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Induction of genetic variability and plant development in palisade grass evaluated in M2 mutants

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The objective of this study was to evaluate the induction of genetic variability and plant development of palisade grass (*Urochloa brizantha* L.) M2 mutants in acclimatization to subtropical climate condition. The mutagenic agent methyl methanesulfonate (MMS), at a dosage of 0.5%, was used to induce mutation in 4,000 seeds of the cultivar Marandu. Thirty five plants survived after the induction of MMS mutagen agent. These plants were isolated transplanted in experimental area to advance the generation. Seeds produced by plants were sown in pots allocated in Biosystems Organized Development incubator and subjected to cold stress at 0°C in seedling stage. Surviving seedlings per family were transplanted to field conditions in Southern Brazil. The plant development was evaluated in the M2 generation during the agricultural years of 2012-2013 and 2013-2014. Genetic variability analyzes were carried out with 21 traits, based on the average Euclidean distance and the relative contribution proposed by Singh (1981). Chemical mutation induction with MMS generates genetic variability in palisade grass, enhancing the selection of superior genotypes in subtropical climate conditions. The chemical induction of mutations with the MMS mutagen provided genetic variability in the population of *U. brizantha* with formation of 18 divergent groups during the research.

Key words: *Urochloa brizantha* L., plant breeding, genetic recombination, forage, multivariate analyses.

INTRODUCTION

The species of genus *Urochloa* are grown in tropical and subtropical regions of the southern hemisphere. The Brazilian cultivated land area has increased due to the species hardiness and adaptation capacity to different climatic conditions. Those characteristics highlight the country as a big exporter of this species seeds, having commercial relations with approximately 40 countries (CARDOSO et al., 2014).

The genetic breeding of this species faces difficulties to increase the genetic variability at the germplasm banks

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due to the influence of apomictic reproduction mode and the presence of different polyploidy levels. A research was carried out with *Urochloa brizantha* accesses in the germplasm bank of the Brazilian Agricultural Research Corporation - EMBRAPA with a total of 222 accesses. The results showed that all accesses are polyploid, which two are diploid, 157 are tetraploid, 41 are pentaploid, and 22 are hexaploid. These factors hamper sexual reproduction due to the chromosome parity and the ploidy level (Resende et al., 2008).

Among the challenges of genetic breeding, the formation of *Urochloa* segregating populations is difficult to reach because of the difficulties in increasing variability in this species (Valle et al., 2009). Currently, the steps of introduction, evaluation and selection are necessary to launch new cultivars of *Urochloa* (Araújo et al., 2008). Research related to the existent genetic diversity on the germplasm banks of *Urochloa* have been conducted by Ambiel et al. (2010) and Garcia et al. (2013). These researchers found that the genetic variability index is considered low. Moreover, Fuzinatto et al. (2012) studied the possibility of crosses between different species of *Urochloa* and indicated that chromosomal irregularity and apomixis are factors that contribute increasing difficulties in exploring the genetic variability and fixing traits of interest.

Studies regarding the induction of mutations present potential for exploitation to expand the genetic variability in *Urochloa*, since the chemical mutation induction is characterized as an alternative to generate genetic variability for species such as *Urochloa*. This species exhibit difficulty in generating new natural recombination because of its narrow genetic basis (Muller, 1927). In Brazil, research with the mutation induction in the Poaceae family species were performed by Coimbra et al. (2004) and Souza et al. (2005), which indicated success in increasing genetic variability and changes in desired traits, standing out as an alternative to generate genetic variability. A mutation is inclined to be a specific change in a gene or act on chromosomes (Allard, 1971), which highlight successive random changes in nucleotides. In this way, it can provide combinations that generate genetic variability amplification (Taiz and Zeiger, 2013).

The use of appropriate methodologies to evaluate *Urochloa* accesses is important to characterize new individuals. The unweighted pair group method with arithmetic mean (UPGMA) is utilized to assess the variability in forage grasses. Totti et al. (2001) utilized the same method in *Paspalum* accesses, describing it as appropriate due to the consistency in distinguishing accessions. Furthermore, studies with ecotypes of *U. brizantha* also observed the efficiency of UPGMA clustering method in the study of genetic diversity (Torres et al., 2015).

In addition to the genetic diversity, it is important to study the canopy growth and development. Thereby, regrowth and vegetative growth are important for assessing the carrying capacity of animals in the pastures (Lenzi, 2012; Souza et al., 2013). However, seed production should also be considered in forage crops breeding since there is a direct positive relation between the inflorescences number and increased seed yield (Bean, 1972). The objective of this study was to evaluate the induction of genetic variability and plant development of palisade grass (*U. brizantha* L.) M2 mutants in acclimatization to subtropical climate condition.

MATERIALS AND METHODS

The experiment was carried out in the Plant Breeding and Plant Production Laboratory of Federal University of Santa Maria located at coordinates of 27°S23'26" and 53°25'43"W, altitude of 461.3 m in the state of Rio Grande do Sul, southern Brazil. Soil structural properties were based on soil analysis carried out at the experiment area. The soil is characterized by 60% of clay, pH 6.3 and 2.3% of organic matter demonstrating good fertility levels for phosphorus, potassium, and micronutrients. A base fertilization with 20 kg ha^{-1} of N, 80 kg ha^{-1} of P_2O_5, and 80 kg ha^{-1} of K_2O (NPK only in rate of 05-20-20) was performed prior to the experiment installation.

Chemical mutation induction on 4,000 viable seeds of *U. brizantha* cv. Marandu was held with the mutagen methyl methanesulfonate (MMS) at a dose of 0.5% according to the methodology adopted by Coimbra et al. (2004) in order to assess the genetic variability. After exposure to the mutagen, the seeds were sown in pots with soil and commercial substrate. The preselection was performed at 21 days after sowing (DAS), when the seedlings were exposed to the controlled temperature of 0°C, which is the frost formation temperature, in B.O.D. incubator. The surviving seedlings per family were transplanted to field conditions on March 15, 2011, forming the basis population that originated the progenies studied in this research, corresponding to the M2 generation.

Transplanted seedlings were exposed to low temperatures condition reaching up to 0°C during winter time, until seed production in the following year. Seeds were collected from 35 individuals that gave rise to M1 generation. The seeds that gave rise to M2 generation were allocated with the check varieties *U. brizantha* MG5, *U. brizantha* BRS Piatã, and *U. brizantha* Marandu sowed in rows on April 3rd, 2012. The plant development was evaluated in the M2 generation during two agricultural years. Experimental units consisted of two meters in length and 0.45 m of distance between genotypes. Potassium nitrate at 0.2% was used at the sowing row to break dormancy. The experimental design used for the accesses allocation was the augmented blocks of Federer, where check varieties were allocated with three repetitions.

Twenty one traits were considered in the analysis to study the genetic variability among different genotypes. The studied traits are final height in the 1st year (FH1), final number of inflorescence in the 1st year (FNI1), final number of tillers (FNT), average value of chlorophyll (AVC), final height in the 2nd year (FH2), final number of inflorescences in the 2nd year (FNI2), regrowth notes index after frost (RNF), number of seeds rows per ear (NRE), length of the last open leaf (LOL), width of the last open leaf (WOL), flower stem length (FSL), length of the inflorescence shaft (LI), racemes number per inflorescence (RNI), length of the basal raceme (LBR), number of spikelets per basal racemes (NSBR), stigma color (SC), anther color (AC), length of the rachis hairiness (LRH), density of limbo hairiness (DLH), density of sheath hairiness (DSH), and density of

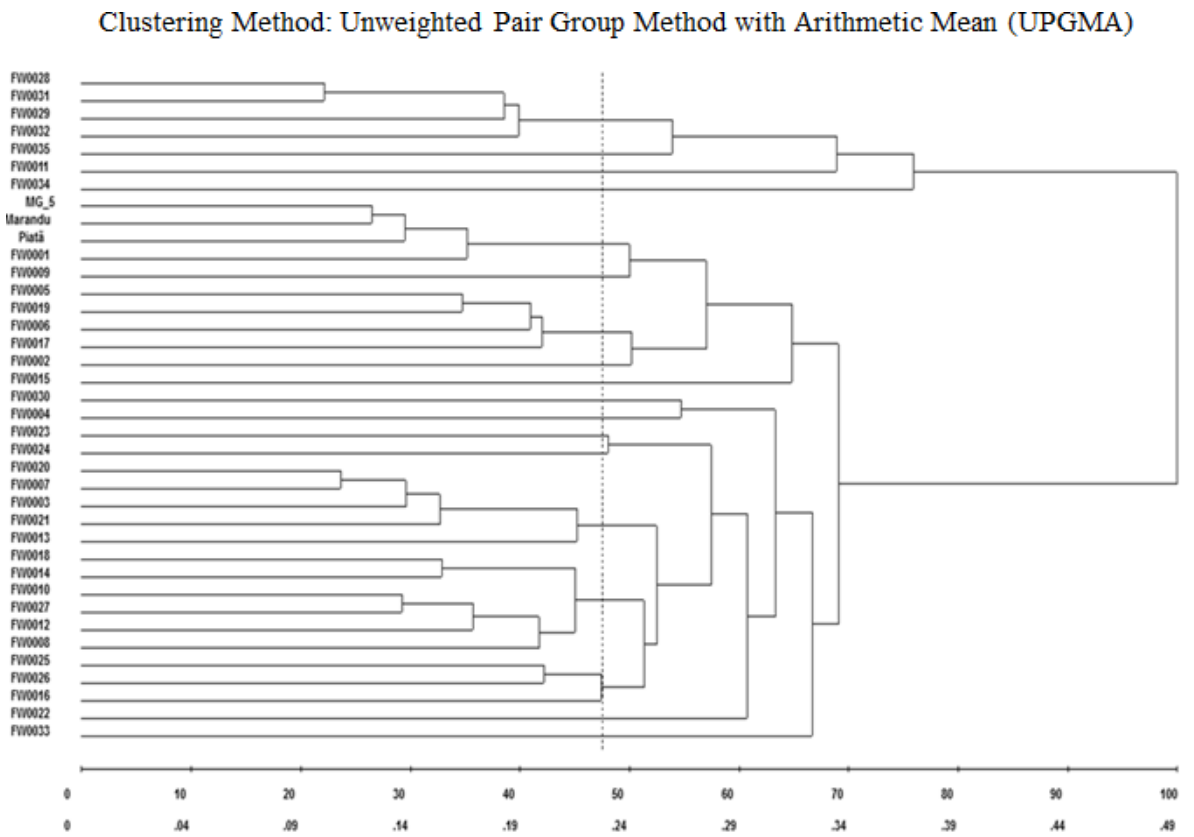

Figure 1. Dendrogram formed by the UPGMA clustering method based on the average eucledian distance, representing the dissimilarity between thirty five M2 mutants accesses of *U. brizantha* and check varieties *U. brizantha* MG-5, *U. brizantha* Piatã, *U. brizantha* Marandu formed from 21 morphological traits carried out in subtropical climate in two agricultural years in Frederico Westphalen, southern Brazil.

the stem hairiness (DTSH).

Evaluations were carried out weekly after the beginning of genotypes emergence to assess the proposed traits FNT, AVC, and RNF. The other assessments to measure the variability were carried out when the genotypes reached full flowering and the final tillers establishment at 140 DAS. The UPGMA method was used for the dissimilarity evaluation and to study the relative contributions of the studied traits.

Assessments of growth and morphological traits were performed weekly to perform the genotypes acclimatization analysis, as follows: Weekly growth rate in the first year: the sowing of the M2 generation was held on April 3rd, 2012, measuring up the weekly development of the studied accesses on observing height in cm, measured from the beginning of the emergency to full flowering of the genotypes; weekly growth rate in the second year: After standardization cut, the weekly development of studied accesses was measured in height (cm). The observations were started before the first frost formation, on July 17th, 2013, corresponding to the start of regrowth until full flowering; appearance of inflorescence in the second year: weekly observation was proceeded to evaluate the appearance of inflorescences, assessing the present inflorescences in the corresponding evaluation units of the week. These evaluations started on September 18th, 2013 being carried out until the full flowering of all genotypes.

Growth variables and inflorescence appearance were submitted to polynomial regression to assess the behavior of accesses in subtropical conditions. Both accesses with better performance and the check varieties were evaluated. Statistical analyzes were performed utilizing Microsoft Office Excel® application and Genes software (Cruz, 2013).

RESULTS AND DISCUSSION

According to Figure 1, there was the formation of 18 distinct groups with average dissimilarity of 23.51% and 17 groups demonstrated dissimilarity in relation to the check varieties. The three check varieties and the genotype FW 0001 were grouped together with unusual characteristics that provide them high similarity. After the measurement of the canopy morphological traits based on 21 studied traits (Table 1), it appears that 97% of the progeny have dissimilarity in relation to check varieties. There was the formation of a group of six genotypes, a group of five genotypes, three groups with four genotypes, one group of three genotypes and 12 groups with one genotype, showing that the UPGMA method was also effective for this research, as also reported in other studies with forage grasses (Totti et al., 2001; Torres et al., 2015).

Detected genetic dissimilarity demonstrates that mutation induction generated high genetic variability,

Table 1. Relative contribution of 21 traits according to the methodology proposed by Singh, referring to population formed by mutants accesses (M2 generation) of *U. brizantha* in acclimatization to subtropical conditions and check varieties *U*. *brizantha* MG-5, *U. brizantha* BRS Piatã, *U. brizantha* Marandu in two agricultural years in Frederico Westphalen, southern Brazil.

Variable	S.j	Relative contribution (%)
Final height in the 1st year	991994.2	5.9599
Final number of inflorescence in the 1st year	664143.1	3.9901
Final number of tillers	12156.93	0.073
Average value of chlorophyll	84783.39	0.5094
Final height in the 2nd year	573419.7	3.4451
Final number of inflorescences in the 2nd year	5261030	31.6081
Regrowth notes index after frost	1749.806	0.0105
Number of seeds rows per ear	3845.444	0.0231
Length of the last open leaf	41354.95	0.2485
Width of the last open leaf	63.18086	0.0004
Flower stem length	4999732	30.0382
Length of the inflorescence shaft	3393389	20.3874
Racemes number per inflorescence	4490.728	0.027
Length of the basal raceme	51446.97	0.3091
Number of spikelets per basal racemes	556985.1	3.3463
Stigma color	1114.222	0.0067
Anther color	1064	0.0064
Length of the rachis hairiness	845.5463	0.0051
Density of limbo hairiness	293.2346	0.0018
Density of sheath hairiness	315.1235	0.0019
Density of the stem hairiness	349.5309	0.0021

S.j. Relative contribution as criterion Singh (1981).

which was also found in other studies with Poaceae family species, as these proceeded in Brazil with oat (Coimbra et al., 2004; Souza et al., 2005). This increase in variability provides high importance to *Urochloa*, mainly due to reproductive difficulties that are provided by the presence of apomixis, present in the genotypes of *U. brizantha* studied in the Brazilian germplasm bank (Resende et al., 2008; Valle et al., 2009) and chromosomal irregularities (Fuzinatto et al., 2012).

The presence of dissimilarity is associated with the efficiency of chemical induction of mutations with the mutagen MMS associated with preselection applied on the progenies. Cold temperatures simulation was applied during preselection and it is characterized as a strategy to eliminate at an early time individuals with low tolerance to cold. At the same time, it allows to select progenies that have cold tolerance, which can provide benefits to the breeding program, selecting promising genotypes for this trait of interest. According to a study performed in Ona, state of Florida, forage selection for cold tolerance was promising as they found herdability of 0.39 to 0.50 for the trait cold tolerance in two crop cycles. These positive results enabled new selections in later generations (Jank et al., 2002).

The use of chemicals for mutation induction provides random nucleotide changes over the genome, which can generate individuals with agronomic interest (Allard, 1971). Among the mechanisms developed by plants throughout evolution to increase cold tolerance, it stands out the greater accumulation of amino acids and sugars (Alcázar et al., 2011), action of anti-freeze proteins (Wang et al., 2006), and greater fluidity of the plasma membrane exposed to stress by cold (Taiz and Zeiger, 2013).

According to the analysis of the 21 variables (Table 1), there was different inputs depending on the studied trait. Among all studied traits, FNI2 (31.60%), FSL (30.03%), LI (20.38%), FH1 (5.95%), FNI1 (3.99%), FH2 (3.44%), and NSBR (3.34%) were highlighted with greater contributions. The remaining 14 variables contribute individualy with values below 1%. The study of dissimilarity among genotypes is important to proceed with the screening of individuals with potential to move forward in a breeding program. In order to register a new cultivar, it must have distinctness from other cultivars by a minimum number of descriptors having homogeneity and stability (Brazil, 2011).

Regarding the analysis of genotypes acclimatization, the greater development (Figure 2) regarding the linear growth of check varieties and genotypes more acclimated to the study conditions was explained by the polynomial regression curve of second degree order, with emphasis

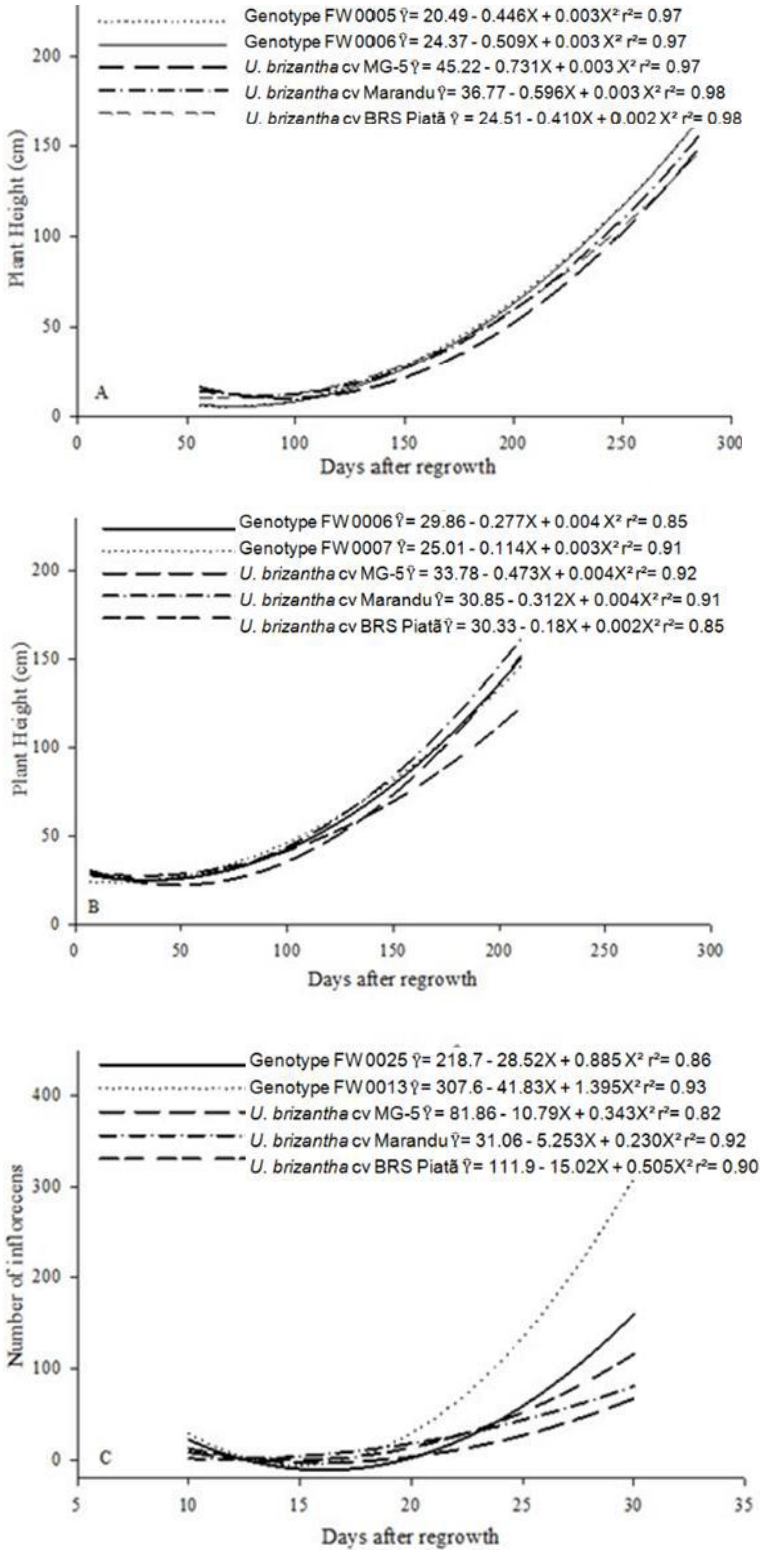

Figure 2. Linear growth of *U. brizantha* genotypes expressed in centimeters with measurements from sowing to full flowering (A), regrowth to full flowering (B), and emergence of inflorescences expressed in units per evaluation plot (C) referring to a population formed by mutants accesses in M2 generation and check varieties *U. brizantha* MG-5, *U. brizantha* BRS Piatã, *U. brizantha* Marandu in subtropical climate in two agricultural years in Frederico Westphalen, southern Brazil.

on access FW 0005 and FW 0006. The proper initial growth before the flowering is important as the initial flowering moment is characterized as the optimal resting point on forage species. At this time, there is a high assimilates translocation from the aerial part to the roots, keeping the reserves available for the forage regrowth. Thereby, grazing performed before or after the optimum point of rest may degrade the pasture (Lenzi, 2012). The experimental precision during the study was considered appropriate due to the crop development from sowing to flowering presented r^2 above 0.97, regrowth to flowering showed r^2 above 0.85, and appearance of inflorescences time exhibited r^2 above 0.82.

The beginning of flowering corresponded to 208 DAS, as the estimated value for growth rate of *U. brizantha* MG-5 was 22.96 cm, *U. brizantha* BRS Piata was 25.75 cm, and for *U. brizantha* Marandu was 42.59 cm. These values are low compared to the better mutant genotypes that expressed 48.29 cm for the FW 0006 genotype and 57.51 cm for the FW 0005 genotype. The estimated values expressed influence of increased cold tolerance in the initial period. Therefore, the main characteristics required for cold tolerance in subtropical regions are the use of more vigorous seedlings, tillering capacity, presence of axillary tillers, and fast regrowth after periods of low temperatures (Souza et al., 2013).

The growth in the second year of evaluation corresponds to regrowth (Figure 2). It is explained by polynomial regression curve of second degree order, where the beginning of flowering was used to estimate the regrowth height with estimated height values of 34.14 cm for *U. brizantha* MG-5, 37.23 cm for *U. brizantha* BRS Piata, 50.36 cm for *U. brizantha* Marandu, 53.54 cm for FW0006, and 53.92 cm for FW0007, that were the better accesses. The mutant genotypes presented greater values than *U. brizantha* Marandu, that was the best check variety for this trait. It is necessary reliable phenotypic expressions that can form the basis for evaluation under field conditions in order to have proper selection and discard of genotypes (Cruz, 2005). This statement can be attributed to the FW0006 genotype, which was expressed among the best individuals related to its growth in two years. This access has potential for further applications in a breeding program.

Regarding the emergence of inflorescences in the second year of evaluation (Figure 2), it showed development explained by the polynomial regression curve of second degree order, with trend line estimated in the full flowering of 66 inflorescences for *U. brizantha* MG-5, 115 inflorescences for *U. brizantha* BRS Piata, 80 inflorescences for *U. brizantha* Marandu, 308 inflorescences for access FW0013, and 159 inflorescences for access FW0025. The planning and selection decision is a vital moment for a breeding program. However, the topic of associating high matter production and seed production of the genotypes should present a balance because genotypes with low seed production may have compromise the economic viability of its commercial release. Moreover, evaluating and selecting the trait number of inflorescences in *Setaria sphacelata* was considered feasible due to the 17% increase in the number of inflorescences in relation to the original population (Jank et al., 2007).

In accordance with analysis of vegetative growth for the studied traits, genotypes with greater growth and regrowth were not in the group with greater seed production. Moreover, the increase in inflorescences mass is a main factor to increase seed production (Bean, 1972). Thus, the selection of individuals that meet market demands for quality forage production combined with proper levels of seed production should be performed during the process of advancing generations.

Conclusion

The chemical induction of mutations with the mutagen methyl methanesulfonate provided genetic variability in the population of *U. brizantha* with formation of 18 divergent groups during the research. The access FW0006 presented proper plant development, standing between the two best accesses during the two years of study. It demonstrates potential for further studies in the breeding program with aim of selecting genotypes with cold tolerance in subtropical regions.

Conflict of Interests

The authors have not declared any conflict of interests.

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