. The media were purchased from Duchefa Biochemie, The Netherlands.

Carbohydrate

Microspores were cultured continuously in the NLN medium supplemented with 13% (w/v) sucrose, maltose, glucose or fructose. For studying the effect of initial short term cultivation with monosaccharides, microspores were isolated and cultured for initial 3 to 14 days in the NLN medium supplemented with 13% glucose or fructose; subsequently, they were collected by centrifugation for 3 min at 100 *g* and cultured in the NLN medium supplemented with 13% sucrose.

Apart from the parameter under study, the other factors were same as in the provisional protocol. For each treatment, microspore cultures were obtained from one isolation. 4 ml of the microspore suspension were cultured in each Petri dish (60 × 15 mm). There were two Petri dishes (60 × 15 mm) for each treatment and each experiment was repeated three times.

In order to examine early microspore development in culture, microspore samples were collected after 2 weeks of incubation and used for DAPI staining and microscope observation (Eclipse E800, Nikon, Japan). At least 100 microspores were examined for each treatment and percentages of microspores containing various numbers of cells were counted. Frequency of callus formation was counted as numbers of calli per Petri dish of cultures, after 9 weeks of incubation. Statistical analysis was performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA).

Plant regeneration from calli and ploidy analysis

Calli that had reached about 3 mm in diameter were transferred onto shoot regeneration medium (MS (Murashige and Skoog,

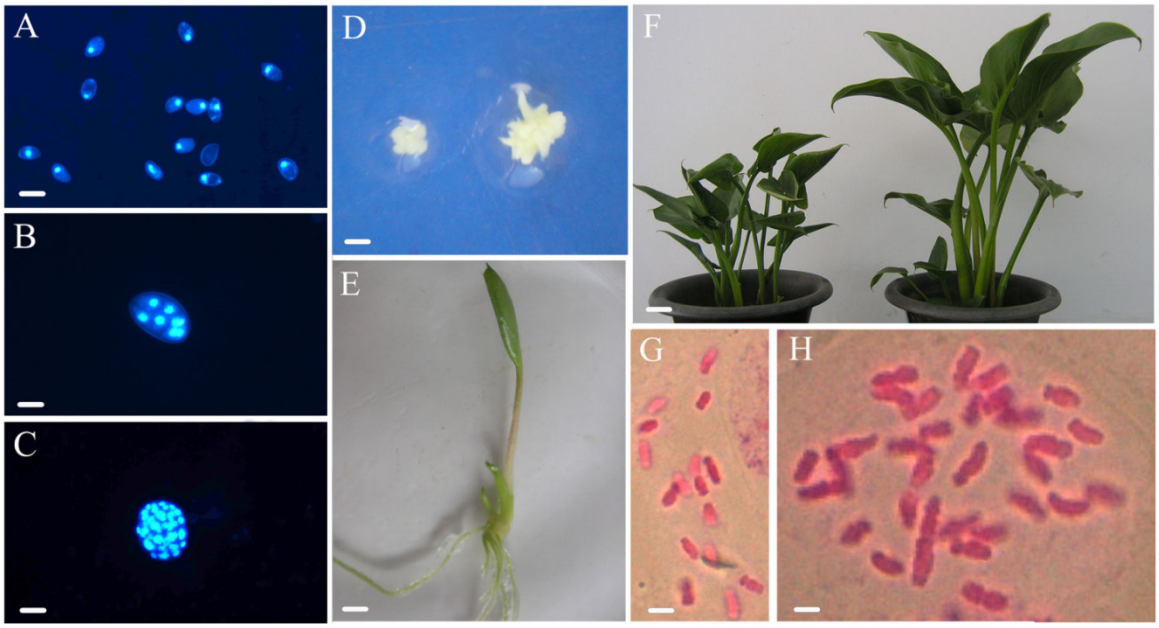

Figure 1. Microspore development in isolated microspore culture of *Z. aethiopica*. A, Late-unicellular microspores freshly isolated for culture; B, microspore with 6 cells after 2 weeks of culture; C, multicellular structure with more than 20 cells after 3 weeks of culture; D, calli formed after nine weeks culture and bud differentiation from a large callus occurred already in the culture; E, rooted plant; F, the sizes of haploid (left) and diploid (right) plants after growing in pots for one year; G, root tip cell nucleus with 2n=X=16 chromosomes in haploid plant; H, root tip cell nucleus with 2n=2X=32 chromosomes in diploid plant. Scale bars = 50 µm for A; 25 µm for B and C; 1.5 mm for D; 0.5 cm for E; 2.4 cm for F; 7.3 µm for G and H.

1962), supplemented with 3% (w/v) sucrose, 0.7% (w/v) plant agar, 1 mg/l 6-benzylaminopurine, 0.2 mg/l naphthaleneacetic acid, at pH 5.8). From then the cultures were kept in a growth room with 16 h light/ 8 h dark cycle at 25℃ (60% humidity, 5000 lux light intensity). Shoots developed (1 to 4 cm in length) were rooted by culturing on MS medium with 3% sucrose, 0.7% agar and 0.1 mg/l indole-3-butyric acid, at pH 5.8.

The chromosome numbers in root tips of the regenerated plants were counted to determine the ploidy level. Root tips were cut, pretreated with 0.002 M 8-hydroxyquinoline at room temperature for 6 h, fixated in a mixture of alcohol and glacial acetic acid (3:1) at 4℃ for 20 h, macerated in 1 M hydrochloric acid at 60℃ for 5 min, then squashed and stained on slides with Cabol Fuchsin reagent (Carr and Walker, 1961). The samples were observed under the microscope (Eclipse E800, Nikon, Japan).

The rooted plantlets were transferred into fertile horticultural soil in 15 cm x 22 cm (diameter x height) pots and grew in open field conditions as described in the section of 'Plant material'. The plant growth was recorded by photograph after growing for one year.

RESULTS

In the initial experiments, we applied the method described for *B. napus* microspore culture (Lichter, 1982; Custers, 2003); however, plant regeneration from the microspores failed with the cultivar 'Hong Gan'. Therefore, we conducted further experiments. To determine a suitable developmental stage for culture, microspores at mid, late-unicellular and early-bicellular stages were cultured. After 2 weeks incubation, percentages of microspores containing various numbers of cells were counted (Table 1). The best early sporophytic response was obtained with late-unicellular stage microspores. The first sporophytic division took place in the first week, usually producing two similarly sized, vegetative-like nuclei. The division of some microspores continued and 0.7% microspores containing 4 to 10 cells were observed after 2 weeks (Table 1, Figure 1b). Starting with mid-unicellular, we did not find microspores containing more than 3 cells. With old microspores at early-bicellular stage, only gametophytic development took place, resulting in a large vegetative nucleus and two small generative nuclei. Therefore, we decided to apply isolated microspores at late-unicellular stage for studying the effect of following factors on the microspore culture.

Effect of temperature stress on early sporophytic response

Table 2 shows the effect of temperature stresses on switching microspore developmental pathway from gametophytic to sporophytic. Continuous incubation of the microspore cultured at 25℃ gave low percentage (0.1%)of microspores containing 4 to 10 cells after 2 weeks. Application of both low (4℃) and high (32℃) temperatures for 2 days in the beginning of the culture increased the percentage. In comparison, heat stress

Table 1. Early sporophytic response of isolated microspores in culture, starting with populations at different developmental stages in *Z. aethiopica*.

Number of cells per microspore	Percentage of microspores containing various numbers of cells after 2 weeks, starting with a population at		
	Mid-unicellular stage	Late-unicellular stage	Early-bicellular stage
0	76.3 ± 9.3	69.7 ± 12.0	75.5 ± 5.5
1	22.1 ± 8.9	18.4 ± 4.7	3.5 ± 1.9
2	1.1 ± 0.3	10.2 ± 7.1	2.1 ± 1.0
3	0.5 ± 0.3	1.0 ± 0.9	18.9 ± 2.9
4–10	0.0 ± 0.0	0.7 ± 0.5	0.0 ± 0.0

At least 100 microspores were examined for each treatment. Each experiment was repeated three times. Mean values ± standard deviation (SD) are given.

Table 2. Effect of temperature stress on early sporophytic response in isolated microspore culture of *Z. aethiopica*.

Number of cells per microspore	Percentage of microspores containing various number of cells after 2 weeks, with temperature stress treatment at		
	25°C continuous	4°C	32°C
0	80.0 ± 8.8	73.5 ± 7.9	65.4 ± 14.3
1	12.4 ± 6.9	19.2 ± 5.9	20.8 ± 6.8
2	6.2 ± 1.7	5.6 ± 1.8	11.7 ± 6.3
3	1.3 ± 0.6	1.2 ± 0.4	1.0 ± 0.7
4–10	0.1 ± 0.2	0.5 ± 0.3	1.1 ± 0.6

At least 100 microspores were examined for each treatment. Each experiment was repeated three times. Mean values ± SD are given.

was more effective, yielding 1.1% microspores containing 4 to 10 cells versus 0.5% after the cold stress.

Effect of culture medium on early sporophytic response

Four culture media, NLN, Nitsch, B5, CHU (N6), were tested for their suitability for the microspore culture. NLN and Nitsch yielded similar amount of microspores (0.7 to 0.8%) containing 4 to 10 cells after 2 weeks incubation, out performing B5 and CHU (N6) (Table 3). In subsequent culture, we observed that the nuclei degraded more quickly with Nitsch than NLN, indicating that NLN was better beneficial for the microspore viability.

Role of different carbohydrates in sporophytic development

Effect of different carbohydrates on microspore culture is shown in Table 4. After incubation for 2 weeks, glucose and fructose gave around 3% microspores containing 4 to 10 cells, whereas sucrose and maltose gave lower percentages of 0.7 and 0.4%. The monosaccharides yielded significantly higher efficiency than the disaccharides. However, multicellular structures subsequently stopped growing and nuclei degraded in continuous culture with each carbohydrate. Further development, such as callus formation, was not obtained. In order to sustain development of multicellular structures, we initially cultivated microspores with glucose or fructose for short term of 3 to 14 days, from then microspores were transferred and cultured in the medium containing sucrose. The results were substantially improved with initial monosaccharide cultivation for 7 days. We observed the multicellular structures with more than 20 cells after 3 weeks (Figure 1c) and calli around 1 to 3 mm in diameter were formed after nine weeks (Figure 1d). Frequency of callus formation was around 2 calli per Petri dish of the culture (Table 4). While 3 days of initial monosaccharide cultivation yielded high percentages of microspores containing 4 to 10 cells in the early stage after 2 weeks of incubation, subsequently callus was not developed. With long time of 14 days, callus formation failed too.

Plant regeneration

In total, 24 calli were obtained in our experiments. For plant regeneration, calli of about 3 mm in diameter were transferred onto shoot regeneration medium. After incubation for one month, 18 out of 24 calli (75%) produced shoots (1 to 4 cm in length). All shoots were rooted on rooting medium after one month incubation

Table 3. Effect of different media on early sporophytic response in isolated microspore culture of *Z. aethiopica*.

Noumbers of cells per microspore	Percentage of microspores containing various numbers of cell after 2 weeks, with culture medium of			
	NLN	Nitsch	B5	CHU (N6)
0	70.0 ± 10.3	67.3 ± 14.3	71.7 ± 13.4	65.1 ± 10.6
1	18.7 ± 8.1	20.6 ± 6.6	19.6 ± 10.2	20.5 ± 7.1
2	9.4 ± 1.7	10.2 ± 6.9	8.5 ± 3.6	14.1 ± 3.6
3	1.1 ± 0.5	1.2 ± 0.5	0.2 ± 0.2	0.3 ± 0.3
4–10	0.8 ± 0.3	0.7 ± 0.3	0.0 ± 0.0	0.0 ± 0.0

Table 4. Effect of different carbohydrates on sporophytic response in isolated microspore culture of *Z. aethiopica*.

Carbohydrate	Incubation time	Percentage of microspores containing 4–10 cells after 2 weeks	Frequency of callus formation after 9 weeks(Number of calli/dish)
Sucrose	Continuous	0.7 ± 0.2^{d}	0.0 ± 0.0^{b}
Maltose	Continuous	0.4 ± 0.2^{d}	0.0 ± 0.0^{b}
Glucose	Continuous	2.7 ± 1.1^{c}	0.0 ± 0.0^{b}
	For 3 days	4.8 ± 0.3^{ab}	0.0 ± 0.0^{b}
	For 7 days	4.2 ± 0.2^{ab}	2.0 ± 1.0^{a}
	For 14 days	3.0 ± 0.2^{bc}	0.0 ± 0.0^{b}
Fructose	Continuous	2.4 ± 1.1^{c}	0.0 ± 0.0^{b}
	For 3 days	5.3 ± 0.4^{a}	0.0 ± 0.0^{b}
	For 7 days	4.5 ± 0.6^{ab}	2.0 ± 1.0^{a}
	For 14 days	2.9 ± 0.5^{c}	0.0 ± 0.0^{b}

Microspores were cultured using NLN with 13% sucrose, maltose, glucose or fructose continuously, or they were cultured with glucose or fructose for the initial 3–14 days followed by changing to NLN medium with 13% sucrose. Each experiment was repeated three times. Mean values ± SD are given. Mean values with a different letter are significant different at $P < 0.01$.

(Figure 1e) and all regenerants were green plants (Figure 1e, f). Chromosome number counting of 18 independent plants, each of which was taken from one callus, indicated that 8 plants were haploid, 6 were diploid and the rest were other numeric chromosomal constitutions. The sizes of diploid plants were larger in comparison to haploid plants (Figure 1f, g, h).

DISCUSSION

The specific role of different carbohydrates in the initiation of sporophytic development of microspores has been previously studied. It is known that disaccharides are more effective than monosaccharides in many crops, for example, sucrose is superior in *Brassica* spp. (Lichter, 1982; Arora and Bhojwani, 1988; Ilić-Grubor et al., 1998; Custers, 2003), whereas, maltose is optimal in many cereal crops (Ferrie et al., 1995; Jähne and Lörz, 1995; Indrianto et al., 1999; Obert et al., 2000). A detailed analysis of the glucose effect in barley show that the microspores do not develop into embryos if glucose is included in the culture during the first two days of incubation (Scott and Lyne, 1994a). Unexpectedly, we found that the monosaccharides were appreciated for early sporophytic response in *Z. aethiopica*. The monosaccharides are metabolized more rapidly than the disaccharides to release energy for cell development. It appeared that the rapid metabolization of carbohydrates was required for the induction of first sporophytic divisions in *Z. aethiopica*. On the other hand, carbohydrates can serve as signaling components (Smeekens and Rook, 1997). The monosaccharides may induce specific gene expression that plays important role in the switch from gametophytic to sporophytic pathway. While monosaccharides gave efficient early sporophytic induction, multicellular structures stopped growing in continuous culture. Sustainable development of callus only took place by combination with beneficial effect of sucrose in later time. Importantly, the role of initialmonosaccharide cultivation depended on length of incubation time. A period of 7 days was effective, whereas 3 or 14 days did not function. Previous studies have revealed that rapid metabolization of carbohydrates leads

to a decrease in osmolality in the medium and the accumulation of toxic products (Scott and Lyne, 1994a, b). These changes are harmful to microspore viability and longer time incubation causes more severe effect. These might be negative factors for microspore development with long time incubation of 14 days. However, it appeared that the failure of callus formation was not entirely due to osmolality change and toxic effect as shorter time of 3 days did not give callus formation too. The exact mechanisms remain to be investigated.

In our procedure, plants were regenerated via a callus phase. The pathway of microspore which gives rise to callus rather than to direct embryo formation is also observed in other crop (Wang et al., 2000). With optimal condition, we can calculate a yield of 3.5 valuable haploid and DH plants produced from one inflorescence giving 500 anthers. In *Zantedeschia* spp., almost the entire length of the spadix is covered with anthers, while hermaphrodite flowers are only present at the lower part. Collecting sufficient number of anthers is simple and yield of microspore isolation is quite high. In view of this, we might consider the microspore culture method as easy and efficient. In addition, obtaining of rooting plantlets took about 120 days in our experiments, shorter than the procedure reported by Ko et al. (1996) which lasts about 300 days.

Finally, we would state that a successful microspore culture method was established in *Z. aethiopica* in this study. Critical parameters were: (1) starting microspores at late-unicellular stage; (2) NLN as culture medium; (3) treatment of the culture at 32°C for 2 days prior to culture at 25°C; (4) an initial one week cultivation in the medium containing glucose or fructose, combined with subsequent beneficial effect of sucrose. The method would have wide potential applications for breeding programs of this ornamental. Application of the monosaccharides in the culture medium for initial short term cultivation is an essential and novel aspect of this method, which might be effective in other recalcitrant genotypes.

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