In vitro Tetraploid Induction of *Chrysopogon zizanioides* (L.) Roberty to Improve Salt Tolerance

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ABSTRACT

Vetiver grass is being used worldwide for soil and water conservation, however, there are no report of recommended salt tolerant germplasms in Thailand. Polyploid induction by colchicine treatment is an efficient technique used to improve abiotic tolerance of many plant species. Therefore, tetraploid of vetiver was induced via *in vitro* shoots according to a factorial in completely randomized design with two factors (four colchicine concentrations; 0, 0.1, 0.2 and 0.3% and four treatment durations; 12, 24, 36 and 48 h) and the DNA content was verified by flow cytometry. It was found that the highest tetraploid induction efficiency was 22.22% at the treatment of 0.3% colchicine for 12 h, providing 93.8% survival rate. Total of 53 tetraploid out of 607 survived shoots were further studied for stomatal characteristics and determined for their salt tolerance. The significant difference of guard cell length between tetraploid and diploid indicated that it can be used as a primary criterion for screening tetraploid vetiver from a large population of colchicine-treated shoots. Salt tolerance examined *in vitro* at 0, 1.75, 2.00, 2.25 and 2.50% NaCl (w/v) revealed that survival rate of most tetraploid accessions was higher than that of the original diploid. To confirm their salt tolerance, four highest salt tolerant tetraploid accessions and the original diploid were transplanted to a natural saline area in Dan Khun Thod District, Nakhon Ratchasima Province for 12 months. The survival rate of selected tetraploid accessions was higher than that of the original diploid and two selected accessions were able to survive throughout the experimental period. This result confirmed that tetraploid induction by colchicine treatment was successfully enhance salt tolerance of vetiver grass.

Key words: Vetiver grass, tetraploid, colchicine, salt tolerance

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Introduction

Vetiver (*Chrysopogon zizanioides* (L.) Roberty) (syn. *Vetiveria zizanioides* (L.) Nash) is a perennial grass belonging to the Poaceae family and has deep root system which able to exploit the subsoil moisture (Truong *et al*., 2002; Srivastava *et al*., 2008). Its roots can grow downward approximately 4 m in the first year and after that penetrate into the deep layers of soil which is effective to stabilize the soil (Truong *et al*., 2002). In Thailand, vetiver has been a long time used for soil and water conservation. However, the utilization of this grass in saline soil had not yet been reported since it does not tolerate to high salinity. As reported by Nanakorn (2005), a survey study of salt tolerant weeds in salt affected soil of 14 sub-locations in 7 provinces of northeastern Thailand demonstrated that vetiver grass was not found under a high salinity (EC > 8 mS cm^{-1}). Furthermore, Thiraporn (1994) reported that growth rate of vetiver was reduced by 75% even at a low salinity of soil ECs about 4.0 dS m^{-1}. However, it has a potential to develop high salt tolerant vetiver since Danh *et al.* (2012) reported that this grass in Australia could survive in saline soil with high EC up to 47.5 mS cm^{-1}.

Polyploidy can be induced by interference to cell cycle. The most common antimitotic agent used for chromosome doubling is colchicine (Dhooghe *et al*., 2011). Chromosome doubling does not produce new genetic material but increase the copies of existing genes and chromosomes. In addition, polyploidy has an overall more tendency to increase gene expression level than diploid (Osborn *et al*., 2003). These changes often make polyploid plants superior to diploid plants with genetic adaptability, anatomical and morphological changes and tolerance to environmental stresses (Ranney, 2006). Several researchers have successfully induced polyploidy in Poaceae family such as *Brachiaria ruziziensis* (sexual tetraploids for breeding program-Ishigaki *et al*., 2009), *Paspalum notatum* (sexual tetraploids for breeding program-Quesenberry *et al*., 2010), *Miscanthus* species (enhanced biomass - Glowacka *et al*., 2010) and *Sorghum bicolor* (high level of soluble carbohydrates - Ardabili *et al*., 2015); nevertheless, the report on polyploidy induction for improving abiotic

stress tolerance was very few. Although polyploid induction to enhance essential oil content in vetiver has been reported by Lavania (1988), there was no indication on the number of polyploid plants and the effective colchicine concentration used. The polyploid induction of vetiver grass needs to be fully elucidated; thus, this study aims to develop high salinity tolerant accessions of vetiver through *in vitro* polyploidization by colchicine.

Materials and methods

Plant materials and establishment of plantlets

The *in vitro* culture of vetiver for establishment of plantlets was followed Suwannachitr (1997). Immature inflorescences enclosed with flag leaf of vetiver grass (*C. zizanioides*) Kampangpetch 2 (KP2) germplasm were used as explants for callus induction. These explants were collected from Nong Phlap, Hua Hin District, Prachuap Khiri Khan Province, Thailand. The surface of explants was disinfected with 70% ethanol and subsequently dipped into 95% ethanol and flamed over for 1-2 times, then the explants were cut into pieces of 1 cm length and cultured onto the callus proliferation medium (CPM), semi-solid Murashige and Skoog (MS) (Murashige and Skoog, 1962) supplemented with 5 μM 2, 4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose and 0.8% agar. The pH of medium was adjusted to 5.8 prior to autoclaving (121 °C and 1.06 kg cm^{-1} pressure for 15 min). The cultures were incubated under a photoperiod of 16 h light/8 h dark at light intensity of 40 μ mol $m^{-2}s^{-1}$ and at 25 ± 2 °C. The callus were sub-cultured onto CPM every 4 weeks. For plantlets regeneration, the compact callus were transferred onto hormone-free MS medium (MS_0) for 4 weeks. Plantlets were then proliferated by transferring onto the shoot proliferation medium (SPM)-MS medium containing 5μM 6-benzyladenine (BA) for 4 weeks and were further sub-cultured at 2 weeks interval.

The effect of colchicine on survival rate and tetraploid induction

For tetraploid induction by colchicine treatment, the experiment was carried out in a completely randomized design using 4x4 factorial with four colchicine concentrations and four

treatment durations, with four replicates. Twelve *in vitro* shoots of each replication were cultured on SPM for 7 days, then transferred to MS_0. Two milliliters of filter-sterilized colchicine at 0, 0.1, 0.2 and 0.3% w/v were applied to spread over the medium surface and incubated for 12, 24, 36 and 48 h under the same culture conditions as previous mentioned. The medium without colchicine was used as control. After that the colchicine-treated shoots were washed 2-3 times with sterile distilled water to remove the remaining colchicine solution and further cultured on colchicine-free MS_0 medium for 1 week and subsequently transferred to SPM medium for 4 weeks. Then, survival rate was determined as follow:

survival rate (%) = [no. of survived shoot/no. of treated shoot] x 100.

After 3 months of culturing on SPM, the putative tetraploids were screened out from all the survived shoots using some leaf morphological changes, *i.e.*, increasing of thickness, width/length ratio and darker green coloration (Liu *et al.*, 2007). The putative tetraploid shoots were then proliferated on SPM medium as mentioned in plant establishment. The proliferated shoots were further subjected to test for their ploidy levels by flow cytometry.

Flow cytometric analysis

Flow cytometric analysis of DNA content was carried out to compare the ploidy levels of the original diploid and putative tetraploid plants followed the method of Quesenberry *et al.* (2010). The fully expanded leaf samples of ~0.5 cm^2 was chopped with a sharp razor blade in a Petri dish containing nuclei extraction and stained using the reagent kit Partec CyStain DNA one step code No. 05-5001 (Partec, Münster, Germany) and 1% polyvinylpyrrolidone (PVP)-40 was added for removing phenolic substances. Then, the suspension of nuclei samples were analyzed immediately using Partec CyFlow Ploidy Analyser II (Partec GmbH; Mnster, Germany).

Evaluation of leaf stomatal characteristics

Leaf samples of diploid (control) and tetraploid vetiver were measured the guard cell length and stomatal density. The procedure was adapted from Carpenter (2005). The lower side of the

fully expanded leaf was placed on glass slide and softly scraped off upper epidermis and mesophyll tissue with a sharp razor blade in 10% w/v potassium hydroxide solution until the layers extremely thin. The leaf sample was then rinsed with distilled water and cut at middle region, treated with commercial bleach for 2-3 sec and rinsed with distilled water. Then, the lower epidermis was turn up and observed under an eye-piece light microscope connected with digital camera (Dino-eye AM4023X, ANMO Electronics, Taiwan). Guard cell length and stomatal density (stomatal number/area) of each ploidy level were determined from 5 selected accessions (with 10 observations for each).

Determination of salt tolerance of selected accessions under *in vitro* conditions

Shoots of diploid (control) and tetraploid were transferred to hormone-free MS medium for 2 weeks for root induction. Afterwards, the rooted shoots (or so call plantlets) were transferred onto MS medium supplemented with 0, 1.75, 2.0, 2.25 and 2.50% NaCl (EC were 5.4, 31.5, 35.7, 39.0 and 42.2 mS cm^{-1}, respectively) and incubated under a light and temperature conditions as mentioned in plantlets establishment. The experiment was conducted with 4 replications, each of 5 observations. After 8 weeks, salt tolerance of the plantlets was determined from the survival rate (Nanakorn *et al*., 1998). Four selected tetraploid accessions which had the highest salt tolerance were propagated under *in vitro* conditions for further confirmation of their salt tolerance under natural saline soil.

Field confirmation of salt tolerance of selected accessions under saline soil conditions

Preparation of the planting material

Rooted plants with three tillers per clump of the original diploid (KP2) and four selected salt tolerant accessions from *in vitro* culture were acclimatized, then, transplanted to polyethylene bag containing mixed soil and grown in greenhouse for 3 months. Thereafter, the vigorous diploid and tetraploid plants were selected for the field experiment. Some plants from all accessions were maintained in pots under non-saline conditions.

Experimental design

The experiment was conducted on-farm in saline soil (Soil Survey and Land Use Planning, Land Development Department, 2005), in Dan Khun Thod District, Nakhon Ratchasima Province, (15 ° 14' 53'' N, 101 ° 43' 27'' E) northeastern part of Thailand. The Randomized Complete Block Design (RCBD) with 4 replications was employed. Five treatments consisted of the above planting materials and the 2.4 x 2.5 m^2 plot size of each treatment were used during June 2014 to July 2015.

Data collection

To monitoring the soil salinity change, electrical conductivity of saturation-extracted soil (EC_{se}) were measured following Rhoades (1982), at before planting in June 2014, 5^{th}, 8^{th} and 12^{th} month after planting (MAP). The survival rate (%) = [(number of clump with survived plants /total number of clump) x 100] and tiller number were monthly recorded.

Statistical analysis

All data were statistically analyzed according to Crop Stat Version 7.2.2007.3 (IRRI, 2007). The experimental data were subjected to analysis of variance, and the differences among means were determined by Duncan's Multiple Range Test.

Results and Discussion

Effect of colchicine treatment on survival rate and tetraploid induction

The survival percentage of *in vitro* shoots was significantly affected by colchicine concentrations and treatment durations (Figure 1). However, both the concentration and exposure time of the colchicine treatments had no interaction on survival percentage of vetiver shoots (Table 1). The combination of colchicine concentrations and treatment durations is the key factor affecting polyploid induction efficiency (Yang *et al*., 2006). High concentration and long duration treatment resulted in plant dead because of the colchicine toxicity (Dhooghe *et al*., 2011). It blocks spindle formation and subsequently results in the production of abnormal and bizarre nuclear configurations and often leads to cell death (Klintschar *et al*., 1999). In the current experiment, 0.1, 0.2 and 0.3% colchicine demonstrated non-significant

survival percentage but lower when compared to the control-colchicine free (Figure 1A). Survival rate at longer treatment durations of 36 and 48 h were significantly lower than that of 12 h (Figure 1B).

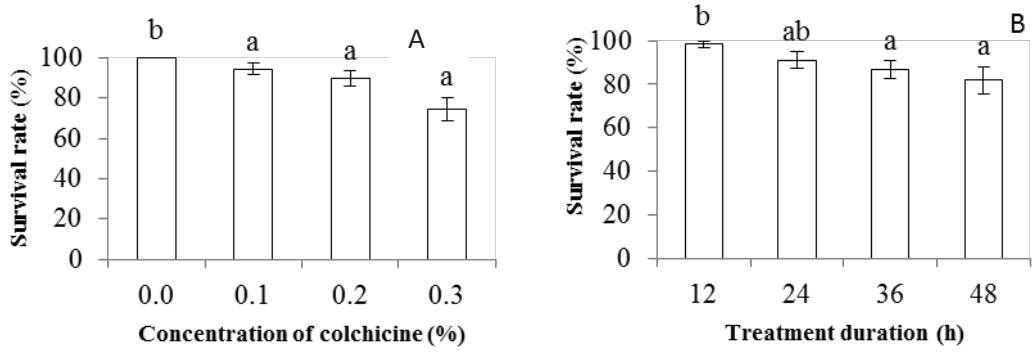

Figure 1 Effects of colchicine concentrations (A) and treatment durations (B) on survival rate of vetiver shoots. Different letters above each bar indicate a significantly different at $P \leq 0.05$ by DMRT Bars indicate the standard error

Table 1 Mean squares from analysis of variance of data for different treatment durations and concentrations of colchicine on survival rate of *in vitro* vetiver shoot

Source	df	Mean Square
Concentration	3.0	1,912.62**
Duration	3.0	792.82*
Concentration x Duration	9.0	174.58ns
Error	48.0	209.06

The survival rate ranged from 87.5±9.9 to 100.0±0.0% under 0.1% colchicine and dropped to 56.3 ± 14.6 to 93.8 ± 6.3% under 0.3% colchicine (Table 2). Higher concentration and longer duration resulted in survival rate reduction that were in agreement with other plants such as *B. ruziziensis* (Ishigaki *et al.*, 2009), *Miscanthus* species (Glowacka *et al.*, 2010) and *Paulownia tomentosa* (Tang *et al.*, 2010).

Representative flow histograms of DAPI-stained nuclei from the original diploid and tetraploid shoots are shown in Figure 2. The tetraploid shoots were found in all treatments except the control and 0.1% colchicine for 48 h (Table 2). The percentages of tetraploid were varied among treatments from 2.5-22.2%, however, the treatment for 12 h was able to induce more tetraploid shoots than other treatment durations at the same colchicine concentrations. The highest induction efficiency for tetraploid was achieved at the treatment with 0.3% colchicine for 12 h with 22.2% base on survived shoot.

The tetraploid shoots exhibited different morphological characteristics from the diploid control as shown in Figure 3. Compared to the diploid control, tetraploid plantlets had visible thicker and broader leaves.

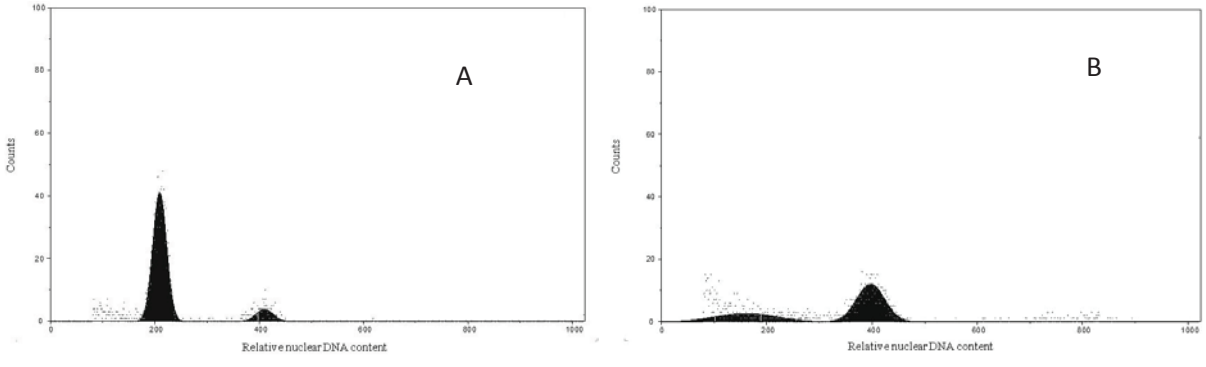

Figure 2 Representative flow cytometric analysis of diploid and tetraploid of vetiver shoot. DAPI fluorescence of nuclei (x-axis) versus number of nuclei counted (y-axis) in diploid (A) and tetraploid (B)

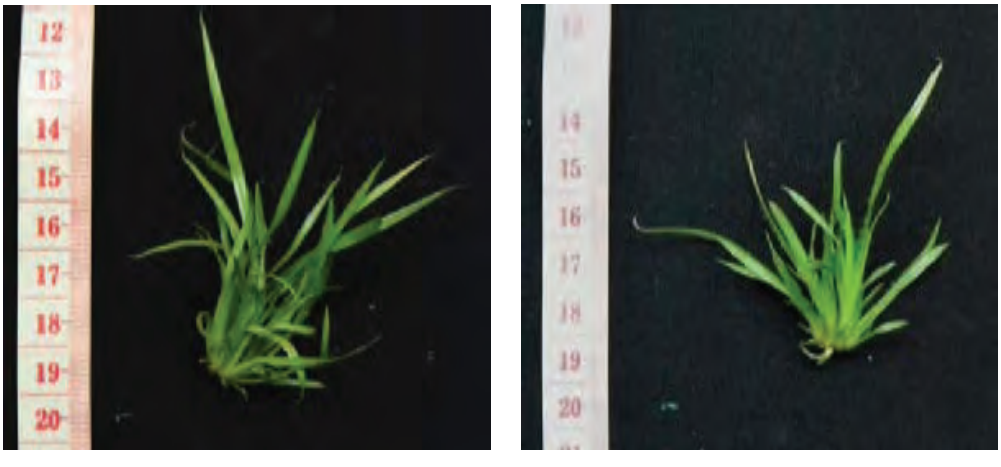

Figure 3 Characteristics of colchicine treated vetiver (A) diploid and (B) tetraploid

Table 2 Effects of different colchicine concentrations and treatment durations on survival rate and tetraploid induction of *in vitro* vetiver shoots

Colchicine concentration (%)	Treatment duration (h)	No. of survived shoot	Survival rate (%)[1/]	Tetraploid shoot	
				Total	%
0	12	48	100.0 ± 0.0	0	0.0
	24	48	100.0 ± 0.0	0	0.0
	36	48	100.0 ± 0.0	0	0.0
	48	48	100.0 ± 0.0	0	0.0
0.1	12	48	100.0 ± 0.0	6	12.5
	24	47	97.9 ± 2.1	3	6.4
	36	44	91.7 ± 5.9	5	11.4
	48	42	87.5 ± 9.9	0	0.0
0.2	12	48	100.0 ± 0.0	9	18.8
	24	43	89.6 ± 10.4	7	16.3
	36	41	85.4 ± 9.9	4	9.8
	48	40	83.3 ± 7.6	1	2.5
0.3	12	45	93.8 ± 6.3	10	22.2
	24	37	77.1 ± 10.4	3	8.1
	36	34	70.8 ± 8.7	4	11.8
	48	27	56.3 ± 14.6	1	3.7
Total		**607**		**53**	

[1/] mean of 4 replications. ± standard error

Leaf stomatal characteristics of tetraploid and diploid vetiver

Stomatal characteristics of vetiver shoots depended on the ploidy level (Table 3 and Figure 4). The average guard cell length of tetraploid was significantly higher than that of the diploid but there was no significant difference in stomatal density. The result of guard cell length was in agreement with many plants such as ruzi grass (Ishigaki *et al.* 2009), *P. tomentosa* (Tang *et al*., 2010) and bahia grass (Quesenberry *et al*., 2010). This result confirmed that the change of guard cell length of tetraploid vetiver can be used as a primary criterion for screening tetraploid. Therefore, in the future experiment which aims to induce tetraploid vetiver for any selection of desired characters, guard cell length will be a useful tool for screening tetraploid from a large population of colchicine-treated shoots. After screening, which the number of variants reduces, the putative tetraploid plants will be verified by flow cytometric analysis for accurate identification.

Table 3 Guard cell length and stomatal density of diploid and tetraploid vetiver shoot.

	Stomatal characteristic	
	Guard cell length (mm)[1/]	**Stomatal density (No. mm^{-2})**
Diploid	0.07 ± 0.07 a	18.3 ± 4.1
Tetraploid	0.09 ± 0.01 b	13.5 ± 9.1

[1/] mean of 5 replicates (±SD)

means in the same column followed by a common letters are not significantly different at 5% by LSD

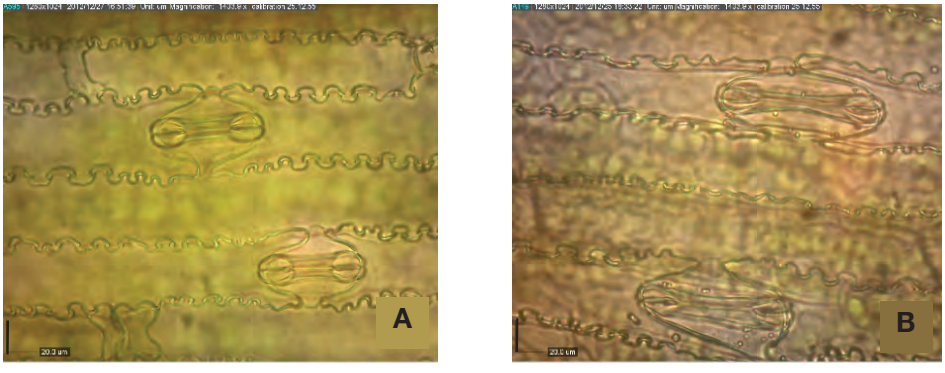

Figure 4 Lower epidermis comparing guard cell size and stomatal density between (A) diploid and (B) tetraploid vetiver shoot. Bar = 20.0 mm.

Determination of salt tolerance of selected accessions under *in vitro* conditions

Under the *in vitro* treatments of NaCl, the survival rate of KP2 (the original diploid) and 53 tetraploid accessions were 100% at 0.00, 1.75 and 2.00% NaCl and decreased with increasing NaCl concentrations to 2.50%. Thirty-one tetraploid accessions had higher survival rate than KP2 at 2.25 and 2.50% NaCl, therefore, their survival rates were presented in Figure 5. The results revealed that most of tetraploid plants tolerated to higher salinity than the original diploid. Similarly to Meng *et al.* (2011) who demonstrated that the tetraploid turnip exhibited better adaptation to high salt concentration than the diploid one. Some mechanism changes that lead to better salt tolerance in tetraploids compare to their diploids have been reported. For example, under salt stress the activity of peroxidase in tetraploid of *Dendranthema nankingenese* was higher than that in the diploid whereas malondialdehyde (MDA), which is a product of lipid peroxidation by free radicals, was maintain at a lower level. Consequently, the tolerant level to salt

stress of the tetraploid was improved (Liu *et al*., 2011). Shafieizargar *et al.* (2013) reported that proline accumulation in leaves of the tetraploid Dez orange (Citrus sinensis (L.) Osb.) was higher than that of the diploid during the increasing NaCl concentrations while MDA and H_2O_2 were lower. It was suggested that these mechanisms led the tetraploid capable of better adapting to salt stress than the diploid one. However in the current study, there were some tetraploid accessions that possessed less salt tolerance than KP2. This might be due to the somaclonal variation which generated during callus induction as also seen in other studies (Bairu *et al*., 2011). Among 31 accessions, there were 6 accessions that survived 80-100% at the highest NaCl concentration of 2.50% namely V12, V23, V24, V52, V75 and V86.1. Out of these, V12 was the most salt tolerant with 100% survival rate at all NaCl concentrations. Four accessions, V12, V23, V52 and V75, were selected to grow under natural saline soil conditions.

Field confirmation of salt tolerance of selected accessions under saline soil conditions

Although *in vitro* technique has been effectively used for selection of desired accessions, the selected plants should be further confirmed under field conditions (Pérez-Clemente and Gómez-Cadenas, 2012). In the field environment, plants are exposed not only to soil

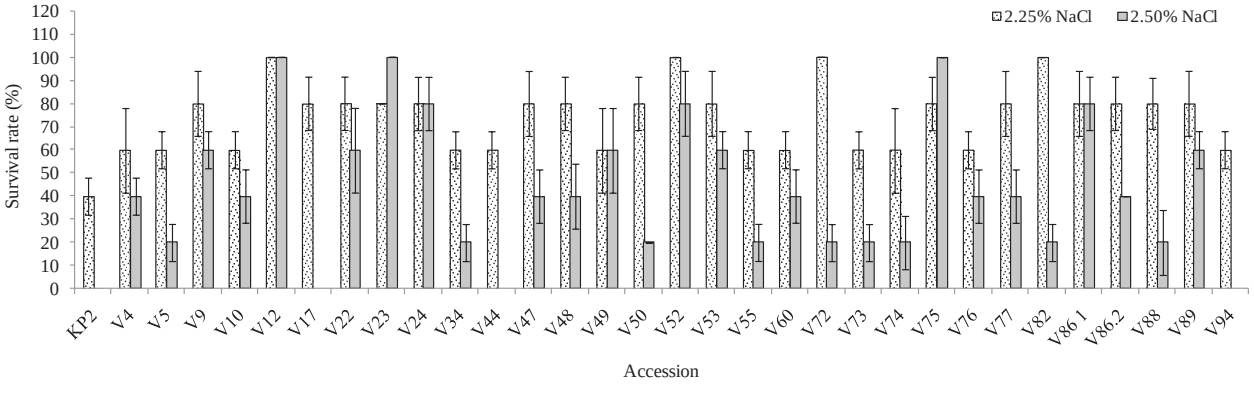

Figure 5 Survival rate of KP2 and 31 tetraploid accessions on MS medium supplemented with 2.25 and 2.50% NaCl for 8 weeks. Error bar indicates standard error; n=4

salinity, but also to heat, drought, light intensity, some toxic trace elements, soil sodicity and pH (Munns and James, 2003). The changes of soil salinity of experimental area at Dan Khun Thod District were 29.00 $\pm$ 4.33 and 16.17 $\pm$ 14.31 mS cm^{-1} at the time before planting and 5th MAP, respectively, and then markedly increased to 64.31 $\pm$ 19.18 and 84.06 $\pm$ 15.21 mS cm^{-1} in 8th and 12th MAP, respectively. The results confirmed that this area is a severely salt affected soil as has been reported (Soil Survey and Land Use Planning, Land Development Department, 2005).

During 12 months of planting, vetiver accessions under non-stress condition displayed 100% survival rate (data not shown) whereas their survival percentage in saline soil gradually decreased. In saline soil conditions, both accessions and blocks did not significantly affect the survival of vetiver all along period as shown in Table 4. However, the results exhibited that all 4 selected salt tolerant accessions had longer survival than KP2. Especially in the 8th month after planting (MAP), KP2 was no longer survived (Figure 6A). V52 shown higher survival rate than other selected accessions during 2nd-6th MAP but died in 9th MAP. During the dry period which soil EC_{se} markedly increased, there were only 2 accessions, V12 and V23, still alive and performed similar survival rate in 9th-11th MAP. In 12th MAP, the survival rate of V12 (14.58 $\pm$ 10.65%) was 7-fold more than that of V23 (2.08 $\pm$ 2.08%), indicated that V12 was more tolerate to salt than V23. These results suggested that the time length of field test should be long enough for plants to express

Table 4 Mean square of survival rate of the original diploid plant (KP2) and 4 selected salt tolerant accessions during 12 months (July 2014-June 2015) of planting in saline field at Dan Khun Thod District, Nakhon Ratchsima Province

S.O.V	*df*	Time after planting in saline soil (month)											
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
Accession	4	211.77^{ns}	178.78^{ns}	367.99^{ns}	498.32^{ns}	512.09^{ns}	270.70^{ns}	38.20^{ns}	413.16^{ns}	342.00^{ns}	196.17^{ns}	177.07^{ns}	161.44^{ns}
Block	3	96.07^{ns}	125.00^{ns}	97.23^{ns}	531.28^{ns}	753.47^{ns}	459.76^{ns}	208.33^{ns}	509.22^{ns}	333.31^{ns}	175.92^{ns}	160.87^{ns}	157.39^{ns}
Error	12	177.10	336.22	317.10	516.77	1,144.09	1,754.89	1,290.50	434.00	272.55	277.19	241.88	177.64
CV (%)	-	15.04	19.51	21.18	28.82	44.82	81.51	130.03	180.10	258.41	312.50	320.26	392.35

ns = non-significant

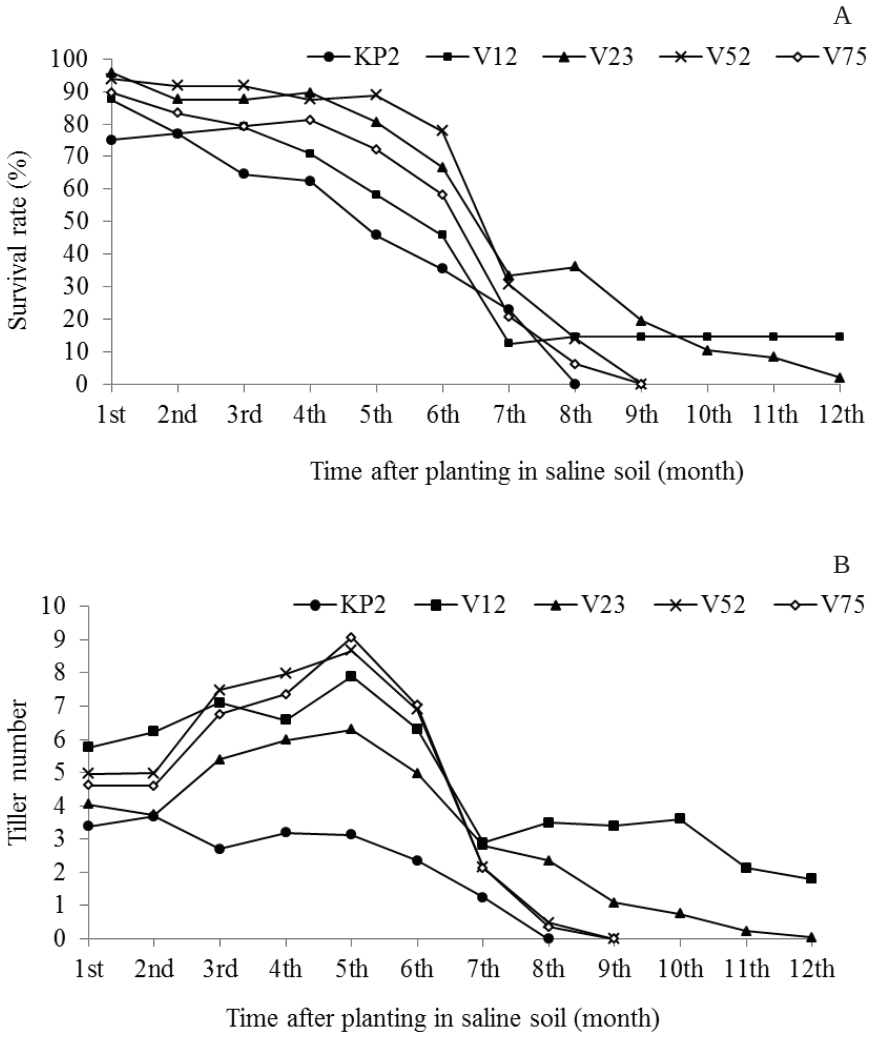

Figure 6 Survival rate (A) and tiller number (B) of KP2 and 4 selected salt tolerant accesions during 12 months (July 2014 - June 2015) of planting in saline field. Rainy period was in Jul 2014 - Oct 2014 (1st - 4th month) and the dry period was in Nov 2014 - Feb 2015 (5th - 12th month)

their tolerant ability to various environment factors.

Both accessions and blocks also did not significantly affect on tillering of vetiver grass (Table 5). Tiller number of KP2 responded to salinity different from selected accessions. Because the tillering of KP2 tended to increase only in first 2 months and then its tiller number decreased. While, the tiller number of all selected accessions slightly raised for 5 MAP in saline soil, from the 6th MAP, their tiller had a trend to decreased (Figure 6B). Furthermore, the tillering of

Table 5 Mean square of tiller number of the original diploid plant (KP2) and 4 selected salt tolerant accessions during 12 months (July 2014-June 2015) of planting in saline field at Dan Khun Thod District, Nakhon Ratchdsima Province

S.O.V	df	Time after planting in saline soil (month)											
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
Accession	4	3.23^{ns}	4.38^{ns}	14.71^{ns}	13.36^{ns}	13.36^{ns}	15.12^{ns}	1.76^{ns}	8.83^{ns}	7.69^{ns}	9.76^{ns}	3.46^{ns}	2.51^{ns}
Block	3	1.19^{ns}	4.55^{ns}	9.28^{ns}	11.63^{ns}	11.63^{ns}	17.59^{ns}	5.43^{ns}	12.30^{ns}	7.82^{ns}	9.40^{ns}	3.39^{ns}	2.49^{ns}
Error	12	0.87	2.00	7.68	10.60	10.60	35.03	16.18	12.08	9.06	11.20	3.72	2.59
CV (%)	-	26.18	36.68	51.99	54.39	54.39	96.06	150.65	245.14	342.82	374.12	403.74	426.75

ns = non-significant

V12 and V23 was better than those of other accessions and conform to survival rate, however, only the tiller number of V12 was stable from 8th-10th (from 3.50 $\pm$ 2.60 to 3.60 $\pm$ 2.43) and gradually decreased in 11th-12th month (2.13 $\pm$ 1.46 to 1.79 $\pm$ 1.22). The better tillering in salt tolerant genotype will reduce salt concentration in growing tissue by dilution effect (Aslam *et al*., 1989). It was corresponded to El-Hendawy *et al*., (2005) who reported that salt sensitive wheat genotypes shown a greater reduction in tiller number than the tolerant one. Besides the survival rate, the tillering of vetiver grass under field conditions also confirmed that all selected accessions were more tolerate to salt stress than the original diploid. It was clearly shown that the most salt tolerant accession, V12, was able to maintain stable tiller number all along 12 months.

The present study emphasized the power of somaclonal variation and tetraploid induction in salt tolerance improvement. Therefore, to widen the opportunity for increasing more salt tolerance than that has been achieved, the most efficient treatment of the present study should be applied to a large population of vetiver. Afterward, the tolerant variants will be introduced for saline soil remediation.

Conclusion

In vitro induction of tetraploid in vetiver grass was effectively achieved using 0.3% colchicine for 12 h. This treatment was able to induce tetraploid plant with 22.22% of the survival shoots. Guard cell length was identified as

useful parameter for distinguishing the tetraploid vetiver accession since this stomata characteristic was significantly higher than that of the diploid. Both *in vitro* and field evaluations indicated that tetraploid vetiver induced by colchicine possessed higher tolerance to salt stress than the original diploid.

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