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Genetic gain for cold tolerance among and within populations of palisade grass

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ABSTRACT

The species of palisade grass presents moderate to low cold tolerance, with development hindered by low temperatures and frost formation that occurs in southern Brazil. The breeding of this species is difficult due to the presence of apomixis, which restrict its genetic basis, providing small number of individuals in the Brazilian germplasm bank. The objective of this study was to generate genetic variability associated with cold tolerance in palisade grass and estimate the genetic gain in surviving individuals at low temperatures within the population (cold levels) and among populations (M2 and M3 generations). The experiment was carried out in Frederico Westphalen, southern Brazil. Activities were developed in the breeding and plant production laboratory of the Federal University of Santa Maria. The climate is humid subtropical, with occasional frost formation during the winter period. Mutation induction occurred with the methyl methanesulfonate (MMS) mutagen and evaluations were carried out in controlled germination chamber. 4000 individuals were evaluated in order to carry out the study, considering the survival rate as the indicative trait of cold tolerance. The experimental design was augmented blocks of Federer with intercalated check varieties, with individuals of the M2 and M3 populations and control treatments submitted to five cold levels of 1°C, 0°C, -1°C, -2°C, and -3°C at 21 days after sowing (DAS). Surviving seedlings evaluation and exposure to extreme cold of -5°C were proceeded at 26 DAS. The chemical mutation induction generated genetic variability for palisade grass individuals and allows genetic selection for cold conditions. Genetic variability in M2 population is greater than M3 population, allowing to obtain greater genetic gains for cold tolerance. The palisade grass mutants can be selected in negative temperatures in controlled germination chamber. The largest number of surviving individuals was observed with the cold levels -1°C (46.85%) and -2ºC (45.68%) associated with extreme cold of -5°C.

INTRODUCTION

The palisade grass growing area in Brazil has increased in comparison with another forage species. Studies on the genetic diversity in palisade grass have been carried out in Brazil (Garcia *et al*., 2013; Torres *et al*., 2015). However, genetic variability is considered low due to apomixis and chromosomal irregularities (Fuzinato *et al*., 2012). In study executed along with the Brazilian germplasm bank, which has 222 accessions of palisade

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grass, it was also identified ploidy level variation with amplitude of 2n to 6n, where only one of the accessions presented sexual reproduction (Resende *et al*., 2008). These factors hamper increasing genetic variability along the germplasm banks, conditioned on research that will propose alternatives to this expansion.

In the first decades of the 20th century, the scientific society found the occurrence of natural mutations, which cause changes in the plant DNA, becoming an important historic mark in understanding evolution. In this sense, the induced mutations can be considered the only hope of plant breeders to get rid of the unique dependence on nature and speed up the process for creation and expansion of genetic variability (Allard, 1971). In Brazil, positive results were reported in oat through chemical mutation induction, which is presented as an alternative to low variability of germplasm banks (Coimbra *et al*., 2004).

Recently in the literature, there are studies carried out in chickpea (Wani, 2011) and in cowpea (Nair and Mehta, 2014), indicating the expansion of genetic variability in traits of interest after mutation induction. In a study with tomato Jahan *et al*. (2016), identified the methyl methane sulfonate as the best inductor, recommending the same to expand the genetic base with the culture. In lentil culture, appropriate doses of inducer also provide increased frequency of desirable mutants, demonstrating their importance to expansion of genetic variability in crops (Amin *et al*., 2015).

This study, come to contribute to the genetic improvement of palisade grass and other forage species, which have the presence of apomixes. To which the chemical mutation induction might be an alternative to the expansion of genetic variability with methyl methane sulfonate (MMS), providing higher gain selection for cold tolerance in subtropical regions.

Studies on mutation induction to increase the genetic variability by selecting individuals for cold tolerant forage species were not found in literature. Thus, the objective of this study was to generate genetic variability associated with cold tolerance in palisade grass and estimate the genetic gain in surviving individuals at low temperatures within the population (cold levels) and among populations (M2 and M3 generations).

MATERIAL AND METHODS

In this paper, we propose a methodology to evaluate the effect of simulating different cold levels in palisade grass mutants, with assessments in M2 and M3 generations. The study was carried out over the 2011-2013 agricultural years in the Breeding and Plant Production Laboratory of the Federal University of Santa Maria - UFSM, located in southern Brazil at coordinates of 27°39'S, 53°42'O and altitude of 461.3 m.

The *U. brizantha* cv. Marandu was the cultivar used for mutation induction. *U. brizantha* cv. Marandu and BRS Piatã cultivars were used without the mutation induction as control treatments. Based on the experimental design, individuals originated from control treatments were compared with individuals generated by mutation induction.

In order to expand the genetic variability, chemical mutation induction was carried out with methyl methane sulfonate (MMS) mutagen at a dosage of 0.5%, using 4000 viable seeds. Those seeds were exposed to the mutagen in the initial germination process, according to the methodology proposed by Coimbra *et al*. (2004). After MMS mutagen exposure on the seeds, sowing was performed in container with soil associated with commercial substrate. Seeds were allocated in a controlled germination chamber (B.O.D.), with alternating temperature of 20ºC to 35ºC. At 21 days after sowing (DAS), plants were exposed to constant temperature of 0°C during one hour. This methodology was developed to simulate periods of frost formation that occur during the winter period.

The surviving progenies were transplanted to the experimental area on March 15, 2011. Those progenies formed the base population composed by 35 individuals, originating the M1 population. Seeds were harvested individually by plant and sown in rows, where each plant corresponded to a sowing row. Moreover, sowing was held on April 3rd, 2012. The experimental design utilized was augmented blocks of Federer (Federer and Raghavarao, 1975).

The utilized experimental design is based on the control treatments repetition in order to proceed the environmental effects estimate. Moreover, it considers the progenies (mutant plants) as unique individuals where the genetic constitution was changed. In the following year, generation with 35 lines was advanced, where the seeds harvested from M1 generation gave origin to M2 generation. M2 generation was sown in rows on March 25, 2013, associated to check varieties in the experimental design of augmented blocks of Federer. Descendants of the 35 mutant individuals gave origin to the seeds that correspond to M3 generation, which were studied under laboratory conditions in different cold levels along with M2 generation and check varieties in B.O.D.

Seeds used to study the simulation of different cold levels were collected during March, 2014. Individual panicle harvest of all plants of M1 and M2 generation was performed, which constitute the seeds forming M2 and M3 generation, respectively. After panicle harvesting, the seeds were manually threshed and stored in seed storage chamber at temperature of 30°C for a period of two months. After this period, seed homogenization was

carried out per generation and samples were randomly selected through the Bulk method. Those seed samples were tested in B.O.D. controlled environment.

The study in B.O.D. was performed with the use of container consisting of Tetra Pak® boxes with area of approximately 1000 cm^3, which were properly washed and sterilized by immersion in sodium hypochlorite at 2% for half an hour. After drying with paper towel, it was proceeded sterilization with ethanol at 70% concentration.

The substrate for the experiment consisted of 50% of the commercial substrate volume with specifications of pH 6.5 and mass density of 500 kg m^3, with 50% of the remaining volume consisting of soil collected from the soil surface horizon of 20 cm, known as an aluminoferric Oxisol. After mixing and allocating the substrate in the experimental units, the container was saturated with distilled water until field capacity. Also, small holes were opened for excess water drainage.

The treatments used in the study were composed of different cold levels, which corresponded to temperatures of 1°C, 0°C, -1°C, -2°C to -3°C and the same treatments associated with the extreme cold -5°C, providing greater accuracy in the selection of individuals. Each cold level was studied with four replications of 50 individuals, totaling 200 individuals. Moreover, 1000 individuals were used for each check variety population (BRS Piatã, Marandu, M2 and M3 generations), totaling 4000 individuals (palisade grass plants, studied until 31 DAS in B.O.D.).

After bulk selection (random selection of these 4000 seeds within a larger sample), these individuals were individually selected with the manual application of pressure on each seed in order to standardize the seeds using only seeds with totally full endosperm, providing seed quality standardization. Each package received 50 seeds, that once planted were irrigated with distilled water solution and potassium nitrate at a concentration of 0.2% to help overcome dormancy.

The experimental units were allocated in B.O.D. and daily randomized to make greater environmental homogeneity. The temperatures were used alternately with 35°C for daylight period of 8 hours and 20°C for an overnight period of 16 hours, until the seventh day. Temperature was stabilized at 20°C from the 8th day until 21 DAS. After 21 DAS, seedling count was performed before treatment application. After that, treatments were established with different cold levels (1ºC, 0ºC, -1ºC, -2ºC, and -3ºC). Counting of surviving individuals was performed 5 days after low temperature stress applied to the plants, with reading of the surviving plants held at 26 DAS. After counting all the different cold levels, seedlings were submitted to extreme cold of -5° and final counting was performed at 31 DAS.

The survival rate of individuals was calculated by the ratio of the counting at 26 and 31 DAS, corresponding to the counting of individuals surviving to the cold levels and extreme cold, respectively. Those counting were compared with evaluations carried out at 21 DAS, before application of cold treatments.

Expressed by the formula:
SR = 26 DAS / 21DAS
SR = 31 DAS / 21DAS

The experimental design utilized in the lab was augmented blocks of Federer. Complementary analysis was performed with the finding of significant minimum differences of mutants *vs* control treatments. Genetic gain analysis of mutants was performed, evaluating the variability in survival individuals at different cold levels and regarding to different generations (M2 and M3).

The estimated genetic gain was expressed by the formula:

$GGE = h^{2\,X}\, s^2_{G}\,{}^{X} i$,,

The GGE corresponds to the estimated genetic gain, h^2 = heritability, s^2_G = genetic variance, i = selection index of 10%. Genetic variation was removed from the phenotypic variance of mutants, where the fraction of the residue mean square (environmental variance) was removed from the mean square variance of the mutant in order to obtain the s^2_G, thereby obtaining the genetic variation.

Data were submitted to analysis of variance by F test, according to the model proposed by the augmented block design, considering the control treatments as fixed. If there was significance in the F, complementary analyzes were carried out by calculating the least significant difference in each cold level at 5% error probability ($p < 0.05$).

RESULTS AND DISCUSSION

Analysis of variance through F test revealed significant effect for all studied cold levels and also to the association of cold levels associated with extreme cold (-5°C), which indicates that significant differences among palisade grass mutants and check varieties.

In the comparison of the survival rate at 1°C among control treatments *vs* M2 and M3 mutants, there was significant difference (Table 1), where the 3-M2 (0.98), 4-M2 (0.75), 5-M2 (1), 7-M3 (0.98), 8-M3 (0.87), and

9-M3 (1) mutant individuals showed higher survival rate compared to the control treatments BRS Piatã (0.64) and Marandu (0.51). The differences were even more pronounced after the association with the extreme cold (1°C + -5°C), when mutants in M2 and M3 generations 3-M2 (0.60), 4-M2 (0.63), 6-M2 (0.36), 7-M3 (0.25), 9-M3 (0.30), and 10-M3 (0.45) were also superior than check varieties BRS Piatã (0.20) and Marandu (0.15). In this regard, there was genetic gain with mutant selection, since cv. Marandu was the genitor that provided seeds used in the mutation induction.

Table 1: Comparison through least significant difference in study with augmented blocks among control treatments *vs* mutants for the variable survival rate to cold in mutants of M2 and M3 generations in relation to check varieties of palisade grass to the level of 1ºC and association to -5°C.

Control treatments/Populations	Temperature ºC	
	1°C	(1°C + -5ºC)
1-BRS Piatã	0.640628	0.2014815
2-Marandu	0.51388875	0.1555555
3-M2	0.98243088*	0.6017095*
4-M2	0.75114738*	0.6374075*
5-M2	1*	0.2239735
6-M2	0.58202087	0.3689945*
7-M3	0.98243088*	0.2555555*
8-M3	0.87614738*	0.1731215
9-M3	1*	0.3035185*
10-M3	0.46513687	0.4512455*

*Least significant difference of 0.2343 and 0.0717 from the left toward the right respectively between control treatments and mutants.

The mutation occurrence may eventually provide from the specific modification of a gene to the modification of the chromosome number and structure (Allard, 1971). Proceeding mutation induction with chemicals causes randomly changes in nucleotides throughout the genome, which can generate individuals with traits of interest to plant breeding (Taiz and Zeiger, 2010).

Regarding the response of genotypes exposed to 0°C and associated with -5°C, there were significant differences among mutants *vs* control treatments (Table 2). The 4-M2 (0.71), 5-M2 (0.67), 6-M2 (1), 9-M3 (0.96), and 10-M3 (1) mutants in temperature 0°C showed a superior survival rate than control treatments BRS Piatã (0.53) and Marandu (0.39). Furthermore, survival rate decreased significantly with the exposure of these plants to extreme cold (0°C + -5°C), both for control treatments and for mutants. Only the M2-6 (0.64) and 9-M3 (0.38) mutants showed significant differences superior to control treatments.

This information has great importance for breeding purposes, as the initial process for the selection of new cultivars tolerant to cold, justifying by the greater presence of surviving individuals to the condition of low temperature, allowing greater variability in the selection of this trait in comparison with control treatments without mutation induction. There was survival to cold of some individuals in control treatments, being justified by the species presence of some level of low temperatures tolerance. Moreover, the same was observed in a study with 16 forage species undergoing evaluation in winter place with low temperatures, where the palisade grass showed moderate cold resistance (Andrade *et al*., 2002).

Table 2: Comparison through least significant difference in study with augmented blocks among control treatments *vs* mutants for the variable survival rate to cold in mutants of M2 and M3 generations in relation to check varieties of palisade grass to the level of 0°C and association to -5°C.

Control treatments/Populations	Temperature ºC	
	0°C	(0°C + -5ºC)
1- BRS Piatã	0.5359063	0.16055718
2-Marandu	0.39360119	0.04166667
3-M2	0.56265584	0.08071566
4-M2	0.71566949*	0.06555474
5-M2	0.67567924*	0.08843911
6-M2	1*	0.64846041*
7-M3	0.50111738	0.0731305
8-M3	0.16951565	0.06555474
9-M3	0.96139353*	0.38605816*
10-M3	1*	0.08596041

*Least significant difference of 0.2791 and 0.1327 from the left toward the right respectively between control treatments and mutants.

Regarding the -1°C cold levels, significant differences were revealed among mutants *vs* control treatments (Table 3). The 5-M2 (1), 8-M3 (0.86), 3-M2 (0.76), 4-M2 (0.74), 6-M2 (0.70), and 9-M3 (0.63) mutants with greater survival rate than the control treatment cv. Marandu (0.51), being only 5-M2 superior than BRS Piatã (0.85). However, when the -1°C cold level was associated with extreme cold (-1°C + -5°C), both control treatments were inferior to the 6-M2 (0.46) and 8-M3 (0.93) mutant individuals.

In research carried out with the forage species Tifton 85, Missioneira Gigante, *Brachiaria* Piatã e Marandu in southern Brazil, in region with soil and weather conditions quite similar to the conditions that this study was

carried out, the authors concluded that both palisade grasses had greater biomass production of dry matter, with greater growth than the other species (Borsuk *et al*., 2013). However, the authors report that during autumn/winter production decline was quite significant. The peculiarity observed meets this study, where even in the early M2 and M3 generations, promising mutants for cold tolerance stand out.

Table 3: Comparison through least significant difference in study with augmented blocks among control treatments *vs* mutants for the variable survival rate to cold in mutants of M2 and M3 generations in relation to check varieties of palisade grass to the level of -1°C and association to -5°C.

Control treatments/Populations	Temperature ºC	
	-1°C	(-1°C + -5ºC)
1- BRS Piatã	0.85024025	0.33641525
2-Marandu	0.51106225	0.0261905
3-M2	0.76768825*	0.05922662
4-M2	0.74492275*	0.06869713
5-M2	1*	0.08887888
6-M2	0.70598025*	0.46904487*
7-M3	0.46768825	0.23077338
8-M3	0.86992275*	0.93130288*
9-M3	0.63974175*	0.08887888
10-M3	0.55042425	0.14904488

*Least significant difference of 0.1233 and 0.2296 from the left toward the right respectively between control treatments and mutants.

For individuals assessed at -2°C, there was superiority of 3-M2 (0.68), 4-M2 (1.00), and 5-M2 (0.66) mutants and for M3 generation 8-M3 (0.58), 9-M3 (0.56), and 10-M3 (0.62) mutants in relation to the control treatment Marandu (0.38). Furthermore, only 4-M2 (1.00) mutant was greater than the control treatment BRS Piatã (0.62) (Table 4). However, when the extreme cold level (-2°C + -5°C) was used, there was superiority of 4-M2 (0.75) and 6-M2 (0.63) mutants, indicating greater cold tolerance of these mutants in comparison with check varieties. The acclimation to environmental stresses has great importance in breeding programs of forage species, showing great potential for the study *Brachiaria* family species (Valle *et al*., 2009).

Table 4: Comparison through least significant difference in study with augmented blocks among control treatments *vs* mutants for the variable survival rate to cold in mutants of M2 and M3 generations in relation to check varieties of palisade grass to the level of -2°C and association to -5°C.

Control treatments/Populations	Temperature ºC	
	-2°C	(-2°C + -5ºC)
1-BRS Piatã	0.6234025	0.1547325
2-Marandu	0.389955	0.0755
3-M2	0.68565875*	0.34486125
4-M2	1*	0.75955625*
5-M2	0.66348375*	0.33046625
6-M2	0.35132375	0.63711625*
7-M3	0.48565875	0.29486125
8-M3	0.58958375*	0.08007625
9-M3	0.56904375*	0.18046625
10-M3	0.62238375*	0.19203625

*Least significant difference of 0.1635 and 0.2707 from the left toward the right respectively between control treatments and mutants.

For the -3ºC cold level (Table 5), 5-M2 (0.84) mutant and M3 generation 8-M3 (0.54), 9-M3 (0.55), and 10-M3 (0.67) mutants demonstrated greater survival rate compared to control treatments BRS Piatã (0.34) and Marandu (0.33). By reducing the temperature to the extreme cold level (-3ºC + -5°C), the control treatments had lower survival rate, providing ratios below 16%. However, the mutants M2-3 (0.26), 4-M2 (0.32), 5-M2 (0.21), and M3-8 (0.34) were greater, especially in relation to the control treatment BRS Piatã (0.04).

Among the mechanisms developed by plants to increase cold tolerance, it is highlighted the greater amino acids and sugars accumulation (Alcazar *et al*., 2011), greater fluidity of the plasma membrane (Taiz and Zeiger, 2010), and greater presence and action of anti-freezing proteins (Wang *et al*., 2006). Thus, these mechanisms may have been favored with the mutation induction.

Table 5: Comparison through least significant difference in study with augmented blocks among control treatments *vs* mutants for the variable survival rate to cold in mutants of M2 and M3 generations in relation to check varieties of palisade grass to the level of -3°C and association to -5°C.

Control treatments/Populations	Temperature ºC	
	-3°C	(-3°C + -5ºC)
1- BRS Piatã	0.34857469	0.04181185
2-Marandu	0.33640351	0.15657895
3-M2	0.45777732	0.26460893
4-M2	0.3934992	0.32192267
5-M2	0.84590743*	0.21200028
6-M2	0.39356045	0.14267366

7-M3	0.22444399	0.03539107
8-M3	0.54804465*	0.3491954*
9-M3	0.55424076*	0.08700028
10-M3	0.6702403*	0.0991954

*Least significant difference of 0.1815 and 0.1685 from the left toward the right respectively between control treatments and mutants.

Based on results revealed by the study, the selection of individuals with exposure to moderate cold expressed greater frequency of surviving individuals, increasing the chances of selection of individuals with cold tolerance associated with agronomic traits of interest as the great biomass production of dry matter. The acclimatization of tropical grasses to cold is evaluated by leaf damage and survival of plants under field conditions (Ludlow, 1980). However, the seedling stage is considered the stage with increased susceptibility to cold (Souza *et al*., 2013), justifying the use of this methodology in a controlled environment to pre-select individuals adapted to environments with low temperatures during winter period.

In research developed to study cold tolerance in different *Oryza sativa* populations, it was observed that the stress provided by the cold is directly linked to sensitivity presented in varieties and among varieties, with assessments carried out with bulk and pedigree revealing variation among studied generations (Bouharmont and Bertin, 1997). This information consistent with the responses found in the current study, which also has observed differences among M2 and M3 generations of palisade grass.

Regarding the genetic gain estimated among populations (GGE_A), there was a higher gain in the M2 (6.59%) to M3 (3.37%) population. Comparing generations (Table 6), the M3 population expressed smaller genetic gain values that the M2 generation, possibly due to the lower genetic variability due to environmental selection provided by the cold condition.

Table 6: Behavior of palisade grass genotypes expressed in estimated genetic gain within and among populations for the factors within the CL population (cold levels) and CL + associated -5°C (cold levels associated with extreme cold), and among populations for individuals in M2 and M3 generation.

	Genetic gain within population				
	1 °C	0 °C	-1 °C	-2 °C	-3 °C
CL	14.15	28.62	0.19	7,11	0,03
CL + -5°C	18.85	19.6	46.85	45,68	9,69
	Genetic gain among populations				
	M2 Population			M3 Population	
CL	6.59			3.37	
CL+ -5°C	26.78			17.33	

The greater estimated genetic gains within the population GGE_W in function of five different cold levels were obtained in the temperature of 0ºC (28.62%), followed by the temperature of 1ºC (14.15%). The GGE_W of the analyzed population in temperature of -3ºC (0.03%) was lower than the other estimates, not corresponding to an adequate temperature to generate genetic variability of cold tolerant individuals in palisade grass breeding programs.

The GGE_W in function of five cold levels at 26 DAS and the exposure to extreme cold -5°C (Table 6) resulted in the greatest selection gains within the population at temperatures of -1°C (46.85%) and -2°C (45.68%). The smaller GGE_W were obtained at temperature of -3°C (9.69%). In general, the use of extreme cold temperatures is important as it provided great gain selection than only the cold levels application.

Regarding the genetic gain among populations, the selection of individuals at an early developmental stage in B.O.D. can be considered a useful tool for breeding programs by eliminating all individuals susceptible to cold stress at an early time. In this way, it optimizes the resources needed for experiments carried out in field conditions.

Increasing the frequency of low temperatures incidence provides the selection of the most tolerant individuals. In this sense, breeding programs should expose genotypes to severe cold conditions, selecting genes for better environment acclimatization (Ramalho *et al*., 2012). Providing the future launching of palisade grass cultivars with great biomass production of dry matter during all periods of the year without going through a sharp decline in the winter period, as observed in studies reported in western Santa Catarina state (Bursok *et al*., 2013).

The presented results indicate that occurred genetic gain (cold exposure), especially for extreme cold conditions where the effects of chemical mutation generated genetic variability associated with cold tolerance traits for all cold levels studied (1°C to -3°C + associations with -5°C). Due to the increased number of surviving individuals at low temperatures, it is recommended to use this methodology in future studies with palisade grass and other forage species in order to increase the frequency of cold tolerant individuals.

Conclusions:

The chemical mutation induction generated genetic variability for palisade grass individuals and allows genetic selection for cold conditions. Genetic variability in M2 population is greater than M3 population, allowing to obtain greater genetic gains for cold tolerance.

The palisade grass mutants can be selected in negative temperatures in controlled germination chamber. The largest number of surviving individuals was observed with the cold levels -1°C (46.85%) and -2ºC (45.68%) associated with extreme cold of -5°C.

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