Induction of Tetraploid Ruzigrass (*Brachiaria ruziziensis*) by Colchicine and Possibility of Using Seed Weight as Screening Method

R. Poungkaew
Nakhonratchasima Animal Nutrition Research and Development Center, Thailand

Y. Mangpung
Nakhonratchasima Animal Nutrition Research and Development Center, Thailand

J. Arananant
Department of Livestock Development, Thailand

P. Chuchuay
Nakhonratchasima Animal Nutrition Research and Development Center, Thailand

S. Thaikua
Department of Livestock Development, Thailand

See next page for additional authors

Presenter Information
R. Poungkaew, Y. Mangpung, J. Arananant, P. Chuchuay, S. Thaikua, and P. Chanpeng

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Poungkaew, R[1]; Mangpung, Y[2]; Arananant, J[3]; Chuchuay, P[4]; Thaikua, S[5]; Chanpeng, P[6]

1 Nakhonratchasima Animal Nutrition Research and Development Center;
2 Nakhonratchasima Animal Nutrition Research and Development Center;
3Bureau of Animal Nutrition Development, Department of Livestock Development,
4Nakhonratchasima Animal Nutrition Research and Development Center,
5 Bureau of Animal Nutrition Development, Department of Livestock Development,
6 Pichit Animal Nutrition Research and Development Center

Key words: Brachiaria breeding, polyploidy induction, thousand seed weight

Abstract

Tetraploid ruzigrass (*Brachiaria ruziziensis*) have been obtained by colchicine treatment of in vitro apical meristems and embryogenic calli. The colchicine treatment consisted of culturing apical meristem on MS basal medium containing 6.0 mg L^{-1} 6-benzylaminopurine (BAP) and 0.05-0.20% colchicine for 8, 14, 20 and 26 h. After treated by colchicine, apical meristems were transferred onto former medium without colchicine for 4 weeks. Surviving apical meristems were induced multiple roots on MS basal medium containing 2.0 mg L^{-1} BAP. The ploidy level of plants after colchicine treatment was determined by flow cytometry. Apical meristems treated with 0.05% colchicine for 26 h. was identified as the optimum treatment and resulted in the highest frequency (13.3%) of tetraploid plants among the treatments tested on apical meristems. Embryogenic calli were induced on MS basal medium supplemented with 5.0 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 1.0 g L^{-1} casein hydrolysate, 0.5 mg L^{-1} kinetin and 5% coconut water. The colchicine treatment consisted of culturing embryogenic calli on MS basal medium containing 1.0 mg L^{-1} kinetin, 0.1 mg L^{-1} 1-napthaleneacetic acid (NAA) and 0.05-0.20% colchicine for 8, 14, 20 and 26 h. After treated by colchicine, embryogenic calli were transferred onto former medium without colchicine for 4 weeks. Shoots from embryogenic calli survived were induced multiple roots on MS basal medium containing 2.0 mg L^{-1} BAP. Two tetraploid plants were obtained from embryogenic calli treated with 0.10% colchicine for 14 h and 0.15% colchicine for 26 h. Total of 16 tetraploid genotypes were clonal propagated and grew in the field, with 1 diploid genotype as the control. It was found that the mean ± standard deviation of thousand seed weight (TSW) of all genotypes, tetraploid genotypes were 8.119 ±1.36 and 8.239 ±1.30 g, respectively, whereas TSW of diploid one was 6.209 g.

Introduction

One of the main pastures in Thailand is ruzigrass, primarily because of its high seed yields and nutritive value. Although the potential of ruzigrass for increasing pasture and animal productivity is well documented, but it is unable to adapt to grow in a long duration of drought, which causes forage crop lacking in dry season. Therefore, ruzigrass breeding for increased yield in dry season will be beneficial for Thai livestock farmers.

The genus of *Brachiaria* mainly consists of tetraploid (2n = 4x = 36) and apomictic species such as *B. decumbens and B. brizantha.* Sexuality is found in diploid species (2n = 2x = 18) such as *B. ruziziensis* (Valle *et al*., 1994). One of the limitations of *Brachiaria* breeding is the ploidy difference between sexual and apomictic plants, which prevents crossings, generates a small number of hybrids, and causes sterility (Valle *et al*., 2004). To overcome this problem, a promising strategy is the artificial tetraploidization of the diploid and sexual species *B. ruziziensis* and the subsequent hybridization with tetraploid and apomictic genotypes of *B. brizantha* and *B. decumbens* (Ishigaki *et al*., 2009). Antimitotic substances, such as colchicine, which is an alkaloid that is widely used in forage species, are used in the induction of polyploidy (Pereira *et al*., 2012). Several duplication studies have been conducted on *B.ruziziensis,* including seeds germinated (Swenne *et al*. 1981; Ishigaki *et al*. 2009; Timbo' *et al*. 2014) and multiple-shoot clums (Ishigaki *et al.* 2009). However, Thailand had no tetraploid ruzigrass yet causing the delay of *Brachiaria* hybridization breeding.

Methods and Study Site

Embryogenic calli formation

Lemmas and paleas were removed from spikelets of diploid ruzigrass, and seed were surface-sterilized in 10% and 5% (v/v) Chlorox (Clorox = 6% sodium hypochloride) for 5 and 10 min, respectively. Followed by three rinses with sterile water. Seeds were germinated on MS solid medium (Murashige and Skoog,1986) supplemented with 0.5 mg L^{-1} 2,4-D and 2.0 mg L^{-1} BAP at 25°C under a 16-h light condition for 1 week. Cultured apical meristems on MS solid medium supplemented with 5 mg L^{-1} 2,4-D, 1 g L^{-1} casein hydrolysate, 0.5 mg L^{-1} kinetin and 5% of coconut water at 25°C in the dark for 2 weeks and transferred to a 16-h light condition for 2 weeks. After that sub-cultured calli to the former medium at a 16-h light condition for 2 weeks.

Increasing embryogenic calli by cultured on MS solid medium supplemented with 0.6 mg L^{-1} 2,4-D and 0.1 mg L^{-1} kinetin at 25°C under a 16-h light condition for 4 weeks.

Colchicine treatment

Four hundred and eighty apical meristems were cultured on MS liquid medium supplemented with 6 mg L^{-1} BAP containing 0.05, 0.10, 0.15 and 0.2% colchicine for 8, 14, 20 and 26 h at 25°C in the dark. Followed by three washings with sterile water. Following colchicine treatment, apical meristem were transferred to MS solid medium supplemented with 6 mg L^{-1} BAP and cultured at 25°C under a 16-h light condition for 30 days for plant regeneration. Regeneration shoots were divided and sub-cultured on MS solid medium supplemented with 2 mg L^{-1} BAP cultured at 25°C under a 16-h light condition for 6 weeks to induced root development. Transferred plantlets to the pots. Four hundred and eighty embryogenic calli (~0.5 cm in diameter) were cultured on MS liquid medium supplemented with 1 mg L^{-1} kinetin and 0.1 mg L^{-1} NAA containing 0.05, 0.10, 0.15 and 0.2% colchicine for 8, 14, 20 and 26 h at 25°C in the dark. Followed by three washings with sterile water. Following colchicine treatment, embryogenic calli were transferred to MS solid medium supplemented with 1 mg L^{-1} kinetin and 0.1 mg L^{-1} NAA and cultured at 25°C under a 16-h light condition for 30 days. Separated shoots from survival embryogenic calli to cultured on MS solid medium supplemented with 2 mg L^{-1} BAP at 25°C under a 16-h light condition for 6 weeks to induced root development. Transferred plantlets to the pots.

Flow cytometry

After 4 weeks, non colchicine-treated ruzigrass plant, colchicine treated ruzigrass plants derived from apical meristems and embryogenic calli were proliferated shoots. Flow cytometry was performed to estimate the ploidy level of plant according to the method of Atichart and Bunnang (2007). Non colchicine-treated ruzigrass plant was used as the internal standard. Approximately 0.5 g of young leaves tissue from non-colchicine treated ruzigrass plant, colchicine-treated ruzigrass plants derived from apical meristems and embryogenic calli were chopped with a sharp razor blade in a 55-mm Petri dish. The hypotonic buffer Cy stain^{R} UV ploidy were added to the leaves tissue (1 mL of one step DAPI staining solution), then filtered through a 30 μm celltrics disposable filter. The samples were analyzed the flow cytometry with Partec PAII. The number and size of guard cells were examined after staining of the epidermis of leaves with safanine solution. Chrolophyll content was determined following the method of Arnon (1959).

Thousand seed weight (TSW)

After estimated the ploidy level of ruzigrass, planted non colchicine-treated ruzigrass (diploid) and various clones of tetraploid ruzigrass on breeding plot (36 plants/breeding plot). Take the seed from each breeding plot to do thousand seed weight determination according to the method of International Rules for Seed Testing (2015). Eight pure seed replicates of 100 seeds must be drawn randomly from the submitted sample. Each replicate weight is recorded in grams to three decimal places and the mean weight determined from these 8 replicates. The mean weight of 100 seeds is then used to calculate the weight of 1000 seeds. Variance, standard deviation and coefficient of variance must be calculated using the formulas. If the coefficient of variation does not exceed 6.0 then the thousand seed weight is accepted. If the limit is exceeded eight more replicates must be drawn and weighed.

Results

Effect of colchicine concentration and treatment duration on survival and chromosome duplication of apical meristem

Induction tetraploid plants from *in vitro* apical meristems, the apical meristems were cultured on MS liquid medium supplemented with 6 mg L^{-1} BAP containing 0.05, 0.10, 0.15 and 0.2% colchicine for 8, 14, 20 and 26 h. Only 32 individuals survived from all treatment. The ploidy level was estimated by flow cytometry, and fourteen out of 32 plants were identified to be tetraploid. Four tetraploid plants were obtained from apical meristems treated with 0.05% colchicine for 26 h, the treatment resulting in the highest frequency (13.3%) among treatments (table 1).

Table 1 Effect of treatment duration and concentration of colchicine on survival and chromosome duplication of apical meristem of diploid ruzigrass

Treatment duration (h)	Concentration (% w/v)	No. of explants treated	No. of explants survived (pieces/ %)	Ploidy*			
				2X	3X	4X (%)**	6X
8	0.05	30	6 (20.0)[a]	2	1	3 (10.0)[a]	0
8	0.10	30	5 (16.7)[b]	2	0	3 (10.0)[a]	0
8	0.15	30	1 (3.3)[b]	1	0	0[b]	0
8	0.20	30	1 (3.3)[b]	1	0	0[b]	0
14	0.05	30	4 (13.3)[a]	4	0	0[b]	0
14	0.10	30	0[b]	0	0	0[b]	0
14	0.15	30	0[b]	0	0	0[b]	0
14	0.20	30	1 (3.3)[b]	0	0	1 (3.3)[b]	0
20	0.05	30	3 (10.0)[a]	1	0	2 (6.7)[a]	0
20	0.10	30	1 (3.3)[b]	0	0	1 (3.3)[b]	0
20	0.15	30	1 (3.3)[b]	1	0	0[b]	0
20	0.20	30	0[b]	0	0	0[b]	0
26	0.05	30	7 (23.3)[a]	2	0	4 (13.3)[a]	1
26	0.10	30	1 (3.3)[b]	1	0	0[b]	0
26	0.15	30	1 (3.3)[b]	1	0	0[b]	0
26	0.20	30	0[b]	0	0	0[b]	0

*Ploidy level was estimated by flow cytometry. **Means in the same column followed by the same lowercase letter are not different at the 0.05 probability level.

Effect of colchicine concentration and treatment duration on survival and chromosome duplication of embryogenic calli

In order to induce tetraploid plants from embryogenic calli, the embryogenic calli were cultured on MS liquid medium supplemented with 1 mg L^{-1} kinetin and 0.1 mg L^{-1} NAA containing 0.05, 0.10, 0.15 and 0.2% colchicine for 8, 14, 20 and 26 h. Only 11 individuals survived from all treatment. The ploidy level was estimated by flow cytometry. Two tetraploid plants were obtained from embryogenic calli treated with 0.10% colchicine for 14 h and 0.15% colchicine for 26 h (table 2).

Table 2 Effect of treatment duration and concentration of colchicine on survival and chromosome duplication of embryogenic calli of diploid ruzigrass

Treatment duration (h)	Concentration (% w/v)	No. of explants treated	No. of explants survived (pieces/ %)	Ploidy*			
				2X	3X	4X (%)**	6X
8	0.05	30	0	0	0	0	0
8	0.10	30	0	0	0	0	0
8	0.15	30	1 (3.3)	1	0	0	0
8	0.20	30	0	0	0	0	0
14	0.05	30	3 (10.0)	3	0	0	0
14	0.10	30	3 (10.0)	2	0	1(3.3%)	0
14	0.15	30	0	0	0	0	0
14	0.20	30	0	0	0	0	0
20	0.05	30	1 (3.3)	1	0	0	0
20	0.10	30	1 (3.3)	1	0	0	0
20	0.15	30	0	0	0	0	0
20	0.20	30	0	0	0	0	0
26	0.05	30	1 (3.3)	1	0	0	0
26	0.10	30	0	0	0	0	0
26	0.15	30	1 (3.3)	0	0	1(3.3%)	0
26	0.20	30	0	0	0	0	0

*Ploidy level was estimated by flow cytometry.**There are no significant differences among different colchicine treatments (P>0.05)

Thousand seed weight (TSW) of non colchicine-treated ruzigrass (diploid) and tetraploid ruzigrass

Follow thousand seed weight test, we were found that tetraploid ruzigrass no.14 had most average TSW 11.17 g. But tetraploid ruzigrass no.4, 13, 16 and diploid ruzigrass had lowest average TSW 6.276, 6.201, 6.268 and 6.209 g, respectively (table 3).

Table 3 Thousand seed weight (TSW) of non colchicine-treated ruzigrass (diploid) and various clones of tetraploid ruzigrass (Moisture content of seeds 10%)

Clones	Thousand seed weight (g)
Diploid ruzigrass	6.209
Tetraploid ruzigrass No.1	8.161
Tetraploid ruzigrass No.2	8.655
Tetraploid ruzigrass No.3	8.278
Tetraploid ruzigrass No.4	6.276
Tetraploid ruzigrass No.5	9.345
Tetraploid ruzigrass No.6	9.232
Tetraploid ruzigrass No.7	9.293
Tetraploid ruzigrass No.8	8.119
Tetraploid ruzigrass No.9	8.303
Tetraploid ruzigrass No.10	8.522
Tetraploid ruzigrass No.11	8.547
Tetraploid ruzigrass No.12	8.366
Tetraploid ruzigrass No.13	6.201
Tetraploid ruzigrass No.14	11.17
Tetraploid ruzigrass No.15	7.080
Tetraploid ruzigrass No.16	6.268

Discussion [Conclusions/Implications]

In this work, we present the first success of tetraploid ruzigrass development in Thailand with various techniques. Previous study, tetraploid ruzigrass have been induced from seeds germinated (Swenne *et al*. 1981; Ishigaki *et al*. 2009; Timbo' *et al*. 2014) and multiple-shoot clums (Ishigaki *et al.* 2009). In this study, we tried to induced tetraploid ruzigrass from embryogenic calli because this techniques were also used for polyploidization in some species successfully such as the research of Chen and Goeden-Kallemeyn (1979), who induced tetraploid plants from cochicine-treated diploid daylily *Hemerocallis flava* L. callus. They found that over 50% of the plants initiated from the colchicine-treated calluses were completely tetraploid. Lan *et al.* (2010) induced polyploidy of Russian wild rye from colchicine-treated embryogenic calli, with the percentage of 53.58 tetraploid cell. However for our study, we could develop only 3.3%, of tetraploid. This low rate of tetraploid may be due to some embryogenic calli were died after treated by colchicine. Moreover some embryogenic calli were difficult to regenerated to plantlet. We also used apical meristems as the material same as the work of Wu *et al*. (2013), which successfully induced tetraploid from apical meristems of *Clematis heracleifolia*, with the percentage of 80. Unfortunately, we could produce only 13.3 % of tetraploid.

Although inducing by seedling is the simplest method, it could not produce the unique genotypes due to the segregation of genes in crossing species. Ishigaki *et al.* (2009) mentioned that multiple-shoot clums could be the appropriate technique to induce tetraploid as it can produce superior and unique tetraploid ruzigrass plants because multiple-shoot clumps can be clonally propagated through additional tissue culture application.

In this study, we also propose the simple screening of tetraploid genotypes using the weight of seeds. With this technique, we can easily decrease clone numbers for the expensive flow cytometry ploidy identification method.

Acknowledgements

This research was financially supported by Bureau of Animal Nutrition Development, Department of Livestock Development.

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