Study on karyotype and *in vitro* micropropagation of *Zephyranthes grandiflora* Lindl.

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ABSTRACT: This research aimed to study the plant morphology of *Zephyranthes grandiflora* and to compare the optimal synthetic medium for plant growth under *in vitro* culture. The Murashige and Skoog (MS) medium supplemented with various concentrations of BA and NAA as plant growth regulators. This experiment was completely randomized design (CRD) with three replications. The first factor (BA) was divided into three levels (0, 2 and 4 mg/L) and the second one (NAA) was comprised three levels (0, 0.5 and 1 mg/L). Both factors were added sugar 30 g/L, agar 8 g/L with pH 5.8-5.9 and cultured at 20 ºC for six weeks. The karyotype test from the root tip of *Z.grandiflora* was carried out by using the Feulgen squash technique. The results showed that the underground stem was bulb-like. The leaf was linear, obtuse, bunchy and large with two jagged edges. The flower was bright pink colour and funnel forms with six perianth petals, six and roecium and one gynoecium. Inferior ovary had three carpels and three loculs. The stigma had three jagged edges and seed was not observed in this time. After culturing for six weeks, the results exhibited that the MS medium supplemented with BA 0 mg/L and NAA 0.5 mg/L could induce the shoot growth averaged Shoot length Leave length number of Roots length. Total Weight 18.85±0.67, 10.37±0.51, 10.25±0.79, 5.23±0.55 and 5.14±0.46, respectively. Based on chromosome investigation, diploid set for *Z. grandiflora* was 2n = 48 and three groups of chromosome were classified including four metacentric (1, 2, 21 and 22), 10 submetacentric (3, 7, 9, 12, 14, 16, 17, 18, 23 and 24) and acrocentric (10 4, 5, 6, 8, 10, 11, 13, 15, 19 and 20) chromosome pairs, respectively.

Keywords: *Z. grandiflora*, morphology, plant tissue culture, karyotype

Introduction

Z. grandiflora is a monocot belonging to the genus *Zephyranthes* with approximately 65 described species. This is now distributed widely in tropical zones throughout the world (Hutchinson, 2003) in Amaryllidaceae. Nowadays, there are about 90 species (Tapia-Campos et al., 2012). It is habituated in the tropical zone of the Western, such as Central America and the Western Islands of India. Additionally, it distributes in the tropical zone and countries: North America, South of Mexico and China. In Thailand, it is imported to cultivate six types: *Z. rosea, Z. tubispatha, Z. candida, Z. ajax, Z. citrine and Z. grandiflora* (Chaisut, 1989) *Z. grandiflora* is one of the beautiful flowering and ornamental plants. It is cultivated easily. *Z. grandiflora* were considered an herb as a treatment for breast cancer (Intakarn et al., 1997). It is also used for plant breeding, that *Z. grandiflora* gene is transferred to the tobacco (Gangopadhyay et al., 2009) in order to resist aphids. Besides being cultivated as an herb, it is cultivated as commerce and earned the agriculturists incomes. Thus, it is cultivated widely and abundantly. In general, *Z.grandiflora* species are usually propagated by bulbs, which is the natural cultivation. This breeding is insufficient for the

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agriculturists' needs as it sometimes causes mutations. Moreover, Some of *Z. grandiflora* hybrids that have been bred for commercial purposes cannot be propagated by asexual or it is difficult to do so. As the results, agriculturists try to find out other means for propagation. *In vitro* micropropagation is one of the techniques that can be propagated plentifully in the short time. Unlikely to the natural propagation, it cannot mutant, be the disease-free plants, and be able to breed. The classification of plants according to taxonomy based on morphology only may be insufficient, so it requires no other manner assist in the classification as a study of the internal structure and to study cell genetics (De Bruyn et al., 1992) the study of chromosome morphology, which is in the classification of the genus. Because life is some kind of genetic variation is a little different, depend on habitat. In the present study, the taxonomy of *Z. grandiflora* use morphology only may not be enough to identify the species. *Z. grandiflora* breeding ensures genetic diversity in order to study the biology of *Z.grandiflora* taxonomy and cytogenetics. Conventional propagation of *Zephyranthes* species and its hybrids by bulb division are slow. This study was described botanical characteristic and cultured in synthetic medium supplemented with growth regulators to get the recipe appropriate by the *Z. grandiflora in vitro* culture. The increase in commercial breeding and conserve species and a *Z. grandiflora.* Study of cytogenetics of *Z. grandiflora* information can help in the classification and indicate the origin of species and evolutionary relationship.

Materials and Methods

Experiment 1

Study of *Z.grandiflora* was described and illustrated botanical characteristic including stems, leaves, flowers of Key for species of *Zephyranthea* present in Colombia (Fernandez-Alonso and Groenendijk, 2004).

Experiment 2

This experiment was 3X3 factorial in CRD. The culture medium contained Murashige and Skoog (1962) supplemented with plant growth regulator were three levels (0, 0.5 and 1 mg/L) of NAA and (0, 2 and 4 mg/L) BA arranged in 3 x 10 complete factorial (Table 1) The first factor (BA) was divided into three levels (0, 2 and 4 mg/L) and the second NAA) was comprised three levels (0, 0.5 and 1 mg/L). Both factors were added sugar 30 g/L, agar 8 g/L with pH 5.8-5.9. All of the media were added with 30 g/L sugar and 8 g/L, and pH was adjusted to 5.9. *Z. grandiflora*. Bulbs were either cut in cross section composed of leaf, bulb top, middle and scale explant on basal plate. Bulbs were trimmed to remove roots, leaves, and outer scale, washed in warm soapy water and wash with 15 % Clorox (Sodium hypercloride) drop of tween 20% 1-2 drops. Shaken for 15 minutes washed and three sterile water rinses. Sterilization Ethanol 70 % burned with fire 3 times, the bulbs were left in petri dish in the hood and bulb were trimmed and outer scales, removed to the medium. All samples were cultivated under light intensity of 2,500 lux at 25 ± 2 ° C for 16 h / day for 12 weeks. The growth explant was meas-

ured as plant height, leaf number, leaf size root length and fresh weight and the results were analyzed statistically using ANOVA. Values are means of three replicates and the mean values presented were separated using Duncan's multiple range tests at $p< 0.05$.

Table 1 Recipes used in medium

Treatment	Medium	Growth Regulator (mg/L)	
		BA	NAA
T1	MS	0	0
T2	MS	2	0
T3	MS	4	0
T4	MS	0	0.5
T5	MS	2	0.5
T6	MS	4	0.5
T7	MS	0	1
T8	MS	2	1
T9	MS	4	1

Experiment 3 Study karyotyping of *Z. grandiflora*

This study was used Feulgen squash technique (Sharma and Sharma, 1980), for cytological analysis root tips were used,pretreated with 2% Para dichrobenzene solution the annealing temperature of 60 °C throughout the night for 6 hours, and stored in frozen 4 °C until further analysis. The root was first washes 3 times for 5 mins. in distilled water, and wash with 70% ethanol, hydrolyzed for 10 mins. in 1N HCl, at 60 °C and stained with Aceto – Orcine 5 mins. the foundation on cover glass covered with crushed tissue of the slide and squashed to count and identify morphology, with light microscope, The term Metaphase cell counts 10 cells. Select chromosome fragmentation in metaphase on overlapping cell were photographed with 1000X measure the length of the chromosome arm .The long and the short chromosome uses occasionally it. These data calculate the Centromeric index (CI).

Results

The morphology of *Z. grandiflora*

Bulb white with brown sheaths, ovate, 2.4 - 3.2 cm in diameter. Blade narrowly linear, apex obtuse, 3-7 leaves per plant, 20 - 39.2 x 0.3-0.9 cm. Perfect flower, pink flowers, spathe dark, lilac. Perigone lage, pink, with a tube of 3 cm in diameter. Inflorescence 6 sepals with parallel edges of leaves per plant. The flowers are shaped funnel, sepals and petals called separable cloves with a total of 6 petals 5 cm. long and 2-3 cm. wide. Anther filament is erect and slender, style 5 cm. long, peduncle long and slender, 16.7 - 20.5 cm in diameter long hollow leaves, inflorescence covered with a thin piece of pollen (**Figure 1**).

Figure 1 *Z. grandiflora* Bar 5µm

Studies on the growth of *Z. grandiflora in vitro*

The results showed that after culturing for six weeks, the results exhibited that the MS medium supplemented with plant growth regulators such as only BA 0.5 mg/L concentration found that the formula can increase shoot length, leave length, leave width, root length and total weight at18.85±0.67, 0.28±0.01 , 10.37±0.51, 5.23±0.55 cm. and 5.14±0.46 g/shoot , respectively. The formula added plant growth regulators 0.5 mg/L NAA combined with 2 mg/L BA can multiply number of leaves the most at 11.45±1.59 leaves/shoot. The formula added 1 mg/L NAA can multiply number of roots the most at 11.60±0.89 cm/root. The formula added plant growth regulators 1 mg/L NAA combined with 4mg/L BA can increase the number of shoot the most at 2.80±0.24 cm/shoot. And increase the leave width the most at0.28±0.01 cm/shoot Figure 2. The formula basal medium without plant growth regulators has the average root length mostly at 5.11±0.51 cm /root (Table 2).

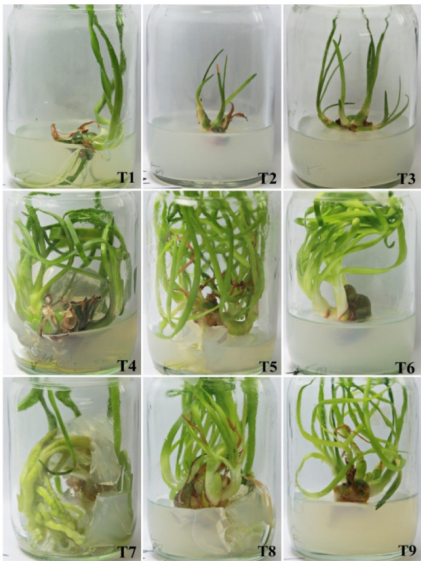

Figure 2 *Z. grandiflora in Vitro after* subculture with modify medium 12 week T1-T9 Modify medium with growth regulator

Study Karyotype of *Z.s grandiflora*

The number of chromosomes in *Z.grandiflora* metallic phase of all 10 cells showed a chromosome number of 2n = 48 chromosome (**Figure 3**) number 2n = 48 1000X magnification (Bar = 5µ). Study of *Z. grandiflora* makes the chromosome by using Feulgen squash Technique result of chromosome number in *Z. grandiflora* 10 cells showed a chromosome number 2n = 48 bar shooting cell meta phase . good disperse on And bring the Rugby Stipe (**Figure 3**) by measuring the average length of arm of chromosome the short (Ls) arm of chromosome side length (LI), length of the chromosome (LT) the Centromeric index (CI) , and type. chromosome 3 cells. For species *Z. grandiflora*, we observed 2n = 48 chromosomes (**Figure 3**)and karyotype with four metacentric, Ten submetacentric, and ten acrocentric pairs with karyotypic formula Metacentric = 1, 2, 21, 22 Submetacentric = 3, 7, 9 , 12 , 14 , 16 , 17 , 18 , 23, 24 Acrocentric 4 , 5 , 6, 8, 10, 11 , 13, 15 , 19, 20.

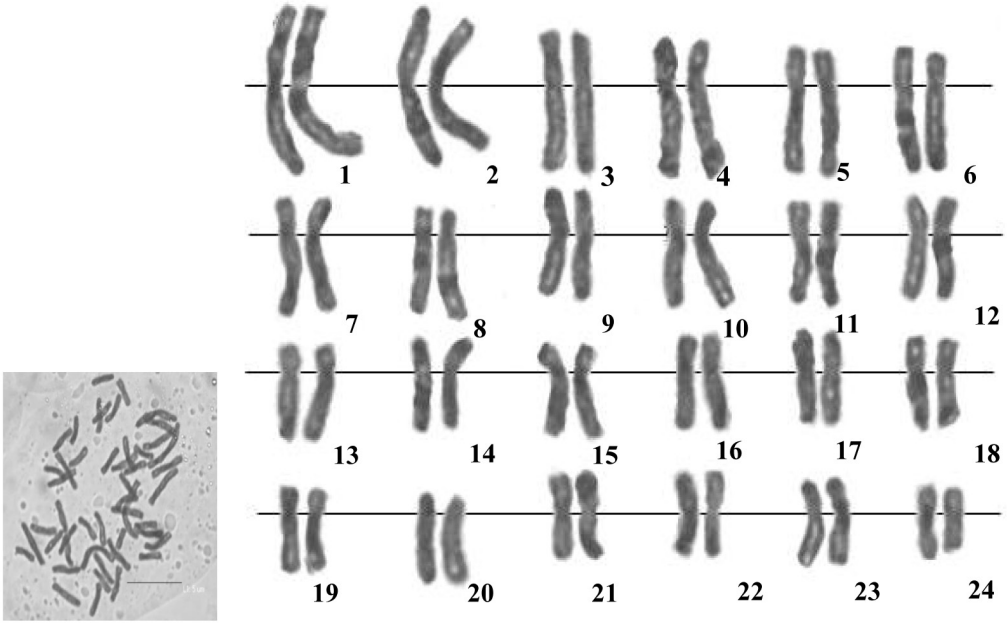

Figure 4 Giemsa-stained metaphase chromosomes of *Z. grandiflora* 2n = 48 Karyogram showed chromosome of *Z. grandiflora* (b) Bar 5µm Metacentric = 1 , 2, 21 , 22 Submetacentric = 3, 7, 9, 12, 14, 16, 17, 18, 23, 24 Acrocentric.4 , 5 , 6, 8, 10, 11, 13, 15, 19, 20

Table 2 Growth of *Z. grandiflora* on MS medium (1962) , including synthetic MS medium with addition of plant growth regulators

Treatments	Mean ± SE (N = 20)							
	Shoot length (cm)	Number 0f Shoot (cm)	Number of Leave (cm)	Leave length (cm)	Leave width (cm)	Number of Roots	Root length	Total Weight (g)
T1	11.31±0.92^{d}	1.70±0.19^{bc}	4.50±0.29^{de}	5.80±0.56^{d}	0.19±0.01^{cd}	5.10±0.57^{b}	5.11±0.51^{a}	0.92±0.07^{c}
T2	6.02±0.85^{e}	1.65±0.15^{bc}	3.95±0.55^{e}	3.02±0.41^{e}	0.18±0.02^{cd}	1.00±0.22^{cd}	0.70±0.18^{cd}	0.66±0.06^{c}
T3	6.245±0.65^{e}	2.70±0.24^{a}	7.15±1.06^{cd}	2.86±0.32^{e}	0.15±0.01^{d}	0.35±0.30^{d}	0.03±0.02^{d}	0.61±0.05^{c}
T4	18.85±0.67^{a}	2.20±0.25^{ab}	7.55±0.80^{cd}	10.37±0.51^{a}	0.28±0.01^{ab}	10.25±0.79^{a}	5.23±0.55^{a}	5.14±0.46^{a}
T5	16.25±1.18^{ab}	2.65±0.35^{a}	11.45±1.59^{a}	9.36±0.78^{ab}	0.24±0.01^{ab}	2.25±0.35c	1.13±0.22^{c}	2.54±0.24^{b}
T6	14.69±1.56^{bc}	2.30±0.25^{ab}	8.35±1.24^{bc}	7.89±0.96^{bc}	0.27±0.02^{ab}	1.95±0.49^{cd}	0.92±0.23^{cd}	3.33±0.52^{b}
T7	14.08±1.61^{bcd}	1.10±0.16^{c}	3.60±0.54^{e}	8.43±0.98^{abc}	0.22±0.02^{bc}	11.60±0.89^{a}	4.19±0.45^{b}	4.72±0.56^{a}
T8	12.45±1.33^{cd}	2.15±0.31^{ab}	9.80±1.82^{abc}	7.15±0.74^{cd}	0.25±0.02^{ab}	3.95±0.65^{b}	1.47±0.39^{c}	3.14±0.56^{b}
T9	15.09±0.73^{bc}	2.80±0.24^{a}	10.95±0.96^{ab}	9.34±0.49^{ab}	0.28±0.01^{a}	1.50±0.25^{cd}	0.44±0.07^{cd}	3.51±0.37^{b}

Means followed by the seam letters in the column are not significantly difference according to

DMRT at $p< 0.05$ (*)

Table 3 Comparison of the number of chromosomes of *Z. grandiflora* 2n = 48 bars.

Research report	Chromosomes of *Z. grandiflora*
Inariyama,1937	48
Coe,1954	48
Flory,1959	48
Flory, in Flagg,1961	48, 49,46-54
Kanyarat Chai. Sut , 1989	48
Kapoor and Tandom,1963	24
Kapoor and Tandom,1965	24
Tandom and Mathur, 1965	24
Tohibi Devi and Borua, 1997	24

The preparation of the chromosomes for analysis Rugby Stipe of *Z. grandiflora*. prepared from somatic cells of the root tip is ideal for the study rather than the end. Because the roots are areas where cell division (Zone of cell division, we find clear and large chromosome that is easily adapted to the study of chromosomes. You can also keep the roots more often than at the end depending on the type of plants used in this study (Chaisut, 1989). Chromosomes by Feulgen squash preparation technique make cells apart easy and fast, but also make the cells spread well that are cells to attach for glass making. It is easy to learn shapes and counts the number of chromosomes (Chaisut, 1989).

Discussion and Conclusion

This study uses key to species of *Zephyranthea* presented in Colombia. The results showed that the underground stem was bulb-like. The leaf was linear, obtuse, bunchy and large with two jagged edges. The flower was bright pink color and funnel forms with six perianth petals, six androecium and one gynoecium. Inferior ovary had three carpels and three loculs. The stigma had three jagged edges and seed was not observed at this time. Compare the plates by leaves color and shape flower separate *Z. robusta* and *Z. grandiflora*. The number of stamens of two or three deferent lengths ; anther more than 0.5 cm. size of Spath ; pergone and anthers *Z. grandiflora* more than *Z.roses* (Fernandez-Alonso and Groenendijk, 2004).

In vitro of *Z.grandiflora* bulb scale was the explant *in vitro* clonal propagation was reported from bulb-scale explants. In the present investigation of *Z.roseus*, callus induction, shoot regeneration, mode of morphogenesis two explant sources (bulb scale and flower bud) were used (Mujib et al., 2014) and later on the regenerated bulbs were encapsulated for conservation of plantlets. The results exhibited that the MS medium supplemented with BA 0 mg/L and NAA 0.5 mg/L could induce the shoot growth averaged shoot length leave length number of Root length.

Total weight 18.85±0.67, 10.37±0.51, 10.25±0.79, 5.23±0.55 and 5.14±0.46, respectively which is consistent with studies of *Z. grandiflora* (Mujib et al., 2014). The use of plant growth regulators, such as (NAA) combine (BAP), was found to be very effective for shoot bud development; maximum shoot number (11.50/ callus mass) was observed in NAA (0.5mg/L) BAP (1.0mg/L) added medium. Organogenesis and plant regeneration in *Z. rosea* Lindl. In other successful cases, bulb scales containing scale leaves with condensed basal stem (Gangopadhyay et al., 2009). In the present study, half scales (from the basal half of the bulb) and segmented bulbs with attached basal plate were found to be efficient sources for regeneration of *Z. grandiflora* shoots. Action of growth regulators, BAP and NAA in particular, in shoot induction has been reported by several workers on micropropagation of bulbous. While working with monocotyledon plants, we previously noticed that these groups of plants responded better to NAA/BAP treatments than with other PGRs applied . The differences in endogenous plant growth regulator level or altered requirement of nutrients may be involved in regulating in vitro morphogenetic responses (Mujib et al., 2014). Micropropagation provides an alternate means of large scale propagation but might induce somaclonal variation, making it mandatory to check genetic integrity (Gangopadhyay et al., 2009).

Histological and chromosomal study the chromosome number of *Z. grandiflora* that have a chromosome number 2n = 48 are consistent with reports that the chromosome number (**Table 3**) and a set of chromosomes is Octoploid (8n). According to Stebbins (1971) explains *Z. grandiflora* 2n = 24 (**Table 3**), the Tetraploid (4n) 2n = 48 of an Octoploid. The study could explain that *Z. grandiflora* 2n = 24, a forerunner of 2n = 48. But the bloom at different times during the making of the reproduction of the species cannot interbreed them that is separated into 2 cytotype is two kinds of different sizes, leaves and flowers, as well as the number of chromosomes. From the preparation Rugby Times in 3 cells showed chromosome 1, 2, 21 and 22 as type Metacentric match 3, 7, 9 , 12 , 14 , 16 , 17 , 18 , 23 and 24 as type. Sub metacentric and a 4, 5, 6, 8 , 10 , 11 , 13 , 15 , 19 and 20 as type Acrocentric which is consistent with reports of presented 2n = 24 with 4M 7SM 1A, however *Z. grandiflora* Lindl (Chaisut, 1989).

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References

Chaisut, K. 1989. Cytogenetics and Cell Taxonomy *Zephyranthes*. Department Botany, Faculty of Science Chulalongkorn University.

De Bruyn, M. H., M. Ferreira, M. Slabbert, and J. Pretorius. 1992. *In vitro* propagation of *Amaryllis belladonna*. Plant Cell Tiss. Org. 31: 179-184.

Fernandez-Alonso, J.L., and J.P. Groenendijk. 2004. A new species of *Zephyranthes* Herb. s. l.. (Amaryllidaceae, Hippeastreae), with notes on the genus in

Colombia. Rev. Acad. Colomb. Cienc. 28:177-186.

Gangopadhyay, M., D. Chakraborty, S. Dewanjee, and S. Bhattacharya. 2010. Clonal propagation of Zephyranthes grandiflora using bulbs as explants. Biol. Plantarum. 54: 793-797.

Hutchinson. 2003. The families of flowering plants. Oxford, Clarendon.

Intakarn, P., L. Eksomtramang, andK. Kanchanapoom. 1997. Karyotyping in *Zephyranthes rosea* Lindl. ants derived from tissue culture. Suranaree J. Sci. Technol. 4: 7-13.

Mujib, A., S. Banerjee, M. Maqsood, and P.D. Ghosh. 2014. Organogenesis and plant regeneration in *Zephyranthes rosea* Lindl.: Histological and chromosomal study. Plant Biosyst. 148: 492-498.

Murashige T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15: 473-497.

Sharma, A.K., and A. Sharma. 1980. Chromosome Techniques: Theory and Practice. 3rd edn. Butterworths, Woburn, MA.

Stebbins, G.L. 1971. Adaptive Radiation of Reproductive Characteristics in Angiosperms, II: Seeds and Seedlings. Annu. Rev. Ecol. Evol. Syst. 2: 237-260.

Tapia-Campos, E., J.M. Rodriguez-Dominguez, M.de los M. Revuelta-Arreola, J.M. Van Tuyl, and R. Barba-Gonzalez. 2012. Mexican Geophytes II. The Genera Hymenocallis, Sprekelia andZephyranthes. Floriculture Ornamental Biotech. 6: 129-139.