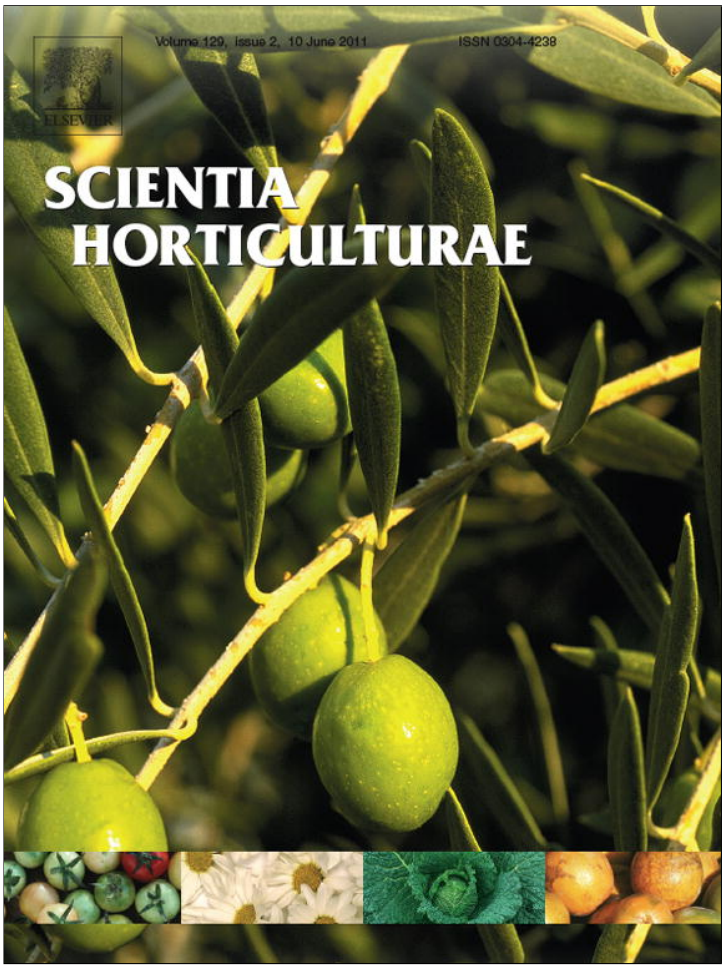

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Haploid plant production in *Zantedeschia aethiopica* 'Hong Gan' using anther culture

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ABSTRACT

This report describes advances in the anther culture of *Zantedeschia aethiopica*. Important factors for improvement as compared to the earlier procedure were: (1) using flowers from inflorescences developed at relatively low temperature during winter, (2) high temperature stress treatment at 32 °C for 2 days in the beginning of the culture, (3) use of Gamborg B5 as anther culture medium, and (4) addition of sucrose at high concentration of 8% in the culture medium. Plants were obtained via a callus phase. Frequency of anthers producing calli was around 4–5%. About 87% of the calli gave regenerants, of which 52% were haploid, 36% were diploid and the rest had other ploidy levels. In addition to chromosome counting, cytological examination of the microspore development and amplified fragment length polymorphism (AFLP) analysis of the regenerants showed that haploid as well as diploid plants originated from the microspores. Finally, 12 doubled haploid (DH) plants could be produced from each inflorescence. One quarter of the DHs equaled the original cultivar in growth vigor, while more than one third showed good fertility, indicating that inbreeding depression was not so severe in this heterozygous species. The improved protocol now enables production of sufficient number of DHs for application of haploid technology in genetic improvement and breeding of *Z. aethiopica*.

1. Introduction

Haploid plants can be generated from microspores through direct embryogenesis or via a callus phase in both anther and isolated microspore culture (Wang et al., 2000). Chromosome doubling, either spontaneous or induced, converts haploids into doubled haploid (DH) plants. In this way, homozygous plants can be obtained in a single generation, which might tremendously facilitate breeding programs (Forster and Thomas, 2005). Haploid technology not only significantly reduces the time needed to develop inbred lines for F_1-hybrid production, but also facilitates selection for recessive and polygenic traits. Haploid plants are also of benefit in genetic transformation and mutation studies (Ferrie et al., 2008).

Calla lily (*Zantedeschia* spp.), a genus of the *Araceae* family, is an important pot and cut flower worldwide (Kuehny, 2000; Snijder, 2004; Wright et al., 2005; Ni et al., 2009). Diploid plants in all *Zantedeschia* spp. contain 32 chromosomes (Yao et al., 1994). The genus consists of two sections, Zantedeschia and Aestivae. *Zantedeschia aethiopica*, the classic white calla lily, belongs to the section of Zantedeschia. This species gives white flowers, has a rhizomatous storage organ and is an evergreen. The species of the section Aestivae give colored flowers, have a tuberous storage organ and require a period of dormancy. Various commercial cultivars have been developed from crosses of different species. Modern cultivars of calla lily are propagated vegetatively by tissue culture and generally have a high degree of heterozygosity. Calla lily is also characterized by a long juvenile phase of 1–2 years. The heterozygosity and long juvenile period 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. Therefore, haploid plant production would be of great benefit to breeding and studying genetics in calla lily. To date, we know only one publi-

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzylaminopurine; IBA, indole-3-butyric acid; IAA, indole-3-acetic acid; KT, kinetin; NAA, naphthaleneacetic acid.

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cation of *Z. aethiopica* on haploid production by anther culture (Ko et al., 1996), but it is a long and quite inefficient procedure. Only one haploid plantlet was obtained from 296 cultured anthers (0.34%) in the paper. Here, we describe a faster and more successful anther culture procedure for haploid production in *Z. aethiopica*. Our preliminary research revealed certain factors that were crucial for haploid plant regeneration from the cultured anthers. This paper presents follow-up experiments that combined the crucial factors, resulting in reproducible yields of haploid and DH plants. Proof is provided that the plants originated from the microspores. Finally, the performance of the DH plants is reported.

2. Materials and methods

2.1. Plant material

Z. aethiopica cultivar 'Hong Gan' was used throughout the experiments. Donor plants were grown in an open field in Kunming, China. They were planted as rhizomes in 2004, and flowers for experiments were taken during 2007 and 2008. Temperatures during plant growth ranged from 16 °C to 25 °C during day and from 3 °C to 12 °C during night in spring (February to April), from 23 °C to 25 °C during day and from 12 °C to 18 °C during night in summer (May to July), from 19 °C to 25 °C during day and from 9 °C to 17 °C during night in autumn (August to October), and from 15 °C to 19 °C during day and from 2 °C to 10 °C during night in winter (November to January). Average photoperiods were 12 h-day/12 h-night, 14 h-day/10 h-night, 12 h-day/12 h-night, and 10 h-day/14 h-night in spring, summer, autumn, and winter, respectively. Water was applied through irrigation tubes at a depth of 1–5 cm, and N:P:K (16:16:16) granule fertilizer was applied every 2 weeks (10 g/m^2). Newly sprouting inflorescences in the length of 5–7 cm were harvested when they emerged for about 2 cm out of leaf sheath. The spadices were excised, dipped for 30 s in 75% (v/v) ethanol, followed by disinfection for 10 min in 1% NaOCl (w/v) with 0.05% (v/v) Tween-20, and then rinsed three times in sterile tap water. Almost the entire length of the calla lily spadix is covered with male flower buds (Fig. 1a), while hermaphrodite flowers are present at the lower part of the spadix. About 500 anthers were isolated from the middle part of each spadix and were used for culture. Staining with 4′,6-diamidino-2-phenylindole (DAPI) showed that these anthers contained about 10% of microspores in the mid unicellular stage and 90% in the late unicellular stage.

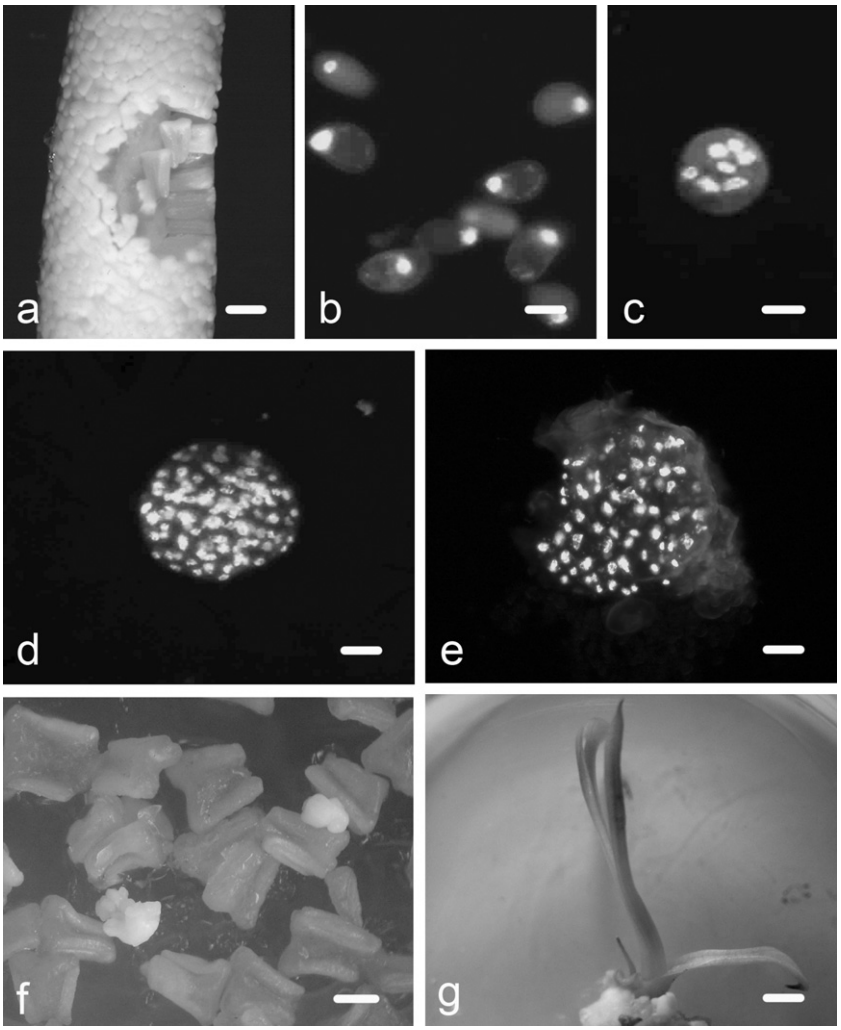

Fig. 1. Microspore development in anther culture of white calla lily (*Zantedeschia aethiopica*). (a) Middle part of inflorescence spadix covered with male flowers; in the hole in the middle are the anthers removed. (b) Late-unicellular microspores isolated from anthers at initiation of culture. (c) Microspore with 7 nuclei after 2 weeks of culture. (d) and (e) Multicellular structures after 3 weeks of culture; (d) round, enveloped in a periderm like a globular embryo, and (e) with irregular surface producing unorganized callus. (f) Visible microspore calli on anthers after 50–70 days of culture. (g) Shoot regenerated from callus after 30 days on shoot regeneration medium. Scale bars = 1.9 mm for (a); 30 μm for (b)–(e); 2 mm for (f); 6.7 mm for (g).

2.2. Callus induction from cultured anthers

Our preliminary research yielded a provisional protocol for the anther culture as follows: (1) spadices developed on plants in the winter are used, (2) anthers are cultured on Gamborg B5 medium (Gamborg et al., 1968) supplemented with 8% (w/v) sucrose, and solidified with 0.7% (w/v) Plant Agar (Duchefa Biochemie, The Netherlands), pH is adjusted at 5.8 before autoclaving, and plant growth regulators, 1.0 mg/l kinetin (KT) and 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) are filter-sterilized and added after autoclaving, (3) cultures are kept in a culture room at 32 °C during the first 2 days and then transferred to 25 °C at 60% humidity in darkness. Unless otherwise indicated the provisional protocol was used for the anther culture.

Early microspore development in cultured anthers was cytologically examined during the first 3 weeks. Anthers were opened with a scalpel and needle, and microspore samples were collected for DAPI staining and microscope observation. Twenty anthers were analyzed at each time point, and at least 100 microspores were examined from each anther. In order to study the effect of various factors on callus production from the anthers, the following parameters of the provisional protocol were tested in more detail:

- *Sucrose concentration* – Sucrose was applied at a concentration of 2%, 4%, 6%, 8%, or 10% (w/v).
- *Plant growth regulators* – Various combinations of cytokinins (KT or 6-benzylaminopurine [BA]) and auxins (2,4-D, indole-3-butyric acid [IBA], naphthaleneacetic acid [NAA], or indole-3-acetic acid [IAA]) were applied.
- *Culture medium* – Six culture media, B5 (Gamborg et al., 1968), MS (Murashige and Skoog, 1962), NLN (Lichter, 1982), S (Bourgin et al., 1979), N6 (Chu et al., 1975), and Nitsch (Nitsch and Nitsch, 1969) were compared. The media were purchased from Duchefa Biochemie, The Netherlands.
- *Temperature stress* – Cultures were kept at 4 °C or 32 °C for the first 1–7 days and then transferred to 25 °C, or they were continuously maintained at 25 °C.
- *Donor plant growing season* – Anthers were isolated from donor plants in three different seasons, spring, autumn, and winter.

Apart from the parameter under study, all the other factors were the same as in the provisional protocol. About 50 anthers were plated per 6.0 cm × 1.5 cm (diameter × height) Petri dish and 10 dishes were used per treatment. Each experiment was repeated 3 times. After 70 days of culture, the frequency of anthers producing calli was determined. Statistical analysis was performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA) for determining significant differences between treatment means.

2.3. Plant regeneration from calli

After 70 days culture, calli were removed from the anther tissue and subcultured on fresh anther culture medium for another 30 days to increase callus volume. Calli that had reached 3–5 mm in diameter were transferred onto shoot regeneration medium (MS medium supplemented with 3% sucrose, 0.7% Plant Agar, pH 5.8), and different concentrations of BA and NAA were tested. From then cultures were kept in a RXZ-380D climate incubator (Ningbo Jiangnan Instrument Factory, Ningbo, China) under a 16 h-light/8 h-dark cycle at 25 °C (60% humidity, 5000 lx light intensity provided by cold-white fluorescent lamps). Shoots developed (1–4 cm in length) after 1 month, and they were rooted by culturing on MS medium with 3% sucrose, 0.7% Plant Agar, and 0.1 mg/l IBA, at pH 5.8, for 30 days.

2.4. Ploidy analysis and chromosome doubling

The ploidy levels of the regenerated plants were determined by counting chromosome numbers in root tips. Root tips were cut off, pretreated with 0.002 M hydroxyquinoline at room temperature for 6 h, fixated in a mixture of alcohol and glacial acetic acid (3:1) at 4 °C for 20 h, macerated in 1 M hydrochloric acid at 60 °C for 5 min, then stained and squashed on slides with Cabol Fuchsin reagent (Carr and Walker, 1961). The samples were observed using a microscope.

Chromosome doubling of haploid plants was carried out by using colchicine. Plantlets with roots were immersed in sterile 0.01% or 0.05% (w/v) colchicine diluted in water for 12–48 h on a shaker, then rinsed 5 times in sterile tap water, incubated on MS medium with 3% sucrose, 0.7% agar, 1.0 mg/l BA, and 0.1 mg/l NAA, and maintained under the same conditions as described in Section 2.3. New shoots developed from axillary meristems and when large enough they were rooted as described above. Typically 2 axillary shoots developed per plant, which increased the chance of successful doubling. Chromosome numbers in root tips were examined again.

2.5. AFLP analysis

Leaf tissue was collected for genomic DNA isolation according to Doyle and Doyle (1990). Amplified fragment length polymorphism (AFLP) was carried out according to the procedure described by Vos et al. (1995). Genomic DNA (50 ng) was digested by *Eco*R I and *Mse* I, and ligated to double-stranded *Eco*R I and *Mse* I adaptors. The ligate was pre-amplified using nonselective primers (5′-GACTGCGTACCAATTCA-3′ and 5′-GATGAGTCCTGAGTAAC-3′). The selective amplification was carried out with selective primers (E-AAC: 5′-GACTGCGTACCAATTCAAC-3′, and M-CAG: 5′-GATGAGTCCTGAGTAACAG-3′). Products were separated on polyacrylamide gel, and silver staining was performed according to Bassam et al. (1991).

2.6. Characterization of regenerants

Sixteen haploid plants, 16 DH plants, randomly chosen, and 16 control plants of cultivar 'Hong Gan' were grown in open field conditions in 15 cm × 22 cm (diameter × height) pots containing a fertile horticultural soil. Temperature conditions and photoperiods were as described in Section 2.1. The plants were watered daily, and N:P:K (16:16:16) granule fertilizer was applied once a week (0.15 g/pot). One shoot was planted per pot, but gradually a number of shoots developed from the rhizome in the soil. At flowering stage, the average length of 3 fully expanded leaf petioles was determined to reflect the plant growth vigor in each pot. In the same way, the lengths of 3 fully expanded flower petioles were measured. Seed set of DH plants was examined and seeds were sown to test germination ability.

3. Results

In initial experiments, plantlet regeneration from 'Hong Gan' anthers in culture was only occasionally found, with an anther response ranging from 0% to 0.8%. However, we determined by close examination of the limited results that certain parameters appeared more important than others. The results could be considerably improved by the combination of some important parameters, namely, stock plant growth in winter, short heat-stress at 32 °C applied to the anther cultures, B5 as culture medium with addition of 8% sucrose and 1.0 mg/l KT + 0.5 mg/l 2,4-D. Using this procedure, callus first developed from the anther and plants thereafter regenerated from the callus.

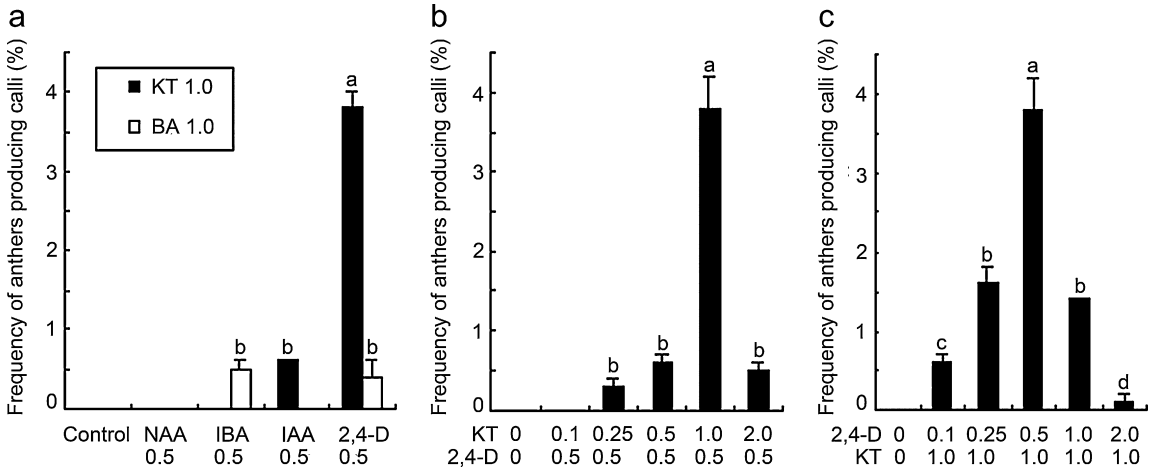

Fig. 2. Effect of different plant growth regulator combinations applied in the medium on callus formation in white calla lily (*Zantedeschia aethiopica*) anther culture. Each experiment was repeated three times. Standard deviations of the means are given on top of the bars. Mean values with a different letter are significantly different at $P < 0.01$. Control: Culture without plant growth regulators. (a) Different cytokinins (KT and BA at 1.0 mg/l) combined with different auxins (2, 4–D, IBA, IAA, and NAA at 0.5 mg/l). (b) Various concentrations of KT with 2,4-D at 0.5 mg/l. (c) Various concentrations of 2,4-D with KT at 1.0 mg/l.

3.1. Early development in culture

To analyze early microspore development, anthers were cultured according to the provisional protocol. Freshly incubated anthers contained primarily late unicellular microspores (Fig. 1b). After 1-week culture, about 20% microspores with 2 nuclei were found. In the majority of microspores, these first divisions were normal gametophytic pollen mitosis, yielding one vegetative and one generative nucleus. In 40% of these microspores, however, two similarly sized, vegetative-like nuclei were found, indicating that a sporophytic division had occurred. We occasionally observed microspores with 3–5 nuclei, resulting from reiterative sporophytic divisions, after 1-week culture. Table 1 presents the increase in numbers of nuclei in the microspores during the first 3 weeks of anther culture. After 2 weeks microspores with more than 5 nuclei were found (Fig. 1c), and after 3 weeks multicellular structures with more than 20 nuclei were formed (Fig. 1d and e). Approximately 30 such multicellular structures were observed per 100 anthers. From then on, development could take two directions: (1) direct embryo formation, if the level of plant growth regulators was decreased to 0, or (2) callus formation with continued plant growth regulator application. Frequency of embryo formation was lower than that of callus formation. Further, successful embryo germination took a long time and plant regeneration from callus was faster. Therefore, we decided to determine the route of plant regeneration via a callus phase.

Table 1
Percentages of microspores containing various numbers of nuclei counted after 0, 1, 2, and 3 weeks in white calla lily (*Zantedeschia aethiopica*) anther culture.

No. of nuclei per microspore	Percentages of microspores containing various numbers of nuclei after			
	0 w[a]	1 w	2 w	3 w
0	6.0	46.4	73.7	94.3
1	94.0	31.9	14	3.0
2	0	20.4	10	2.0
3	0	0.9	0.8	0
4	0	0.3	0.6	0
5	0	0.1	0.6	0.3
6–10	0	0	0.3	0.1
11–20	0	0	0	0.2
>20	0	0	0	0.1

[a] w: week.

Histological examination revealed that the anther wall tissue quickly died, and never produced callus. The callus always originated from the inside of the anther. Thus, we concluded that the microspores were the source of the callus formation. For the sake of convenience, however, in this paper the callus formation will be referred to as callus formation from anthers.

3.2. Effect of various parameters on callus formation

Sucrose concentration – Callus formation from anthers did not occur at the lowest (2%) and highest (10%) sucrose concentrations screened (Table 2). Frequency of anthers producing calli increased with increasing concentration from 4% to 8% sucrose, and the highest anther response of 4% occurred at 8% sucrose.

Plant growth regulators – Various combinations of cytokinins (KT and BA) and auxins (2,4-D, IAA, IBA, and NAA) were studied, at various concentrations. Combined application of a cytokinin plus an auxin was necessary for callus formation. The combination of 0.5 mg/l 2,4-D with 1.0 mg/l KT was the most effective, yielding a high anther response of 3.8% (Fig. 2a). In the combination of 2,4-D with BA, such a high response was not observed. The other auxins, IBA, IAA, and NAA, in the combination with the two cytokinins resulted in anther responses of 0.6% or lower (Fig. 2a). In the presence of 0.5 mg/l 2,4-D and varying concentrations of KT, an optimum anther response was found at 1.0 mg/l KT (Fig. 2b). The same optimum was found with KT at a constant concentration of 1 mg/l and varying concentrations of 2,4-D (Fig. 2c).

Culture medium – Six culture media, B5, MS, NLN, S, N6 and Nitsch, were compared for the anther culture. Callus formation from anthers occurred in four media, B5, MS, N6, and Nitsch, yielding 4.3%, 0.6%, 0.2%, and 0.2% anther response, respectively

Table 2
Effect of sucrose concentration on callus formation from white calla lily (*Zantedeschia aethiopica*) anthers in culture.

Sucrose (%, w/v)	Mean no. of anthers	Frequency of anthers producing calli (%)
2	554	0.0 ± 0.0 d
4	515	0.4 ± 0.1 c
6	614	1.0 ± 0.2 b
8	514	4.0 ± 0.3 a
10	542	0.0 ± 0.0 d

Note: Means ± SD are given. Mean values with a different letter are significantly different at $P < 0.01$.

Table 3
Effect of six different media on callus formation from white calla lily (*Zantedeschia aethiopica*) anthers in culture.

Medium	Mean no. of anthers	Frequency of anthers producing calli (%)
B5	514	4.3 ± 0.6 a
MS	672	0.6 ± 0.1 b
NLN	635	0.0 ± 0.0 b
S	502	0.0 ± 0.0 b
N6	498	0.2 ± 0.0 b
Nitsch	508	0.2 ± 0.1 b

Note: Means ± SD are given. Mean values with a different letter are significantly different at $P < 0.01$.

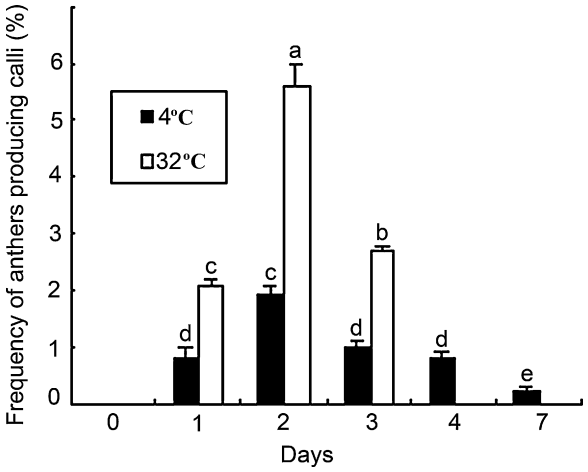

Fig. 3. Effect of temperature stress on callus formation in white calla lily (*Zantedeschia aethiopica*) anther culture. Cultures were kept at 4 °C or 32 °C for the first 1–7 days and then transferred to 25 °C, or they were continuously at 25 °C. The experiment was repeated three times. Standard deviations of the means are given on top of the bars. Mean values with a different letter are significantly different at $P < 0.01$.

(Table 3). B5 medium was optimal, resulting in significantly higher percentage of callus forming anthers than the other media.

Temperature stress – Both low (4 °C) and high (32 °C) temperature stresses for various durations (1–7 days) in the beginning of the anther culture were studied. Application of temperature stress was a prerequisite for the anthers to produce calli (Fig. 3). Both 4 °C and 32 °C were effective, each showing an optimum by 2 days treatment. Heat stress, however, highly outperformed the cold stress, yielding 5.6% anther response versus 1.9% after the cold.

Donor plant growing season – Anthers collected from donor plants grown in spring, autumn, and winter were compared for their ability to produce calli (Table 4). The most favorable period was the winter season, resulting in an anther response of 5.1%, significantly higher than during spring (0.2%) and autumn (0.3%).

Table 4
Effect of plant growing season on callus formation from white calla lily (*Zantedeschia aethiopica*) anthers in culture.

Season	Mean no. of anthers	Frequency of anthers producing calli (%)
Spring	514	0.2 ± 0.1 a
Autumn	673	0.3 ± 0.1 a
Winter	635	5.1 ± 0.5 b

Note: Anthers were collected from donor plants growing in spring, autumn, and winter. Means ± SD are given. Mean values with a different letter are significantly different at $P < 0.01$.

Table 5
Effect of plant growth regulator (PGR) application on shoot regeneration from white calla lily (*Zantedeschia aethiopica*) callus obtained in anther culture.

PGR treatment (mg/l)	Mean no. of calli	Frequency of calli producing shoots (%)	Shoot length (cm)
BA 0.5 + NAA 0.1	30	16.7 ± 1.9 cd	1–2
BA 0.5 + NAA 0.2	30	13.3 ± 5.1 cd	2–3
BA 1.0 + NAA 0.1	30	86.7 ± 8.8 a	3–4
BA 1.0 + NAA 0.2	30	73.3 ± 10.7 ab	3–4
BA 2.0 + NAA 0.1	30	56.7 ± 5.1 abc	2–3
BA 2.0 + NAA 0.2	30	43.3 ± 5.1 bc	2–3

Note: Calli that developed shoots were counted after 30 days cultivation and shoot length was measured. Means ± SD are given. Mean values with a different letter are significantly different at $P < 0.01$.

3.3. Plant regeneration

Quite large masses of calli appeared from the anthers after 50–70 days culture, while the anther wall tissue had turned brown and died (Fig. 1f). Regularly, frequency of anthers with calli was 4–5%. To facilitate plant regeneration, the visible calli were grown to a larger size of 3–5 mm by subculturing on anther culture medium for another 30 days. Proliferated calli were then cultured on MS medium supplemented with BA and NAA at different concentrations in order to induce plant regeneration. Shoots had already developed from the calli after 1 month (Fig. 1g), and combination of 1 mg/l BA with 0.1 mg/l NAA gave the highest frequency of shoot producing calli (86.7%; Table 5). For rooting of shoots, addition of IBA at 0.1 mg/l to the medium yielded 100% rooted plants after 30 days (Fig. 4a). In total, 245 independent plants were regenerated from experiments; only one plant was taken per callus. All regenerants were green plants and 90% survived transfer into soil.

3.4. Ploidy and AFLP analysis

Chromosome number determination indicated that 128 plants (52.2%) were haploid (inset in Fig. 4a), 89 (36.3%) were diploid, and 28 (11.4%) had other numeric chromosomal constitutions. For chromosome doubling, best results were obtained when haploid plants were immersed in 0.05% colchicine solution for 36 h. Fifty percent haploid plants yielded DH offspring (Table 6).

Amplified fragment length polymorphism analysis of randomly chosen diploid plants indicated that these plants had a haploid origin (Fig. 4b). While control donor plants showed similar pattern of AFLP bands, patterns of diploid regenerants from the anther culture showed polymorphic bands, implicating that they derived from the genetically segregating microspore population. The number of AFLP bands in the regenerants was reduced as compared to the number in the controls, indicating that regenerants were homozygous individuals versus the heterozygous cultivar.

3.5. Phenotypic characterization

The full-grown microspore-derived plants showed large phenotypic variation (Fig. 4c). Plant growth vigor based on leaf and flower petiole lengths widely differed (Table 7). In general, haploids were smaller than DH plants. A 25% part of the DHs reached similar sizes as the donor plants. Six out of 16 DHs (37.5%) produced seeds, and showed seed germination, indicating that the plants were fertile (Table 7, Fig. 4d), and as expected all haploid plants were sterile.

4. Discussion

Zantedeschia spp. belong to the *Araceae* family. Data on haploid production in this family are rare. Ko et al. (1996) reported on ant-

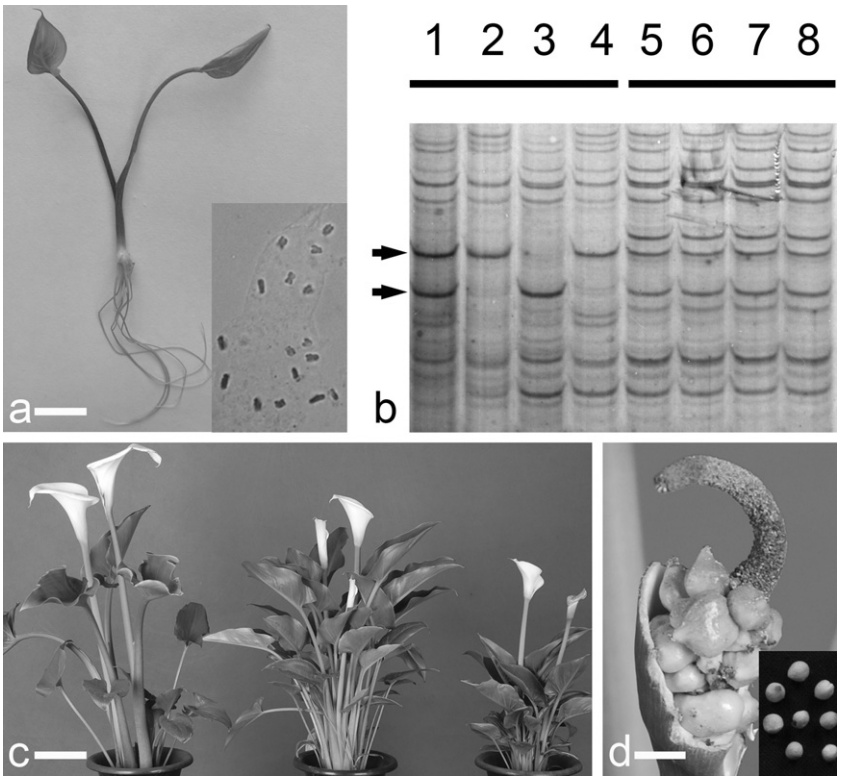

Fig. 4. Characterization of white calla lily (*Zantedeschia aethiopica*) plants obtained from anther culture. (a) Rooted haploid plant; inset shows root tip cell nucleus with $2n = X = 16$ chromosomes. (b) A section of AFLP banding patterns; lanes 1–4 are from 4 diploid plants from anther culture, and 5–8 from 4 individual donor plants. Arrows indicate polymorphic bands. (c) Phenotypes of 3 individual DH plants. (d) DH plant with fruit bodies at the base of the spadix; inset shows isolated seeds. Scale bars = 2 cm for (a); 12 cm for (c); 0.67 cm for (d).

Table 6
Chromosome doubling of haploid plants of white calla lily (*Zantedeschia aethiopica*) using colchicine.

Colchicine treatment		No. of plants treated	No. of surviving plants	No. of plants developing DHs
Colchicine concentration (%, w/v)	Immersion time (h)			
0.01	12	10	10	0
	24	10	9	1
	36	10	8	3
	48	10	7	3
0.05	12	10	9	2
	24	10	7	3
	36	10	6	5
	48	10	4	3

her culture of *Z. aethiopica* and Eeckhaut et al. (2001) published a paper on ovule culture in *Spathiphyllum wallisii*, but in both cases, numbers of haploid and doubled haploid plants produced were low. The paper by Ko et al. (1996) mentioned only the production of one haploid plant, and although diploids were produced as well, their genetic constitution was not studied in order to prove that they might be useful DHs. So, this former procedure had low practical value. In comparison, our paper increased the yield of haploid plant production, but also included essential studies and analyses, making the protocol be a well-proven tool for enhancing the breeding of *Zantedeschia*.

The most obvious differences between the Ko et al. (1996) protocol and ours are medium composition and sucrose concentration applied. Ko et al. (1996) used MS medium, but we found that anther response on B5 was roughly 7 times higher than on MS. The other four media tested, NLN, S, N6, and Nitsch, gave even lower anther responses. Superiority of B5 over MS has also been reported, for instance, in anther cultures of *Lilium longiflorum* (Arzate-Fernández et al., 1997) and *Cyclamen persicum* (Ishizaka and Uematsu, 1993). It has been speculated that the lower ammonium concentration

Table 7
Growth vigor and fertility comparison of white calla lily (*Zantedeschia aethiopica*) microspore-derived plants with donor plants grown in pots under open field conditions.

Plant generation	No. of plants in successive leaf petiole length classes				No. of plants in successive flower petiole length classes				No. of fertile plants
	10–15 cm	16–25 cm	26–35 cm	36–45 cm	15–30 cm	31–40 cm	41–50 cm	51–60 cm	
Haploid	2	14	0	0	9	7	0	0	0
DH	0	7	5	4	2	6	4	4	6
Donor	0	0	0	16	0	0	0	16	16

in B5 as compared to MS would enhance callus formation from the microspores. However, a low ammonium concentration is also present in the other media, N6, NLN, and S, which we used in our experiments, indicating that the excellent performance of B5 is not simply due to low ammonium level. Another component, for which B5 medium distinguishes itself from the other media, is thiamine HCl. B5 contains 10 mg/l of this vitamin, much more than in the other media. Thiamine functions as an enzymatic cofactor in carbohydrate metabolism to produce energy (Goyer, 2010). We found that 8% sucrose in the medium was optimal for anther response in 'Hong Gan', which concentration is much higher than the 3% earlier applied by Ko et al. (1996). Likely, both thiamine HCl and sucrose at high concentration play an important role in the energy management of the *Z. aethiopica* microspore cells.

The third parameter with a great impact on success of *Z. aethiopica* 'Hong Gan' anther culture was 32 °C heat stress treatment. Stress in various forms is commonly recognized as a main trigger to convert microspores from the gametophytic to the sporophytic development and thereafter initiation of embryogenesis (Shariatpanahi et al., 2006). Cold treatment at 4 °C was less efficient in our research, but the combination of low temperature and high temperature stresses might have an additional positive effect in *Z. aethiopica*, as recently was found in *Brassica oleracea* microspore culture (Yuan et al., 2011).

One drawback with anther culture is that the regenerants may originate from anther somatic tissue (Hidaka, 1984). This did not appear to be the case in our experiments. Firstly, cytological analysis of the early microspore development during anther culture, supplemented with histological observation of the anther wall tissue, indicated that the calli in our study originated from the microspores. Second, 52.2% regenerated plants were haploids, showing that they derived from haploid microspores. Third, amplified fragment length polymorphism analysis of the diploid plants obtained indicated that they were spontaneous doubled haploids. Thus, the plants obtained in our procedure derived originally from segregating gametophytes, and both haploids and diploids are valuable for breeding practice, as they both contribute to exhibiting the genetic variation of the original heterozygous donor.

In the chromosome doubling experiments, the optimal colchicine treatment was found to be also very phytotoxic, as 40% of the treated plantlets died. This loss of haploid and potentially DH plants reduces the efficiency of our protocol for *Z. aethiopica*. In order to minimize this disadvantage, following approaches are suggested: (1) to micro-propagate haploid shoots prior to the treatment with colchicine, and (2) to change colchicine for a less toxic chromosome doubling agent such as oryzalin (Van Tuyl et al., 1992).

Problem with heterozygous species is that homozygosity leads to inbreeding depression. Usually, the higher the degree of heterozygosity is, the stronger the inbreeding problems are. In our study, one quarter of the DHs equaled the original cultivar in plant growth vigor and over one third set seeds with germination ability, indicating that these plants were well fertile. Obviously, inbreeding depression was not so severe in *Z. aethiopica*. Due to this, fortunately, a reasonable part of the DH offsprings can immediately be used for breeding purposes, without need for first a longtime intercross and selection program aimed at restoring fertility of the homozygotes.

Our procedure includes a range of subcultures, each with specific success rate. Taking these into account, one could calculate a yield of 2.4 DHs per 100 anthers in the optimum protocol. This means that we can produce 12 DHs from one inflorescence giving 500 anthers. In the case of *Zantedeschia*, isolation of the anthers from the spadix is a simple operation, so collecting sufficient number of anthers is easy. Assuming that 200 DHs are needed for breeding application from each interesting heterozygous genotype, we might judge the present anther culture procedure as efficient. Condition, however, is that the described procedure works for a wide range of *Z. aethiopica* genotypes, or easily can be adapted to them.

Finally, we would state that the anther culture method described here would be of great interest for genetic improvement of *Z. aethiopica*. Now, our research will continue to develop haploid technology for the Aestivae section, which includes many economically more important colored cultivars (Snijder, 2004). In the anther cultures of *Z. rehmannii* and *Z. elliottiana*, we already observed that increasing sucrose concentration from 3% to 8% in the medium stimulated the occurrence of sporophytic divisions instead of the normal gametophytic pattern of the microspore development.

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References

Arzate-Fernández, A.M., Nakazaki, T., Yamagata, H., Tanisaka, T., 1997. Production of doubled-haploid plants from *Lilium longiflorum* Thunb. Anther culture. Plant Sci. 123, 179–187.
Bassam, B.J., Caetano, A.G., Gresshoff, P.M., 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. 196, 80–83.
Bourgin, J.P., Chupeau, Y., Missonier, C., 1979. Plant regeneration from mesophyll protoplasts of several *Nicotiana* species. Physiol. Plant. 45, 288–292.
Carr, D.H., Walker, J.E., 1961. Carbol Fuchsin as a stain for human chromosomes. Biotech. Histochem. 36, 233–236.
Chu, C.C., Wang, C.C., Sun, C.S., Hsu, C., Yin, K.C., Chu, C.Y., Bi, F.I., 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18, 659–668.
Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12, 13–15.
Eeckhaut, T., Werbrouck, S., Dendauw, J., Bockstaele, E.V., Debergh, P., 2001. Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis. Plant Cell Tissue Org. Cult. 67, 181–189.
Ferrie, M.R., Taylor, D.C., Mackenzie, S.L., Rakow, G., Raney, J.P., Keller, W.A., 2008. Microspore mutagenesis of *Brassica* species for fatty acid modifications: a preliminary evaluation. Plant Breed. 127, 501–506.
Forster, B.P., Thomas, W.T.B., 2005. Doubled haploids in genetics and plant breeding. Plant Breed. Rev. 25, 57–88.
Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrients requirements of suspension culture of soybean root cell. Exp. Cell Res. 50, 151–158.
Goyer, A., 2010. Thiamine in plants: aspects of its metabolism and functions. Phytochemistry 71, 1615–1624.
Hidaka, T., 1984. Induction of plantlets from anthers of 'Trovita' orange (*Citrus sinensis* Osbeck). J. Jpn. Soc. Hort. Sci. 53, 1–5.
Ishizaka, H., Uematsu, J., 1993. Production of plants from pollen in *Cyclamen persicum* Mill. through anther culture. Jpn. J. Breed. 43, 207–218.
Ko, J.A., Kim, Y.S., Eun, J.S., 1996. Embryogenesis and plant regeneration by the anther culture of *Zantedeschia aethiopica* spp. J. Kor. Soc. Hort. Sci. 37, 468–474 (In Korean).
Kuehny, J.S., 2000. Calla history and culture. HortTechnology 10, 267–274.
Lichter, R., 1982. Induction of haploid plants from isolated pollen of *Brassica napus*. Z. Pflanzenphysiol. 105, 427–434.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.
Ni, L., Li, X., Custers, J.B.M., Zhang, K., Zhang, L., 2009. Response of arum lily calli to culture filtrate of *Pectobacterium carotovorum* subsp. Carotovorum. Afr. J. Biotechnol. 8, 5362–5366.
Nitsch, J.P., Nitsch, C., 1969. Haploid plants from pollen grains. Science 163, 85–87.
Shariatpanahi, M.E., Bal, U., Heberle-Bors, E., Touraev, A., 2006. Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. Physiol. Plant. 127, 519–534.
Snijder, R.C., 2004. Genetics of Erwinia resistance in Zantedeschia: impact of plastome-genome incompatibility. Dissertation, ISBN:90-5808-975-4, Wageningen Univesiteit, The Netherlands.
Van Tuyl, J.M., Meijer, B., Van Diën, M.P., 1992. The use of oryzalin as an alternative for colchicines in *in vitro* chromosome doubling of Lilium and Nerine. Acta Hort. (ISHS) 325, 625–630.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T.V.D., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 4407–4414.

Wang, M., Bergen, S.V., Duijn, B.V., 2000. Insights into a key developmental switch and its importance for efficient breeding. Plant Physiol. 124, 523–530.

Wright, P.J., Triggs, C.M., Burge, G.K., 2005. Control of bacterial soft rot of calla (*Zantedeschia* spp.) by pathogen exclusion, elimination and removal. N. Z. J. Crop Hort. Sci. 33, 117–123.

Yao, J.L., Rowland, R.E., Cohen, D., 1994. Karyotype studies in the genus *Zantedeschia* (*Araceae*). S. Afr. J. Bot. 60, 4–7.

Yuan, S.X., Liu, Y.M., Fang, Z.Y., Yang, L.M., Zhuang, M., Zhang, Y.Y., Sun, P.T., 2011. Effect of combined cold pretreatment and heat shock on microspore cultures in broccoli. Plant Breed. 130, 80–85.