

Inducing Salt Tolerance in Purple Guinea Grass (*Panicum maximum* TD58) via Gamma Irradiation and Tissue Culture

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ABSTRACT

The seeds of *Panicum maximum* TD58 were cultured on MS medium supplemented with 15% coconut water, 5 mg/l kinetin and 100 mg/l casein hydrolysate to induce multiple shoots. The gamma irradiated shoots grown in 0–2.0 % NaCl gave 58 clones of purple guinea grass. All clones were transferred to grow in the natural field. Ten clones of grass with good morphological properties were selected and subsequently grown in the salt stress environment at Maha Sarakham province, Northeastern Thailand. Under salt stress condition, seven out of ten selected clones showed salt tolerant trait. However, there was no difference in AFLP fingerprinting patterns found as compared with the controlled non-irradiated guinea grass. The salt tolerance character might be due to the mutation at a certain location or on a specific gene which could not be distinctly detected by the available AFLP primers.

Key words: *Panicum maximum*, gamma ray, tissue culture, salt tolerance

INTRODUCTION

The uprise in population growth has made the high demand of food supply throughout the world. Cattle farmings, therefore, have been promoted to alleviate the food shortage in Thailand. The subsequent insufficiency of animal feed has led to the high cost of feed import as seen from the record of the Department of Livestock Development (1995) showing the alarming increase of its cost each year. Purple guinea grass (*Panicum maximum* TD58) is one of the cattle feed introduced into Thailand from Ivory Coast, Africa. The high protein content in this grass guaranteed its quality as one of the best nitrogen sources for

animal growth. Purple guinea grass is a perennial plant which can be grown in a wide range of soil texture. Had it been modified to tolerate salt stress, it would be considered high potential for the harsh saline soil of Northeastern Thailand which covers more than 6 million hectare. This waste infertile land could be transformed into cattle farming land and hence bring about the economic growth and more income to the local population of the Northeastern.

Gamma irradiation through tissue culture has been used for modification and selection of specific traits in many kinds of plant, e.g. rice, cereal crops, and some forage crops. Recent studies on the quality improvement of guinea grass in

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tissue culture and protoplast culture have been reported (Saura *et al.*, 1987; Akashi and Adachi, 1991, 1992, 1993; Krishna and Vasil, 1995; Herrera, 1997; Shivashanker *et al.*, 1988; Lajonchere *et al.*, 1993). The outcome has led to a successful protoplast fusion of doris grass and guinea grass (Akashi *et al.*, 1995). However, there is no report on purple guinea grass (*P. maximum* TD58) development especially on the attempt to find its appropriate medium formula for multiple shoot induction and the effect of gamma rays and tissue culture on the growth of this grass in the salt stress condition.

MATERIALS AND METHODS

Explant preparation

Purple guinea grass (*P. maximum* TD58) seeds were selected and surface sterilized with 95% ethanol. The multiple shoots of this grass were induced by culturing the seed on MS (Murashige and Skoog, 1962) medium supplemented with 15% coconut water, 100 mg/l casein hydrolysate and different combinations of growth regulators (BAP, kinetin and NAA) in 19 cultured formulae. The seeds were cultured at 25±2 °C with light intensity of 2000 lux for 6 weeks. The medium furnished with the highest number of shoots was selected after 6 weeks of culture.

Gamma irradiation

The multiple shoots of *P. maximum* TD58 were irradiated with ¹³⁷Cs at the levels of 0, 10, 20 and 30 Gray (Gy). After irradiation, shoots were transferred to a selected medium and added with different concentrations of NaCl (0, 0.5, 1.0, 1.5 and 2.0 %). They were maintained at 25±2 °C with light intensity of 2000 lux for 4 weeks.

Field observation

Plantlets of *P. maximum* TD58 were selected based on the good traits such as forage crop properties and/or salt tolerant trait. The plantlets were duplicated and grown at The

National Corn and Sorghum Research Center, Nakhon Ratchasima province for 45 days. The grasses with good characteristics were selected and transferred to grow in the field having high salt stress condition at Borabue district, Maha Sarakham province, Northeastern Thailand, for a year.

DNA fingerprinting pattern

Amplified Fragment Length Polymorphism (AFLP) technique was used to identify the DNA fingerprinting pattern of the 10 selected clones of salt tolerant trait grown at The National Corn and Sorghum Research Center. Ten pairs of primers were chosen for DNA identification.

RESULTS AND DISCUSSION

Selection of seed culturing formulae

Four distinctive medium formulae giving high total shoot induction (>100 shoots) and high number of shoot per seed (>3.00) were MS supplemented media containing either 1-2 mg/l BAP or 5 mg/l kinetin or 4 mg/l kinetin + 1 mg/l NAA (Table 1). Although BAP and NAA seemed to contribute the high shoot induction to a certain extent, good characteristics of plantlets were clearly seen in the medium having kinetin at 5 mg/l (Figure 1). Kinetin is a cytokinin that helps promote cell division and stimulate the initiation and growth of shoots *in vitro* (Trigiano and Gray, 2000). The unfolding of a complex gene expression program in tissue culture is promoted by cytokinin that results in the formation of shoots (Howell *et al.*, 2003). Based on the published reports of Lu and Indra (1985), Akashi and Adachi (1993) and Ross *et al.* (1995) as well as our own investigation (unpublished data), the positive effect of kinetin on shoot induction in *Gramineae* such as *Panicum* species and *Brachiaria ruziziensis* (Ruzi grass) were shown. In this experiment, multiple shoot initiation began after approximately 6 days in culture. The result showed that the higher

concentration of kinetin, the more induction of multiple shoots occurred (Table 1) which means that *P. maximum* TD58 could respond to kinetin

better than to BAP or the combination of kinetin and NAA or even the combination of BAP and NAA.

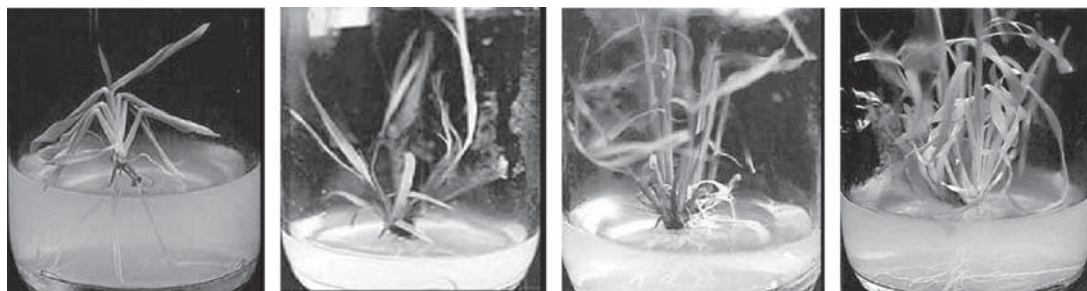


Figure 1 The characteristics of multiple shoots induced from seeds cultured on MS medium supplemented with 15 % coconut water, 100 mg/l casein hydrolysate and different combinations of growth regulators. The shoots were cultured for 6 weeks. A) 1.0 mg/l BAP, B) 2.0 mg/l BAP, C) 5.0 mg/l kinetin, D) 4.0 mg/l kinetin and 1.0 mg/l NAA.

Table 1 Multiple shoot induction of seeds cultured on MS medium supplemented with 15% coconut water, 100 mg/l casein hydrolysate and different combinations of growth regulators. The shoots were cultured for 6 weeks.

MS medium supplemented with 15% coconut water, 100 mg/l casein hydrolysate and different growth regulators (mg/l)			Number of cultured seed	Number of total shoot induced	Number of shoots per seed
BAP	Kinetin	NAA			
-	-	-	36	9	0.25
0.5	-	0.5	36	7	0.19
1.0	-	0.5	36	10	0.27
1.5	-	0.5	36	10	0.27
2.0	-	0.5	36	6	0.16
0.5	-	-	36	13	0.36
1.0	-	-	36	108	3.00
1.5	-	-	36	16	0.44
2.0	-	-	36	161	4.47
-	1.0	-	36	20	0.55
-	2.0	-	36	23	0.63
-	3.0	-	36	30	0.83
-	4.0	-	36	27	0.75
-	5.0	1.0	36	202	5.61
-	1.0	1.0	36	68	1.88
-	2.0	1.0	36	30	0.83
-	3.0	1.0	36	31	2.58
-	4.0	1.0	36	127	3.52
-	5.0	1.0	36	69	1.91

Therefore, the most suitable medium for multiple shoot induction was found to be MS medium supplemented with 15% coconut water, 5 mg/l kinetin and 100 mg/l casein hydrolysate. This medium could induce total shoots of 202 and produced as high as 5.61 shoots/seed.

Gamma irradiation via tissue culture

Multiple shoots of *P. maximum* TD58 which had been grown in the most suitable medium for 6 weeks were irradiated with gamma rays at 0, 10, 20 and 30 Gy, and transferred to the same medium formula with different concentrations of NaCl (0, 0.5, 1.0, 1.5 and 2.0 %) for another 6 weeks. It was found that the controlled non-irradiated shoots could grow well on the selected medium having no NaCl added giving as high as 9 shoots per plant. However, having been exposed to the salt the number of shoots per plant declined considerably to only 3.99 shoots per plant which was well below all other irradiated shoots in the same salt condition. It was conceivably clear that different doses of gamma irradiation caused the lower production of shoots per plant in salt-free medium but these irradiated shoots could perform well in the salt stress conditions. The best adapted ones were found on the 20 Gy irradiated grasses, with 5.33, 5.00 and 4.66 shoots per plant in the medium containing 0.5, 1.0 and 1.5 % NaCl,

respectively (Figure 2). It should be noted that the average percentage of NaCl in the natural saline soil was approximately 0.8 % (Department of Land Development, 1998). The grass irradiated with 20 Gy giving approximately 5.00 shoots per plant certainly could survive in the harsh saline soil of infertile land of Northeastern Thailand. Guinea grass is known to avoid salt stress condition through some special mechanisms like having the salt accumulated in roots (Greenway and Munns., 1980) or by shedding the older leaves (Thompson, 1975). In this experiment, we also observed the formation of salt gland in the non-irradiated grass which had been cultured on the 1.5 % NaCl medium for 8 weeks (Figure 3).

Field observation

The irradiated shoots of purple guinea grass which had been grown on different salt concentrations for 6 weeks were transferred to the natural field at The National Corn and Sorghum Research Center for 45 days (Figure 4). There were only 58 clones (approximately 6.97% of the total plantlets) that could survive under this condition. Ten clones out of these 58 clones were selected based on their superior characteristics of forage crop. Their morphological properties, i.e., number of tillers per plant, leaf width, leaf length, and plant height were recorded (Table 2). These clones were

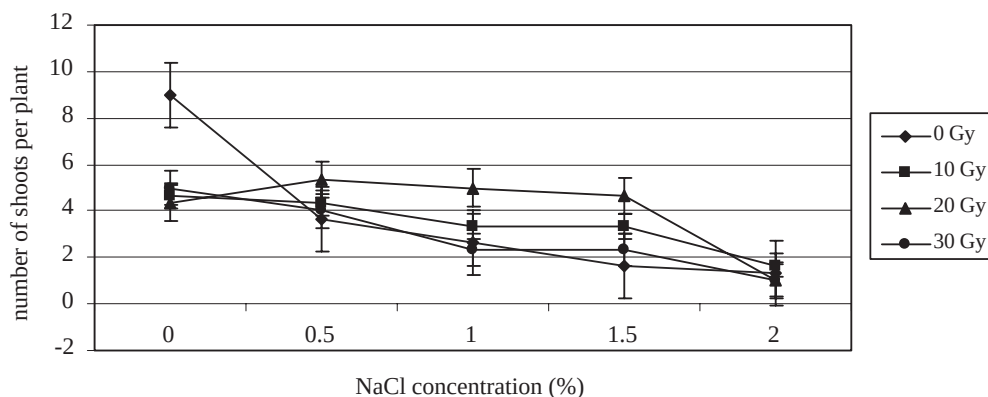


Figure 2 The average number of shoots per plant after being irradiated with gamma rays and cultured on the selected medium supplemented with NaCl at different concentrations.

duplicated in five replications and transferred to grow in the salt stress condition at Borabue district, Maha Sarakham province, Northeastern Thailand, for a year. Seven out of the ten clones were selected as salt tolerant purple guinea grass.

DNA fingerprinting pattern

DNA fingerprinting patterns of thirty purple guinea grasses collected from several places in Thailand were preliminary investigated. They were from Kanchanaburi, Khon Kaen, Nakhon Ratchasima, Phrae, Prachuap Khiri Khan and Sa

Kaew provinces. Ten primers, i.e., E-AAG/M-CAT, E-AAG/M-CAG, E-ACA/M-CAC, E-ACA/M-CTA, E-ACA/M-CTC, E-ACT/M-CAA, E-AAC/M-CAT, E-AAG/M-CAC, E-ACT/M-CTT and E-ACC/M-CAG were chosen for amplifications. The result showed that DNA patterns of these thirty purple guinea grasses were similar. This confirmed the apomixes nature of purple guinea grass where homogeneity is

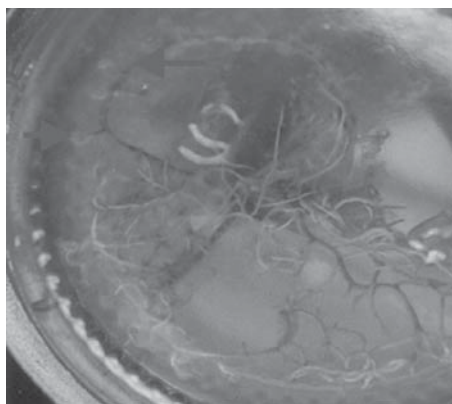


Figure 3 Characteristic of salt gland (red arrow) found in non-irradiated grass cultured on 1.5 % NaCl.



Figure 4 *P. maximum* TD58 after irradiation with gamma rays and cultured on selective medium supplemented with different concentrations of NaCl and grown in the field at The National Corn and Sorghum Research Center, Nakhon Ratchasima province for 45 days.

Table 2 Characteristic of irradiated and non-irradiated *P. maximum* TD58 grown at The National Corn and Sorghum Research Center, Nakhon Ratchasima province for 45 days.

Sample	Doses of gamma rays	% NaCl	# tillers per plant	Leaf width (cm)	Leaf length (cm)	Plant height (cm)
1	10	0.5	114	2.56	57.56	85.50
2	20	1.0	104	2.63	59.14	99.44
3	0	1.5	83	2.41	55.15	92.28
4	1.5	0	105	2.32	54.26	97.80
5	0	1.5	83	2.40	55.00	92.30
6	0	0	91	2.61	60.58	100.00
7	20	0	114	2.53	60.00	96.00
8	30	1.0	105	2.60	56.33	105.00
9	0	0	90	2.63	61.00	101.00
10	10	1.0	73	2.73	63.04	99.00



Figure 5 The ten selected clones of purple guinea grass grown in the salt stress field for a year.

expected (Figure 6). However, as the ten selected survival clones of irradiated grass grown at Borabue district were subjected to the same AFLP analysis, the same patterns of DNA fingerprinting were found (Figure 7). The undetectable polymorphism among these plants having distinctively different morphological properties (Table 2) suggested that silent mutation or specific gene mutation could be affected by gamma radiation and therefore, other methods need to be employed to detect the causes of alteration.

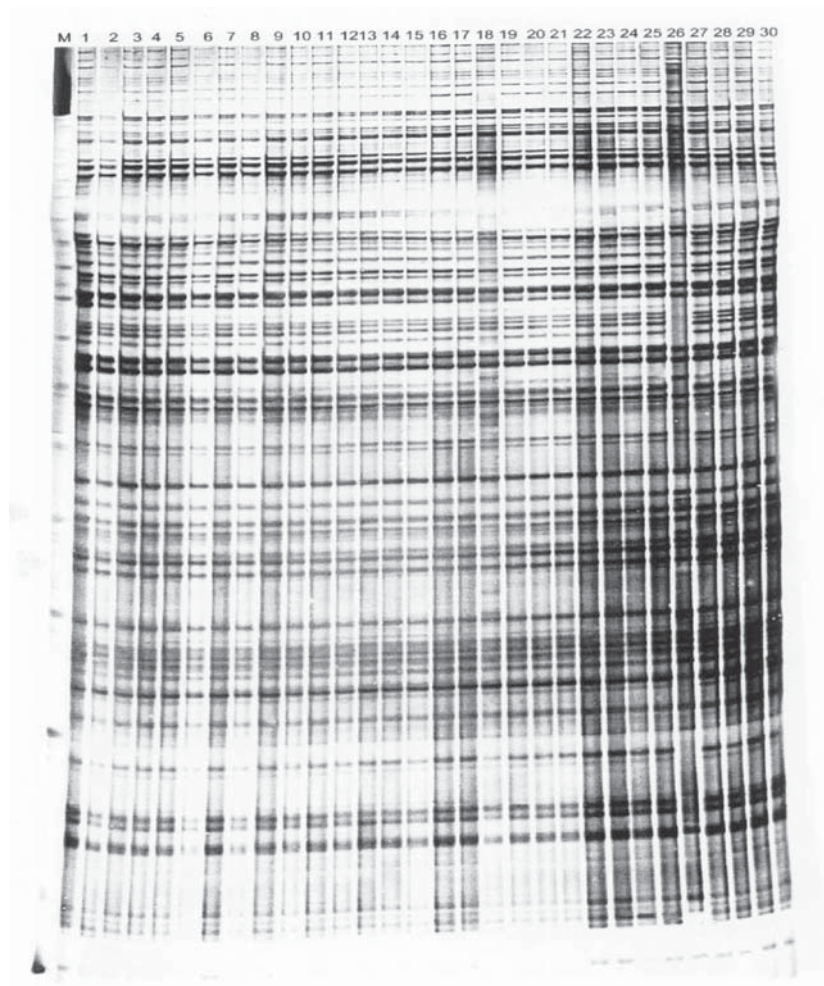


Figure 6 DNA fingerprinting patterns of purple guinea grass collected from several places in Thailand.

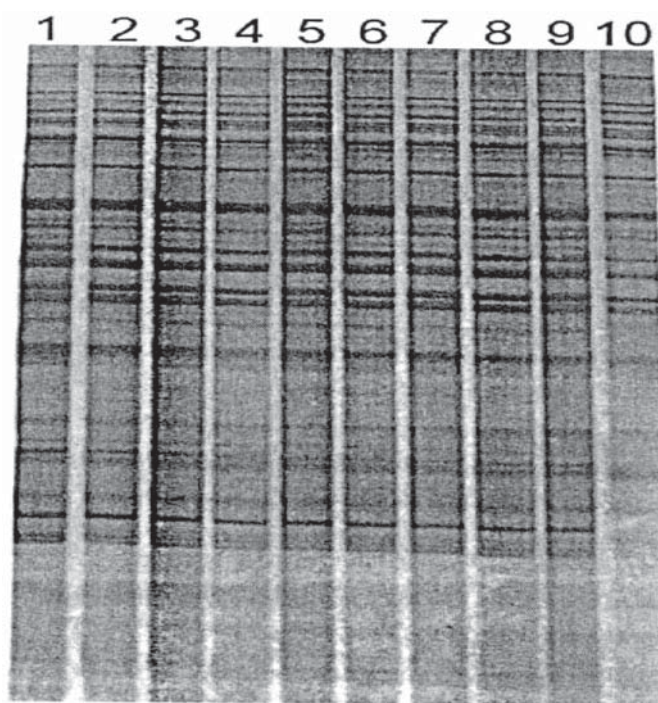


Figure 7 DNA fingerprinting pattern of ten selected clones.

CONCLUSION

Purple guinea grass (*P. maximum* TD58) is an apomixes plant which produces genetically homogeneous seeds. The multiple shoots induced from seeds in tissue culture also showed the homogeneity (the same morphological trait) when cultured on the suitable medium (MS supplemented with 15 % coconut water, 5 mg/l kinetin and 100 mg/l casein hydrolysate). After being irradiated and transferred to grow in the selected medium supplemented with 0–2.0 % NaCl, only 58 clones of plantlets could survive in the field. The morphological properties of grasses were different considering from the number of tillers per plant, plant height, leaf width and length. The duplicated clones were grown at Borabue district, Maha Sarakham province, which is a natural high salt field in the Northeastern Thailand to observe the salt tolerant trait. The good characteristics were observed in ten clones, of

which seven of them showed the salt tolerant trait. Even though the physical characteristic of these grasses were different, the DNA fingerprinting patterns using AFLP technique were similar to the controlled non-irradiated sample. This suggested that the mutation took place at a specific location which could not be detected by this AFLP method. Yet, the tolerance can be the result of temporary adaptation imposed by salt stress and this can be proven by continual seed propagation of the tolerant lines. The mutant trait should remain the same while the physiological adaptation should lose its resistant property soon upon a number of seed propagations.

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