CHANGES IN ALLELE FREQUENCIES IN COLCHICINE-TREATED RYEGRASS POPULATIONS ASSESSED WITH RAPD MARKERS

ALTERAÇÕES NAS FREQÜÊNCIAS ALÉLICAS DE POPULAÇÕES DE AZEVÉM SUBMETIDAS A TRATAMENTO COM COLCHICINA

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ABSTRACT

shown that besides chromosomal duplication, ryegrass genotypes treated with colchicine present a significant amount of novel traits, suggesting that colchicine also has point mutagenic effects. Molecular approaches were used to evaluate diversity and relatedness in two annual ryegrass populations treated with colchicine. Colchicine treatment generated differences beyond the levels of inter and intrapopulational variability. Contrary to expectations, populations subjected to higher colchicine doses did not always contain more diversity than untreated populations. The use of Random Amplified Polymorfic DNA (RAPD) markers allowed the comparison of colchicine effects on different genome regions, which strongly support the findings at the morphological levels.

Key words: mutagenic, AMOVA, variability.

INTRODUCTION

The alkaloid colchicine is a potent polyploidy-inducing mutagenic agent. Since its discovery, in 1937, researchers have been using it either to directly induce chromosomal duplication (autopolyploids), or to restore fertility in interespecific hybrids (amphidiploids), after gene exchange between distinct species (DEWEY, 1980).

In ryegrass (*Lolium* sp) breeding, colchicine is commonly used to develop tetraploid cultivars (BORRIL, 1986). Also, it is frequently used to duplicate hybrids obtained from *Festuca* sp. x *Lolium* sp. (THOMAS & HUMPHREYS, 1991) or *L. perenne* x *L. multiflorum* crosses (JONES & HUMPHREYS, 1993).

An enlargement of vegetative organs is a common feature of autopolyploids (ALLARD, 1971). This phenotypic effect has been described in ryegrass (MYERS, 1939), sorghum (FRANZKE & ROSS, 1952), flax (DIRKS et al., 1956), peanut (CONAGIN, 1972), sunflower (DOWNES & MARSHALL, 1983) and cotton (LUCKETT, 1989).

Recently, studies comparing untreated and colchicine-treated ryegrass genotypes, without the occurrence of chromosomal duplication, have shown significant differences in many traits, e.g., leaf area, flowering date and flower number (HAGUE & JONES, 1987); tillering ability and flowering time (HASSAN et al., 1989); tiller number and leaf weight (FRANCIS & JONES, 1989) and mesophyll cell size and chloroplast number (FRANCIS et al., 1990; HASSAN et al., 1991). These investigations suggest a mutagenic action independent of changes at ploidy levels.

RAPD analyses have given valuable information on the characterization and genetic structure of ryegrass populations

Colchicine has been commonly used to duplicate plant chromosomes. Some studies have

(HUFF, 1997). This study was carried out aiming to analyse the effects of colchicine as a point mutagen, at the molecular level, using RAPD markers in two annual ryegrass populations subjected to different doses of the alkaloid.

MATERIAL AND METHODS

Annual ryegrass populations were obtained from the grass gene bank of the Universidade Federal de Pelotas. Accessions were chosen to represent a breed genotype (uruguayan cultivar “La Estanzuela-284” , LE-284) and a landrace (Pantano Grande-RS, Brazil, PG).

Seeds treated with colchicine solution at 0.2 and 0.4% were kept for three hours at room temperature, along with a distilled water control. Seeds were rinsed with distilled water for one hour and placed in a germinator at 20 ^{o}C. On the fifth day, 10 seedlings from each treatment were randomly chosen and transferred to pots with sterilized soil in the greenhouse.

DNA was extracted from 20 to 25-day-old seedlings by a procedure similar to that used by SAGHAI-MAROOF et al. (1984). From each individual, two independent DNA extraction were carried out. For each gram of plant tissue, 2.5 mL of buffer were added and samples were heated for 30 min at 60 ^{o}C. Afterwards, 1.25 mL of chloroform: isoamyl alcohol (24:1) was added and samples stirred for 15 min and centrifuged for 10 min at 2500 rpm. The supernatant was collected and 1.5 mL of isopropanol were added. Precipitated DNA was washed with 70% ethanol, allowed to air dry and resuspended in TE pH 8.0. DNA quantification was performed in 0.8% agarose gel stained with ethidium bromide. Bands were compared to λ/*Hind*III marker bands.

We previously tested twenty primers from University of British Columbia (UBC 1-20). Ten primers showing consistent amplification in ryegrass (UBC 1, 2, 3, 4, 9, 12, 13, 15, 16 and 18) were employed.

Genomic DNA was pipetted into a microcentrifuge tube to a final concentration of 2.5 ng/µL. Reactions were run in 0.2 mM dNTPs, 0.2 mM cresol red, 0.5µ M primer, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.0, 0.1% Triton X-100 and 1 unit of *Taq* polymerase (Pharmacia Biotech Inc.). Amplification reactions were initiated by a denaturation step at 94 ^{o}C (180 sec), followed by 44 cycles at 94 ^{o}C (60 sec), 38 ^{o}C (60 sec) and 72 ^{o}C (90 sec). After that samples were subjected to 72 ^{o}C for 5 min. Amplified fragments were separated on 1.4% agarose gels, stained with ethidium bromide.

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Each band was scored as present (1) or absent (0) for a given genotype. Each group of individuals subjected to different colchicine doses was considered a new population; A, B and C for the doses 0, 0.2 and 0.4% of colchicine, respectively, applied to population LE-284. For genotypes from population PG, groups were designated as D, E and F, for 0, 0.2 and 0.4% of colchicine, respectively.

The genetic distance between individuals was estimated as Euclidian distance (*E*), according to EXCOFFIER et al. (1992) and HUFF et al. (1993), $E = n[1-2n_{xy}/2n]$, where *n* is the total number of polymorphic bands, and n_{xy} is the number of bands shared by the individuals *x* and *y*. Populations were compared two by two using the Arlequin software (SCHNEIDER et al., 1996). From a matrix obtained from the square of the Euclidian distance, genetic variability between and within populations was estimated, through an analysis of molecular variance (AMOVA). The "AMOVA" calculates the ϕ_{st} value, which is equivalent to the proportion of total variation that is shared between the two populations (EXCOFFIER et al., 1992). The genetic distance between any two populations was represented by its ϕ_{st} value, and referred to as interpopulational distance. Statistical package SAS (SAS Institute, 1990) was used for analyses of principal components. The cluster analyses were performed with the NTSYS software (ROHLF, 2000) using the unweighted pair group method arithmetical means (UPGMA) based on the DICE coefficient (NEI & LI, 1979).

RESULTS AND DISCUSSION

The results indicate that colchicine is capable of generating changes in allele frequencies, and that RAPD markers are informative for assessing genetic variability. Analysed data generated from two independent DNA extractions of plant material from each population were registered. Bands that were not amplified with DNA from both extractions were not included in polymorphism scores. The ten primers used in this study amplified 59 polymorphic bands. However, no band was found to be unique to any population. Also, when plants from PG and LE-284 were compared, no unique band was found. All the relevant analyses performed in the present study were based on differences in allele frequency between populations. The absence of distinct bands in each annual ryegrass population analised is consistent with LOOS (1994), who evaluated 60 perennial ryegrass populations with five isoenzymatic systems but no band unique to a population was found. HUFF (1997) also assessed 18 perennial ryegrass populations with RAPD markers and did not find any unique band.

The result from the analysis of molecular variance showed that 94.76% from total variation was present within the six populations, and only 5.24% was found between populations (Table 1). This result is consistent with the reproductive system of ryegrass, which is allogamous with gametophytic self-incompatibility (FEARON et al., 1983).

Pairwise population comparisons are presented in Table 2. The results showed that among individuals from population LE-284, the only significant difference was found between untreated genotypes and those treated with the highest colchicine dose. However, when individuals belonging to the PG population treated with different doses (0, 0.2 and 0.4%) were compared, no significant differences were found. It suggests that the PG population had never been subjected to artificial selection, thus expressing a broader genetic basis than LE-284. Thus, the chances of some changes in nucleotides caused by colchicine being considered as natural variation are increased.

Table 1 - Analysis of molecular variance (AMOVA) of 60 individuals of *Lolium multiflorum* subjected to different doses of colchicine based on 59 RAPD markers.

Source of Variation	DF	SS	MS	Component of variance	Percentage of total variation
Between populations	1	26.267	26.267	0.546	5.24*
Within populations	58	572.933	9.878	9.878	94.76
Total	59	599.200	36.145	10.424	100

DF: Degrees of freedom; SS: Sum of squares; MS: Mean square. *Significantly different, $\alpha = 0.05$.

Table 2 - Summary of the comparison analysis of annual ryegrass populations, two by two, treated with different doses of colchicine obtained by AMOVA. The percentage of total molecular variation existing between populations (ϕ_{st}) is a measure of genetic distance between populations (bellow diagonal). The test of significance of each value ϕ_{st} was calculated as the probability that a value ϕ_{st} found is higher than the observed value (above diagonal).

Population	A	B	C	D	E	F
A	-	0.075	0.000*	0.071	0.000*	0.000*
B	3.9	-	0.073	0.000*	0.000*	0.000*
C	9.89	3.7	-	0.558	0.000*	0.035*
D	3.1	4.55	-0.005	-	0.188	0.367
E	13.03	9.8	0.061	0.019	-	0.071
F	13.51	13.4	0.050	0.018	0.024	-

Population: "A", LE-284 without colchicine; "B", LE-284 with colchicine 0.2%; "C", LE-284 with colchicine 0.4%; "D", Population PG without colchicine; "E", PG with colchicine 0.2%; "F", PG with colchicine 0.4%.
*Significant difference, $p < 0.05$.

Untreated individuals of PG population did not differ significantly from untreated accessions of LE-284 and also from LE-284 samples subjected to the highest dose. However, they did differ significantly (4.55%) from LE-284 accessions treated with 0.2% colchicine. It was expected that these accessions would also differ significantly from those treated with 0.4% colchicine, however, a negative distance was observed between the LE-284 accessions treated with 0.4% colchicine and the untreated PG accessions.

The data also reveal that the number of shared bands between individuals subjected to both treatments was higher than the characteristic bands from each treatment, suggesting that 0.4% colchicine treatment on the LE-284 individuals originated alleles common to those found in the PG population. Nevertheless, the 0.2% colchicine concentration, gave rise to different alleles. This fact could be explained by the random nature of mutation, however, the idea that increasing the dose from 0.2% to 0.4% would cause a higher reversion of alleles to their native state can not be generalised.

Applying colchicine to PG accessions, both at 0.2 or 0.4%, induced significant differences in these genotypes making them different from all LE-284 accessions. The largest distances found were between accessions of LE-284 without

colchicine and PG accessions with 0.2 and 0.4%, showing molecular variation of 13.03% and 13.51%, respectively. Another large difference, 13.4%, was found between LE-284 accessions treated with colchicine 0.2% and PG accessions 0.4%.

The differences between populations found in the AMOVA can be easily visualized in the principal components analysis, where the first two components explain 16.45% of total variation (Figure 1). On the first component, in general, separation of populations A and C, and of E and F from A, B and C, D being intermediate between these two groups and separated from B, was observed. The second component, however, does not separate the populations, except some genotypes from the PG population (D, E and F). This shows a higher variability within the PG population than within LE-284, which is consistent with the former conclusions.

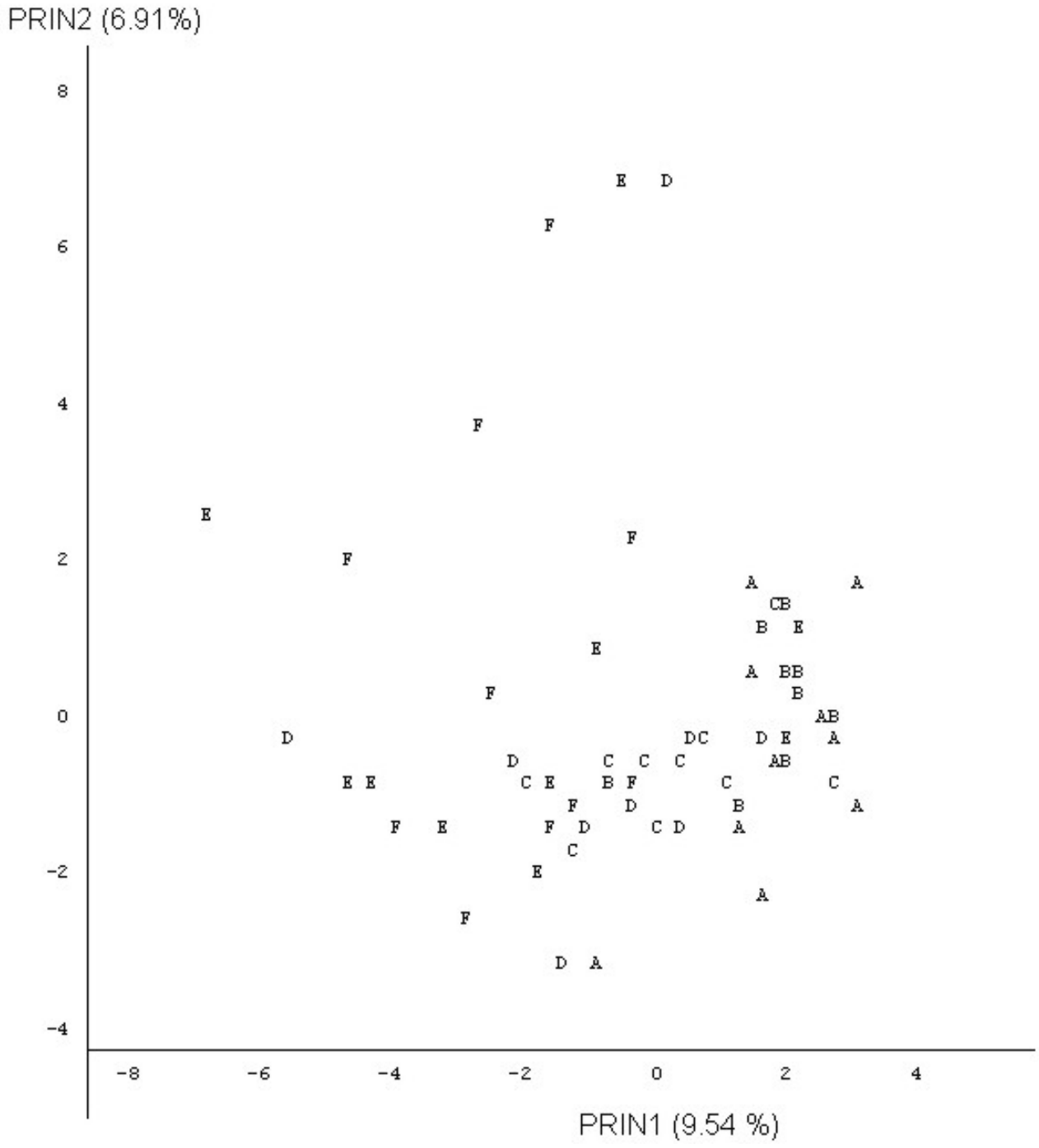

Figure 1 - Principal components analysis of six ryegrass populations treated with colchicine. Populations: “A”, LE-284 without colchicine; “B”, LE-284 with colchicine 0.2%; “C”, LE-284 with colchicine 0.4%; “D”, PG without colchicine; “E”, PG with colchicine 0.2%; “F”, PG with colchicine 0.4%.

In the cluster analysis, considering a cut off point of 0.49 % of similarity, which is equivalent to the average pairwise similarity, seven groups are formed (Figure 2). The first one is divided in two sub-

groups, 1A and 1B. The sub-group 1A is predominantly composed by individuals from population LE-284 (61%). By the other hand, the sub-group 1B is equally formed by individuals from populations PG and LE-284. Individuals from population LE-284 treated with the higher dose of colchicine are dipersed along all the first group. With the exception of group 3, all the other groups formed are composed by individuals from population PG. The dendrogram illustrates the results of the analysis of molecular variance. Individuals from E and F are more distantly related to the others. The fact that the PG population treated with colchicine (E and F) was significantly different from individuals from LE-284 with and without colchicine (A, B and C), suggests that the mutagenic efficiency to generate new alleles is higher when a broader genetic basis exist.

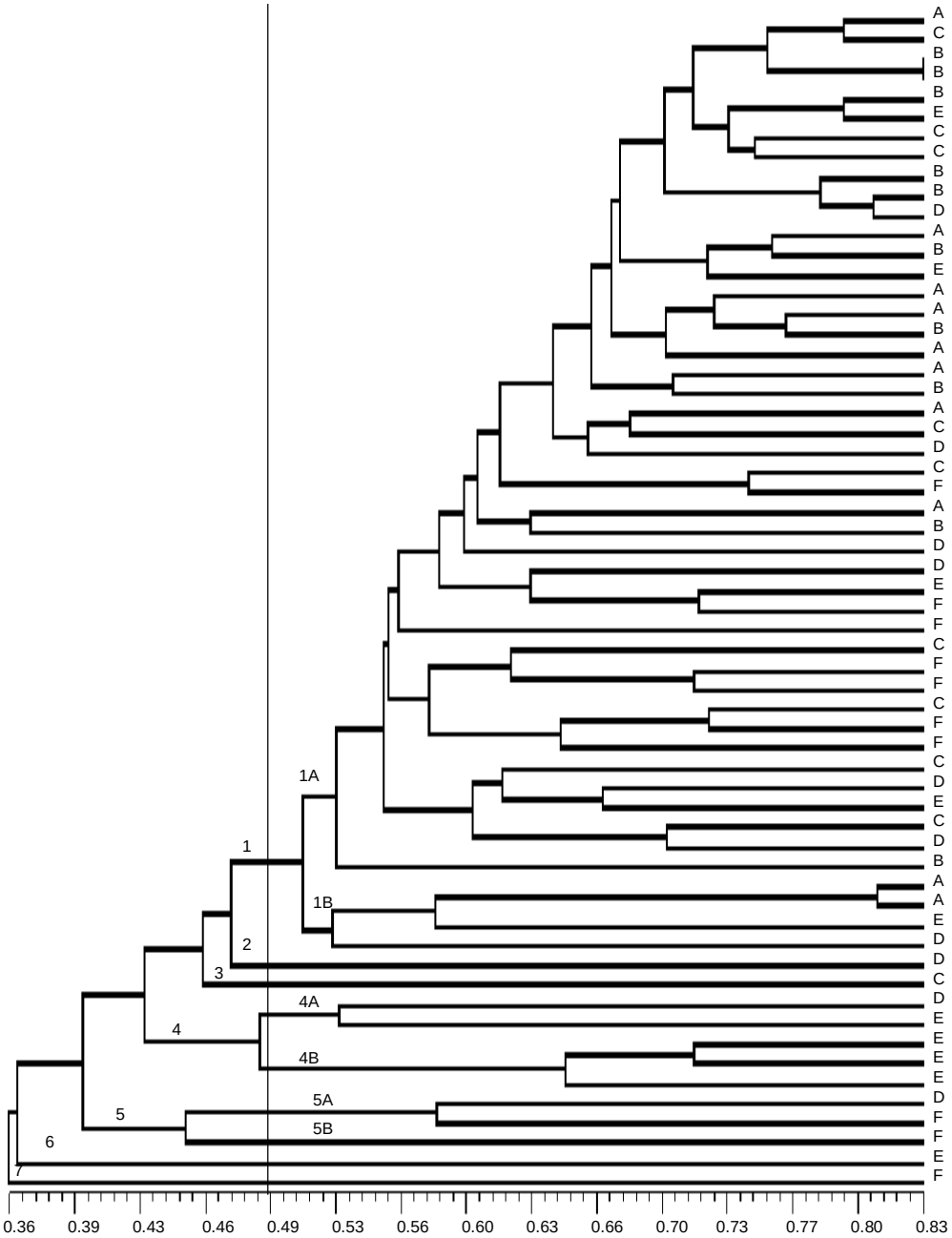

Figure 2 - Clustering using the UPGMA method based on the DICE coefficient (NEI LI, 1979) of annual ryegrass populations. Populations: “A”, LE-284 without colchicine; “B”, LE-284 with colchicine 0.2%; “C”, LE-284 with colchicine 0.4%; “D”, PG without colchicine; “E”, PG with colchicine 0.2%; “F”, PG with colchicine 0.4%. Vertical line indicates the avarege paierwise similarity among genotypes.

The use of colchicine has always been linked to chromosomal duplication, although evidence exists that this substance induces mutations in multiple points of the genome being observed in sorghum (FRANZKE & ROSS, 1957) and barley (GILBERT & PATTERSON, 1965).

Colchicine caused significant effects in the frequency and occurrence of DNA markers, which are consistent with variations found among treated vs. untreated ryegrass plants for agronomic traits. Such variations have been reported for leaf area, heading date and flower numbers (HAGUE & JONES, 1987), tillering ability and heading time (HASSAN et al., 1989), number of tillers and leaf weight (FRANCIS & JONES, 1989), size of mesophyll cells and chloroplast numbers (FRANCIS et al., 1990; HASSAN et al., 1991), and, for cytogenetic traits such as number of univalents per cell, bivalent variance and pattern of chiasmata distribution in bivalents (HASSAN & JONES, 1994; 1995).

The increase in differentiation among accessions due to higher doses of colchicine seems to be genotype dependent. Comparing the differences between accessions belonging to the same population, that were not treated with colchicine with those treated with the higher dose (0.4%), and those with the lower dose (0.2%), the percentage of differentiation between populations “A” x “C”, is much greater then “A” x “B” . For the other set of populations , however, colchicine effects do not follow the same pattern, “D” x “E” showing a slightly higher differentiation than “D” x “F” (Figure 3).

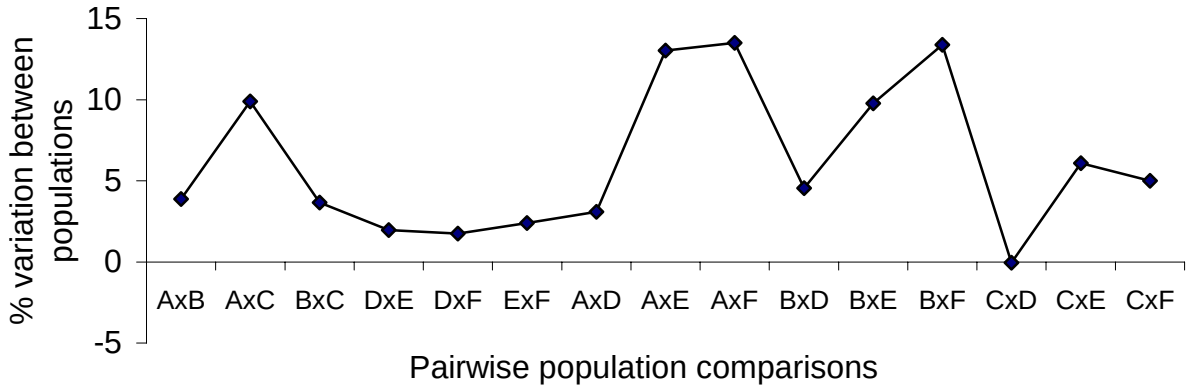

Figure 3 - Percentage differentiation between populations calculated by the analysis of molecular variance (EXCOFFIER et al., 1992). Populations: “A”, LE-284 without colchicine; “B”, LE-284 with colchicine 0.2%; “C”, LE-284 with colchicine 0.4%; “D”, PG without colchicine; “E”, PG with colchicine 0.2%; “F”, PG with colchicine 0.4%.

The real function of colchicine as a point mutagen is still unknown, although it has been suggested that an alteration in genome organization, e.g., amplification of ribosomal DNA, cistrons or other families of repetitive DNA has been observed (FRANCIS & JONES, 1989). Parallel effects on agronomic traits induced by colchicine in ryegrass and 5-azacytidine in other plant species have been found. The latter substance causes hypomethylation at specific sites in repetitive DNA (BEZDEK et al., 1991).

It was showed that colchicine has a powerful effect on changing allele frequencies. Nevertheless, new studies must be carried out to elucidate effects of colchicine on DNA sequences that make ryegrass populations exposed to colchicine different from their untreated counterparts.

RESUMO

A colchicina é muito utilizada para duplicar cromossomos em plantas. Alguns estudos têm mostrado que independentemente da ocorrência de duplicação cromossômica, genótipos de azevém submetidos ao tratamento com colchicina apresentam diferenças significativas em determinadas características de interesse agronômico, sugerindo que este alcalóide também tem um efeito mutagênico de ponto. Neste trabalho foi avaliada a diversidade genética com base em marcadores moleculares em duas populações de azevém submetidas a diferentes doses de colchicina. O tratamento com colchicina gerou diferenças entre os níveis de variabilidade intra e inter-populacional. Contrário às expectativas, populações submetidas a uma maior concentração de colchicina nem sempre apresentaram maior diversidade genética em relação às populações não tratadas. O uso de marcadores RAPD permitiu a comparação dos efeitos da colchicina em diferentes regiões do genoma, os quais estão plenamente de acordo com os registros verificados nos níveis morfológicos.

Palavras-chave: mutagênico; AMOVA; variabilidade;

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