

Production of Salt Tolerant Ruzi Grass (*Brachiaria ruziziensis*) by Tissue Culture

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ABSTRACT

The best medium formula for multiple shoot induction was determined for seed culture of Ruzi grass (*Brachiaria ruziziensis*). Murashige and Skoog (MS) medium containing 10 mg/l BAP gave the highest average of 7.49 shoots. These shoots were irradiated at different doses of 0, 10, 30, 50, and 70 Gy and transferred to grow on the selected multiple shoot induction medium having different concentrations of NaCl at 0, 0.5, 1.0, 1.5 and 2.0%. It was found that the percentages of shoot survival decreased as the radiated doses increased. However, at the high salt concentration of 2.0% the growth was completely inhibited.

All 173 clones of surviving plant were transferred to grow in the natural field at the National Corn and Sorghum Research Center, Nakhon Ratchasima province. A set of replicated plants from this field was also grown under high saline soil at Borabue district, Maha Sarakham province for comparison. Fifteen healthy clones were chosen from these two sites and grown in the high saline soil for one year. Only ten surviving clones were collected from this second growth and propagated as salt tolerant Ruzi grass. Upon testing the DNA fingerprints of the original 15 healthy clones using AFLP technique with 10 pairs of primers, there was no differences in the banding patterns found among them.

Key words: Ruzi grass, AFLP, multiple shoot, gamma irradiation, salt tolerance

INTRODUCTION

Ruzi grass (*Brachiaria ruziziensis*) was originally found in Africa (Sudjipanontha, 1989) but distributed mainly in Zaire (or Congo) (Sampetch, 1989). It was grown in Thailand for the first time in 1968 at Thai-Denmark Dairy Farm, Saraburi province, and at Pak-Chong Animal Nutrition Research Center, Nakhon Ratchasima province (Jamsavad, 1994). The second set of Ruzi grass was from Ivory Coast and grown at Animal

Breeding Extension Division, Armed Forces Development Command, Nakhon Ratchasima province. Propagation of Ruzi grass was quite successful. They produced good quality seeds at the average of 437.5 kg/hectare.

Ruzi grass belongs to genus *Brachiaria*. This genus has been classified into almost 100 species, but only 5 species are commonly known, i.e., *Brachiaria brizantha* (cv. La Libriede, Marandee), *B. decumbens* (cv. Basilisk), *B. humidicola* (cv. Llanero, Tully), *B. mutica* and *B.*

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ruziziensis (cv. Kenedy). They are all perennial plants and propagated by seeds with apomixis type of reproduction. However, only *B. ruziziensis* or Ruzi grass is a cross-fertilized type. Its chromosome number is $2n = 2x = 18$ (Crowder and Chheda, 1982; Valle and Savidan, 1996). Ruzi grass gives high productive yield and positively responds to nitrogen fertilizer. The plant itself contains high protein which serves well as animal feed. Moreover, Ruzi grass can be grown in diverse types of condition. Although it prefers high fertility soil, it also tolerates drought and acid soil but can not be grown in the flooded area.

At present, cattle ranching is greatly promoted especially in the vast unused areas of the Northeastern part of Thailand. Ironically, the majority of these 2.85 million hectare land is saline-soil with different degree of salinity (Yuvaniyom, 1999). Salt tolerant grass could be obtained through the use of irradiation and tissue culturing in high salt medium. The aim in the production of salt tolerant Ruzi grass would provide not only high protein food source for cattle but also helped making the best use of high salinity soil in the Northeastern Thailand.

MATERIALS AND METHODS

Multiple shoot formation

The surface-sterilized seeds of Ruzi grass (*Brachiaria ruziziensis*) were cultured on four different formulae of multiple shoot induction MS medium containing 0, 6, 8 or 10 mg/l of BAP at $25 \pm 2^\circ\text{C}$ for 6 weeks. The number of shoots produced in each medium formula was recorded. Each shoot was individually separated to repeatedly grow in the same medium formula for 6 weeks to increase shoot formation. The medium giving the highest number of shoots was then selected for further investigation.

Multiple shoot production from a single seed

Since Ruzi grass is a cross-fertilization plant, each seed is also genetically different. To

obtain a large number of shoots of the same genetic composition, a single seed culture in the selected medium formula was performed. The shoots from the same seed were then replicated in the same medium for further evaluation.

Induced mutation

The shoots from a single seed culture were subject to gamma irradiation at different doses of 0, 10, 30, 50 or 70 Gy. They were then grown in the selected medium containing NaCl at 0, 0.5, 1.0, 1.5 or 2% for six weeks. The number of surviving shoots was recorded.

Field experiment

The surviving shoots were cultured in the root induction MS medium for four weeks. They were transferred to grow in the plastic bags for one month. The healthy plants were then selected to grow in the natural field at the National Corn and Sorghum Research Center. After 45 days of field growth, their height, number of tillers per plant, leaf length, and stem structure were evaluated. Plants with superior characters were chosen for further use.

Selection for salt tolerance

The selected grasses with good characteristics were singly separated from the clump and replicated to increase the number of plants. They were transferred to grow in the saline soil at Borabue district, Maha Sarakham province for one year. The surviving plants were collected as salt tolerant Ruzi grass.

DNA fingerprinting by AFLP

Fifteen clones of irradiated plant grown at the National Corn and Sorghum Research Center were analyzed using AFLP technique with ten pairs of primers, i.e., E-ACC/ M-CAC, E-AAC/ M-CTT, E-AAG/ M-CAA, E-AAG/ M-CAC, E-ACA/ M-CTA, E-ACA/ M-CTC, E-ACC/ M-CAA, E-ACC/ M-CTT, E-ACG/ M-CAG, and E-ACG/ M-CTT. Their DNA fingerprint patterns

were determined and compared.

RESULTS AND DISCUSSION

Multiple shoot formation

Ruzi grass seeds cultured in multiple shoot induction MS medium containing different amount of BAP for six weeks gave the highest average of 3.19 shoots/seed in the medium supplemented with 6 mg/l BAP while those supplemented with 8 or 10 mg / l BAP also gave quite satisfactory average numbers of 2.80 and 2.73 shoots / seed compared to the control of 0 mg / l BAP of only 1.12 shoots / seed (Table 1 and Figure 1). Further culturing of these single shoots in the same MS media containing either 0, 6, 8, or 10 mg/l BAP for four weeks gave the averages of 2.00, 5.21, 6.94 and 7.49 shoots, respectively (Table 2). This result supported the finding of Watanabe *et al.* (1999) on culturing of lateral buds of *Populus euphratica* in half MS medium containing 0.25 mg/l of BAP confirming the

positive effect of BAP on multiple shoot induction. It was clearly seen that the number of shoot produced from each individual single shoot increased correspondingly to the increasing amount of supplemented BAP, thereby, making the MS medium containing 10 mg/l BAP the most effective formula for multiple shoot induction.

Multiple shoot production from a single seed

After transferring single shoots to different MS medium formula for four weeks, multiple shoots were obtained. Table 2 shows that MS medium supplemented with 10 mg / l BAP gave the highest average number of 7.49 shoots per mother shoot. These shoots contained the same genetic composition and were used for mutation induction in the subsequent experiment.

Induced mutation

Shoots resulted from a single seed culturing exposed to gamma irradiation at 0, 10, 30, 50 or 70 Gy were cultured on MS medium

Table 1 Seed culturing of Ruzi grass on MS medium supplemented with BAP for six weeks.

MS medium supplemented with BAP(mg/ l)	Number of seeds		Total number of shoots		Average shoots per seed		Average total shoots per seed
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2	
0	3	4	3	5	1.00	1.25	1.125
6	25	17	83	52	3.32	3.06	3.190
8	20	25	60	65	3.00	2.60	2.800
10	19	21	66	42	3.47	2.00	2.735

Table 2 Multiple shoot production from a single shoot transferring to the same MS medium formula after four weeks of culturing.

MS medium supplemented with BAP (mg/ l)	Number of single shoots	Total number of shoots	Average number of multiple shoots per mother shoots
0	3	6	2.00
6	29	151	5.21
8	32	222	6.94
10	37	277	7.49

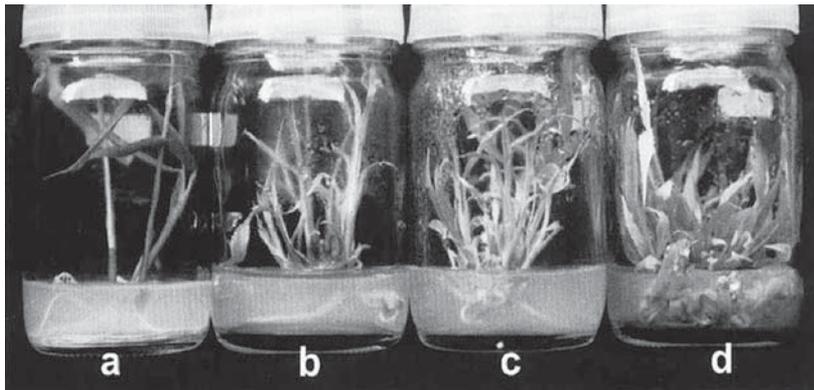


Figure 1 Multiple shoot formation of Ruzi grass seed cultured on different medium formulae.

- a. MS medium (control)
- b. MS medium supplemented with 6 mg/ l BAP
- c. MS medium supplemented with 8 mg/ l BAP
- d. MS medium supplemented with 10 mg/ l BAP

supplemented with 10 mg/ l BAP and different NaCl concentrations of 0, 0.5, 1.0, 1.5 and 2.0%. The medium having 2.0% NaCl could not support growth of the irradiated shoots. However, the 10-Gy-irradiated shoots could sustain in the saline medium better than those of the controlled non-irradiated shoots. As for the irradiated shoots at higher levels of 30, 50 and 70 Gy, they became weak and obviously could not survive as the percentage of NaCl was increased to 2% (Table 3). This agreed with the results obtained by Aranananth (2001) using five levels of gamma irradiation to induce salt tolerance in dwarf Napier grass.

Field experiment

The surviving shoots from the saline culturing were grown in the root induction MS medium containing no growth regulator until they reached their mature stage and transferred to grow in plastic bags. All 173 surviving clones were transferred to the field at the National Corn and Sorghum Research Center, Nakhon Ratchasima province, for 45 days. The plants were diversely different considering the height, the number of tillers per plant, stem types (straight, semi-

climbing, climbing), colors of leaf sheath (green, light red, deep red), and leaf types (hairy, not hairy). As for the width and length of the leaf, they were in the range of 1.0 - 2.2 cm and 25-30 cm, respectively. The most distinctive feature of elliptical leaf having the width of only 1 cm wide was found in the clone irradiated with high dose of 70 Gy and transferred to grow in MS medium without NaCl (Figure 2). This typical narrow leaf grass was a preferred type to make hay for animal feed and was selected for further propagation in



Figure 2 Narrow-leaf character of Ruzi grass resulted from high dose (70 Gy) gamma irradiation and cultured in MS medium without NaCl.

the cattle farm. The narrow leaf character was suspected to be the result of gamma irradiation.

Selection for salt tolerant grasses

All 173 clones of irradiated surviving grasses were separated into clumps and transferred to grow at Borabue district, Maha Sarakham province, where the soil was highly saline as seen

from whitish covering of salt on the soil surface. Fifteen clones with preferred characters were selected from the two growing sites with five replicates of each clone were transferred to grow at Borabue district to confirm their salt tolerant quality. Ten clone numbers 3, 5, 6, 7, 8, 10, 12, 13, 14 and 15; (Figure 3) were finally selected for having distinctive characters, i.e., clone number

Table 3 Survival percentage of irradiated Ruzi grass shoots grown on multiple shoot induction medium having different levels of NaCl concentration.

Irradiation dose (gray)	NaCl (%)	Number of shoots	Number of surviving shoots	Surviving percentage	Average of surviving percentage
0	0.0	72	64	88.89	56.28
	0.5	89	70	78.65	
	1.0	85	64	75.29	
	1.5	83	32	38.55	
	2.0	102	0	0.00	
10	0.0	99	71	71.72	59.36
	0.5	101	91	90.10	
	1.0	89	70	78.65	
	1.5	87	49	56.32	
	2.0	100	0	0.00	
30	0.0	102	65	63.73	40.07
	0.5	101	65	64.36	
	1.0	83	46	55.42	
	1.5	95	16	16.84	
	2.0	101	0	0.00	
50	0.0	102	62	60.78	24.55
	0.5	101	23	22.77	
	1.0	90	28	31.11	
	1.5	99	8	8.08	
	2.0	101	0	0.00	
70	0.0	109	27	24.77	9.36
	0.5	103	10	9.71	
	1.0	88	9	10.23	
	1.5	95	2	2.11	
	2.0	102	0	0.00	

15 had wide leaf while clone number 10 gave narrow leaf of only 1 cm in width. The remaining clones gave either high number of plants per clump, fast growing, or high stem. These chosen clones were then further propagated and cross-fertilized. The seeds of this growth are considered synthetic variety and could be distributed as salt tolerant grass seeds.



Figure 3 Surviving salt tolerant Ruzi grass collected from high saline soil at Borabue district, Maha Sarakham.

DNA patterns of irradiated salt tolerant Ruzi grass

The AFLP markers determined from 15 selected clones were not different (Figure 4). The ten pairs of primers used in this DNA identification could not classify their genetic difference even their morphology were clearly observed. The mutation of this sort could possibly take place at certain or specific gene location which was yet to be identified. More primer combinations were suggested to use for detecting their fingerprinting difference. Other molecular technique with co-dominant banding patterns, i.e., SSCP and SSR can also be used to elucidate their genetic change as well.

CONCLUSION

MS medium containing 10 mg/l BAP gave the best multiple shoot induction for seed culturing of Ruzi grass. After gamma irradiation and transferring to grow in the multiple shoot induction media supplemented with NaCl, the survival rate of shoots declined with the increasing dose of irradiation but none of the irradiated shoots

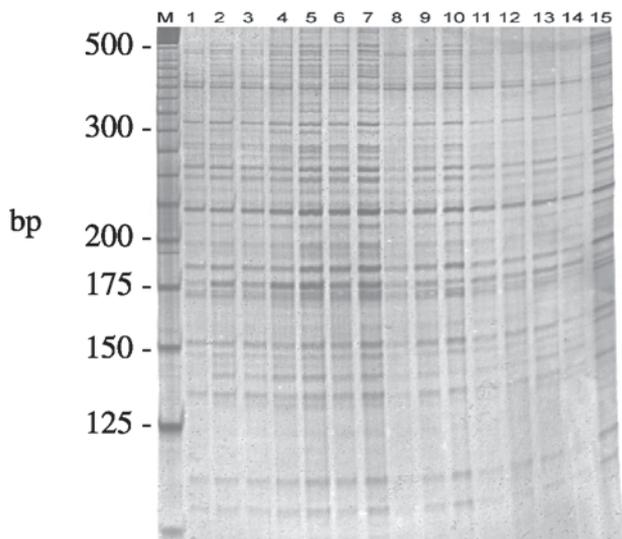


Figure 4 DNA fingerprinting pattern of 15 selected clones of irradiated and cultured Ruzi grass using AFLP technique and E-ACC/M-CTT primers.

could survive in the MS medium supplemented with 2 % NaCl. The field growth of surviving irradiated and salt-cultured plants gave diverse characters of plant height, the number of tillers per plant, stem type, leaf-sheath color, hair-type of leaf, leaf width and length. However, the preferred type of good characters, i.e., narrow leaf and fast growing grass were selected for further propagation. Ten clones were selected from high salinity field growth as salt tolerant grasses. There was no difference in DNA pattern found in the selected salt tolerant clones of Ruzi grass using AFLP technique and 10 pairs of primers.

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