CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Comparison between doubled haploid lines and lines obtained via the bulk method in tobacco

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Crop Breeding and Applied Biotechnology
22(4): e42992249, 2022
Brazilian Society of Plant Breeding.
Printed in Brazil
http://dx.doi.org/10.1590/1984-70332022v22n4a44

Abstract: *This study aimed to compare recombinant inbred lines (RIL) obtained via the bulk method with doubled haploid lines (DHL). For comparison, 190 DHL and 194 RIL were evaluated simultaneously at two different locations. Phenotypic and genetic parameters were estimated for the traits green leaf yield (GLY) and alkaloid content (ALK). The results of the RIL and DHL evaluations were similar for ALK. Conversely, estimates of genetic variation and heritability in the DHL were higher than those in the RIL for GLY. However, the mean estimate of the RIL was 13.3% higher than that of the DHL and thus, the annual gain with selection was higher for the RIL. The use of DHL in a breeding program program will be efficient in comparison to RIL if a large number of lines are obtained, which must undergo a preliminary selection before the most intensive evaluation to identify the lines to be recombined.*

Keywords: Nicotiana tabacum L.*, recombinant inbred lines, plant breeding*

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is a cash and industrial crop that is commonly grown worldwide. This plant is important for the agriculture sector, with high-quality varieties produced in different regions globally (Camlica and Yaldiz 2020).

The most time-consuming step in the genetic improvement of most cultivated species is obtaining homozygous individual lines. This is because, from an F_2 or S_0 generation, it is necessary to carry out successive self-fertilization until most of the loci become homozygous. One of the most researched alternatives to accelerate this process is the use of doubled haploids (DH), which generates haploid individuals with duplicated chromosomes to obtain DH lines (DHL) (Chaikam et al. 2019, Lenaerts et al. 2019, Atlin and Econopouly 2021, Marques et al. 2022). The use of DH has been widely researched and adopted in some crops such as wheat (Eliby et al. 2022), rice (Naik et al. 2017), corn (Mishra and Rao 2016, Maqbool et al. 2020), and tobacco (*Nicotiana tabacum* L.) (Hancock et al. 2015, Ma et al. 2020).

Although the procedures for haploid induction and chromosome duplication to obtain DH have been improved, their frequencies remain low (Hancock et al. 2015, Boerman et al. 2020, Trentin et al. 2020). Thus, it is questionable whether there is preferential segregation during the DH-obtaining process, that is, whether only specific genotypic combinations can generate DH. If this is true, it would be a restriction on the DH methodology use because the variability generated

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Received: 15 July 2022
Accepted: 01 October 2022
Published: 06 December 2022

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in the crossings would not be fully explored.

There are no reports in the literature on whether the constitution of DHL differs from that of lines obtained using conventional inbreeding methods. Melchinger et al. (2017) evaluated DHL with single-nucleotide polymorphism (SNP) markers on corn and did not detect any restriction in its genetic variability, whereas Zeitler et al. (2020) used the same genomic data to observe losses in DH variability. In some crops, such as triticale, barley, and corn, the performance of the lines obtained via DH and a conventional method, was not similar (Charmet and Branlard 1985, Bjornstad et al. 1992, Ma et al. 1999). To date, no studies have compared the use of DH and inbred tobacco lines in Brazil. This information is important because the use of DH in the breeding of this crop is strongly encouraged, and greater efficiency is expected.

Therefore, the purpose of this study was to verify if from the same tobacco gene pool, the recombinant inbred lines (RIL) obtained by the bulk method differ from those derived by the DH method. Furthermore, this study aimed to verify the feasibility of using DH in breeding programs under cultivation conditions.

MATERIAL AND METHODS

The data used in this study were provided by British American Tobacco (BAT), Brazil. This study was conducted in two steps. The first was to obtain the RIL and DHL via the bulk and DH methodologies, respectively, and then compare the lines under field conditions.

Three populations of tobacco from the Virginia group, named A, B, and C, were used in this study. These populations were obtained through several biparental crossings of the best lines available at the company. Two strategies were followed to obtain the lines from the plants of generation F_2: one was the bulk method, which entailed successive self-fertilization up to generation $F_{6:7}$, and the other was the generation of DH via anther culture.

To obtain the RIL, the seeds of F_1 plants were sown, and the descendants generated F_2. The F_2 plants were harvested individually, and a sample of an equal number of seeds from each plant was collected to start the next generation; this procedure was repeated until generation F_4. In this case, the method used to conduct the population was bulk, with the difference that each plant contributed a similar number of descendants to the next generation. This was performed to avoid natural selection.

From the F_4 generation, plants were harvested individually to generate progenies. These progenies were planted during the 2017/2018 crop season, and one plant from each progeny was used to start the next generation, $F_{5:6}$. The same procedure was performed in 2018/2019 to obtain seeds of progenies $F_{6:7}$. All individual plants were selected based on visual evaluation. As the average heterozygosity of the plants in this generation is only 1/64 of the loci, they are considered virtually homozygous lines. After F_1, six years were required to obtain the RIL.

Simultaneously with the development of the RIL, the DHL were obtained in the Tissue Culture Laboratory of BAT, located in Rio Negro, Paraná, Brazil.

The DHL was obtained by *in vitro* induction of F_2 plants using anther culture. The methodology adopted was that recommended for tobacco crops (Kasperbauer and Collins 1974). The steps were as follows:1) haploid induction; 2) identification of the haploid seedlings; 3) duplication of the chromosomes; 4) identification of the DH; and 5) multiplication of the DH seeds.

Haploid induction was performed by picking flowers from blooming plants of generation F_2 from each population separately. For this purpose, five flowers from each plant were selected at the initial stage of development and sent to the laboratory. The flowers were disinfected with 70% ethanol for 30 s and a 5% sodium hypochlorite solution for 10 min. The anthers were then extracted without the fillets and inoculated in Petri dishes in an androgenesis induction culture medium (A-medium) (Kasperbauer and Collins 1974). The dishes were incubated for 24 h in a Bio-Oxygen Demand incubator at 35 ± 1 °C and then placed in a growth chamber at 25 ± 1 °C with a photoperiod of 16 h. Approximately three weeks later, shoots containing at least two primary leaves were excised from the anthers and transplanted to an MS medium (Murashige and Skoog 1962) for root induction.

As the seedlings obtained in the culture medium may have been regenerated not only from the haploid cells of the pollen grain but also from the anther wall tissues, which are diploid, it was necessary to confirm the ploidy of the

obtained individuals. The haploid individuals were identified by quantifying seedling DNA using flow cytometry.

Upon confirmation of ploidy, the chromosomes of the haploid individuals were duplicated following the BAT protocol. Then, the ploidy verification stage was carried out again using a flow cytometer, and only individuals that were effectively duplicated were selected. The selected plants were acclimatized, and seedlings were produced in a greenhouse. Afterward, the seedlings were transplanted to the field to develop and provide DHL seeds.

The experiments for the evaluation of the DHL and RIL were carried out during the 2019/2020 crop season in two locations in Mafra, the northern region of Santa Catarina, Brazil. Location 1 was situated at lat 26º 09' 58.20" S, long 49º 48' 08.10" W, and alt 824 m asl, and Location 2 at lat 26° 10' 13.30" S, long 49° 56' 20.40" W, and alt 824 m asl.

Initially, the RIL and DHL seeds were sown in a polyethylene tray to obtain seedlings using a float system in the greenhouse. Approximately 60 d after sowing, the seedlings were transplanted into the experimental area.

Three contiguous experiments were conducted at each location, with one for each population. From populations A and B, 70 lines of each origin and four controls were evaluated. In this case, the adopted experimental design was a 12 × 12 triple lattice. In population C, 58 lines of each type and five controls were evaluated in an 11 × 11 triple lattice. The experimental plot consisted of a 10-meter line, spacing 0.50 m between plants and 1.20 m between lines. The crop management in the experiments was the same as that adopted by BAT. The topping of the plants, which was the removal of the inflorescence, was carried out on a plot basis depending on the productive potential of the plants. The harvest of the leaves started 15–30 days after topping and was carried out successively based on the point of physiological maturity.

The traits evaluated were green leaf yield (GLY, in kg plot^{-1}) and total alkaloid content (ALK). ALK was measured in the BAT laboratory using near-infrared reflectance spectroscopy from a sample of leaf dry mass and calculated as a percentage of the total sample (%). All data were obtained on a plot basis.

Statistical analyses were performed in the R environment (R Core Team 2022). Initially, the GLY and ALK data were analyzed considering each population separately, and then all lines were analyzed regardless of the population.

To obtain the variance components estimates of the RIL and DHL effects separately, we performed another analysis using a model similar to the following.

$$Y = X\beta + Z_1 I_{RIL} + Z_2 I_{DHL} + Z_3 b + Z_4 g + e,$$

where Y is the vector of the mean of phenotypic data; β is the vector of the fixed effects, mean, location, and repetition; I_{RIL} is the random RIL effect, in which $I_{RIL} \sim N(0, I\sigma^2_{RIL})$; I_{DHL} is the random DHL effect, in which $I_{DHL} \sim N(0, I\sigma^2_{DHL})$; b is the random block within the repetition effect, in which $b \sim N(0, I\sigma^2_b)$; g is the random interaction lines × environments effect, in which $g \sim N(0, I\sigma^2_g)$; X, Z_1, Z_2, Z_3, and Z_4 are the incidence matrices for β, I_{RIL}, I_{DHL}, b, and g, respectively; and e is the vector of the residual effects (random), in which $e \sim N(0, I\sigma^2_e)$.

Estimates of the variance components of the random effects were obtained using the restricted maximum likelihood method. The likelihood-ratio test (LRT) was used to test the significance of the estimated variance components.

From the variance, the heritability estimates (h^2) were obtained by considering the selection of the lines of each origin in each population and considering all lines regardless of the population. In all cases, the same estimator was used:

$$h^2 = \frac{\sigma^2_L}{(\sigma^2_L + \sigma^2_{LE}/_k + \sigma^2_E/_{rk})},$$

where σ^2_L is the genetic variance between the lines, σ^2_{LE} is the variance of the line × environment interaction, σ^2_E is the residual variance, k is the number of locations, and r is the number of repetitions. The variance components used in each case were those estimated in the specific model for each situation, that is, considering all lines or the DHL and RIL separately. Confidence intervals of the h^2 estimates were obtained in accordance with the method described by Knapp et al. (1985).

The percentage of the expected gain with the selection of the ten best lines of the general mean (GS) was obtained using the following estimator.

$$GS\ (\%) = \frac{\overline{BLUPs}}{\overline{Y}} * 100,$$

where $\overline{BLUPs}$ is the best linear unbiased prediction (BLUP) means of the selected lines and $\overline{Y}$ is the BLUP mean of all lines. All estimates were made considering the RIL and DHL of all lines regardless of the population. For all situations, the percentage of the expected annual genetic selection gain (GS_A) was also estimated, considering that the time required to obtain the RIL and HL was six and three years, respectively.

RESULTS AND DISCUSSION

The focus of this study was to verify whether the efficiency of obtaining good lines using the DH methodology is the same as that of the conventional breeding method. The main challenge was determining how to test the above hypothesis. One alternative would be to use genomics, as was done by Melchinger et al. (2017), who compared open-pollination varieties of corn with DHL derived from these varieties based on molecular analyses with SNP markers. The authors stated that obtaining the DHL did not result in systematic directional selection in specific genomic regions. However, using the same genomic data, Zeitler et al. (2020) obtained contradictory results; that is, there was a restriction in the variability when DH was used.

Another option would be to compare the lines obtained using the two methods in field experiments, which was adopted in the present study. For corn, there are some reports comparing the performance of maternal DHL with the conventional lines obtained via single seed descent (SSD) (Lashermes et al. 1988, Bordes et al. 2007). In these studies, the lines were evaluated based on their performance in topcrosses, and no difference was detected in the traits evaluated. However, when this method is adopted, the tester line can influence the performance of the assessed lines, thereby affecting the detection of differences. In other crops, such as wheat (Ma et al. 1999), barley (Bjornstad et al. 1992), and triticale (Charmet and Branlard 1985), the DH and SSD lines have also been compared for some agronomic traits and have presented different performances, as observed in this study.

Initially, the estimates of genetic/phenotype variances were considered for the comparison between the DHL and RIL. The variance between all the lines was significant for the two traits evaluated (Table 1). The same was found for the genetic variance between the RIL ($\sigma^2_{G_{RIL}}$) and DHL ($\sigma^2_{G_{DHL}}$). Specifically, $\sigma^2_{G_{DHL}}$ was higher than $\sigma^2_{G_{RIL}}$ in all cases. This difference was proportionally more expressive for the ALK trait, where $\sigma^2_{G_{DHL}}$ was 1.7 times greater than $\sigma^2_{G_{RIL}}$. The h^2 involving all lines can be considered medium to high for all traits, varying from 0.72 for ALK to 0.88 for GLY (Table 1). Furthermore, h^2 involving only the DHL was higher than that involving the RIL for all traits.

The genetic variation for the traits can also be seen by the frequency distributions of the BLUP means of the variables GLY and ALK involving all lines (Figure 1). For GLY, the range of variation in relation to the general mean was high (73%), whereas, for ALK, it was low (37%) (Figure 1). When the origin of the lines was considered, the variation range for GLY in relation to the mean for all lines was virtually equal, with 47% for RIL and 49% for DHL. Similar results were observed for ALK, with 25% for the RIL and 31% for the DHL.

Because the results were obtained with the estimation of variances and h^2, the DHL seemed more efficient at first because it displayed higher variability. For all estimates of genetic and phenotypic parameters, there is an associated error. Thus, in some situations, this inference may not be correct even if the estimates per point are different. Because the confidence intervals of the two estimates overlap, there is a possibility that they are equal.

Furthermore, there is some variation beyond the expected value, exclusively due to genetic recombination.

Table 1. Estimation of genetic and phenotypic parameters between lines considering all populations simultaneously, obtained in the joint analyses of the traits green leaf yield (GLY) (kg plot^{-1}) and total alkaloid content (ALK) (%). The data are from 194 recombinant inbred lines (RIL) and 190 doubled haploid lines (DHL) of tobacco from the Virginia group, evaluated in two locations

Parameter	GLY	ALK
$\sigma^2_{G_L}$ [1]	4.454**	0.025**
$\sigma^2_{G_{RIL}}$	2.357**	0.017**
$\sigma^2_{G_{DHL}}$	3.420**	0.029**
σ^2_{gl}	0.000	0.005**
σ^2_E	3.816	0.044
h^2_L	0.88 (0.85–0.89)[2]	0.78 (0.75–0.82)
h^2_{RIL}	0.79 (0.74–0.83)	0.70 (0.67–0.78)
h^2_{DHL}	0.84 (0.81–0.87)	0.80 (0.77–0.85)

[1] Genetic variance among all lines ($\sigma^2_{G_L}$), only conventional ($\sigma^2_{G_{RIL}}$) and DHL ($\sigma^2_{G_{DHL}}$), lines × environment interaction variance (σ^2_{gl}), residual variance (σ^2_E), heritability (h^2) for the selection of the mean involving all lines (h^2_L), only RIL (h^2_{RIL}), or DHL (h^2_{DHL}). [2] Values between parentheses correspond to the confidence intervals.** Significant ($p<0.05$) based on the LRT, respectively.

The induction of the DH by anther cultures may generate unexpected variability (Nichols and Rufty 1992). Methylation of pollen grains has been suggested as the reason for this variation (Devaux et al. 1993, Oakeley et al. 1997). This variability can produce both deleterious and favorable effects on selection (Witherspoon et al. 1991, Nichols and Rufty 1992).

Mean estimates were also used to compare the differences between lines of different origins. In this case, the mean of the RIL was 13% higher for GLY (18.86 kg plot^{-1}) than for the DHL (16.65 kg plot^{-1}) (Table 2). Although there was an overlap between the means of the DHL and RIL for this trait, the DHL means were concentrated at the lower end of the distribution, whereas the RIL means were concentrated at the upper end (Figure 1). When considering ALK, the opposite occurred, with the mean of the DHL (2.30%) being slightly higher than that of the RIL (2.26%). Considering the ten best lines, the mean GLY for the RIL was 12.5% higher than that for the DHL. For ALK, the mean of the ten best DHL was 3.2% higher than that for the best RIL.

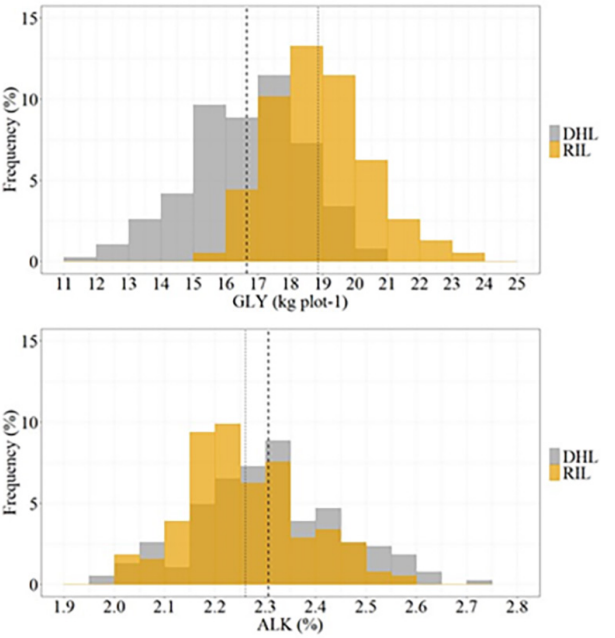

Figure 1. Distribution of the mean frequencies considering all recombinant inbred lines (RIL) and doubled haploid lines (DHL) for the traits green leaf yield (GLY) (kg plot^{-1}) and total alkaloid content (ALK) (%). DHL Mean (dashed line), RIL Mean (dotted line).

The reason why the RIL performed better than DHL in terms of means in relation to GLY could be questioned. A probable explanation is the sampling effect, which implies that the difference in favor of the RIL was due to chance. However, considering that 190 DHL and 194 RIL were evaluated, the probability of sample error was small. Another explanation is residual heterozygosity in the RIL. In generation $F_{6:7}$, the average homozygosity of the loci in the RIL was 98.44% and heterozygosity was 1.56%. Loci in heterozygosity contribute only to a higher estimate of the mean when there is dominance (Bernardo 2020). The dominance effect may occur in tobacco (Pscheidt et al. 2021, Carvalho et al. 2022); however, its contribution to phenotype expression is much lower due to additive allele interaction, which is common in autogamous plants (Bernardo 2020).

The difference between the performance of the lines from different origins may be related to the existence of preferential segregation while obtaining the DHL, that is, whether there would be a restriction on the occurrence of some genotypes because of the process. In corn, some quantitative trait loci which are situated in different chromosomes are involved in the genetic control of the haploid inductor (Prigge et al. 2012, Hu et al. 2016). Linkage and pleiotropy

Table 2. Estimation of the general mean and the mean of the ten best lines and controls considering all lines (All), only RIL or DHL, and the total (GS*) (%) or annual (GS_A) (%) gain with the selection of the ten best lines for the traits green leaf yield (GLY) (kg plot^{-1}) and total alkaloid content (ALK) (%). The data were obtained in the evaluation of 194 RIL and 190 DHL in two locations

Origin	GLY			ALK		
	Mean	GS*	GS_A**	Mean	GS*	GS_A**
All	17.76	25.64	-	2.28	14.10	-
RIL	18.86	25.64	4.27	2.26	10.24	1.71
DHL	16.65	11.67	3.89	2.31	13.97	4.66
Controls	15.79	-	-	2.46	-	-
MS_{RIL} [1]		22.30			2.52	
MS_{DHL}		19.82			2.60	

[1] Mean of the ten best lines selected within RIL (MS_{RIL}) and DHL (MS_{DHL}). * GS: Gain selection percentage in relation to the overall mean considering all lines. ** GS_A: Gain selection per year considering RIL obtaining cycle: six years; DHL: three years.

may occur between these genes and consequently, restrict the occurrence of some genotype combinations. Although there was no inducer when the DH was obtained via anther culture, genes involved in the induction of haploids when using this method have also been detected. Thus, some differences in the performance of the lines are due to linkage or pleiotropy between the genes involved in the ability of the gametic cell to origin the haploid and in its duplication, and other genes, especially those controlling the traits of the highest agronomic or industrial interest.

Another way to assess the efficiency of the DHL and RIL, which is of most practical interest and involves variances and means, is to estimate the gain with selection. The estimated total gain (GS) or GS_A with the selection of the ten best RIL or DHL is presented in Table 2. For GLY, the GS estimate considering all lines was equal to the gain with the selection considering only the RIL, indicating that the ten lines with the best performance were obtained using the conventional breeding method. The GS for GLY was 14% higher for the RIL than for the DHL. The same pattern for this trait was also observed for GS_A. For ALK, the estimated gain obtained was higher with the selection of the DHL, with its GS_A being 2.7 times higher than that of the RIL.

The advantage of using DH is the acceleration of the improvement process (Atlin et al. 2017, Chaikam et al. 2019), as it makes it possible to obtain lines in a shorter period. For tobacco, where it is only possible to have one harvest per year, it takes six years to generate the evaluated conventional lines since obtaining F_1. However, it takes only three years to develop and evaluate the DH. Thus, the DHL was obtained twice as fast as the RIL. Therefore, the GS_A was expected to be higher in the DHL, as it presented higher estimates of genetic variance and h^2; however, this did not occur. The GS_A for GLY with the selection of the RIL was 9.76% higher than that obtained for the DHL (Table 2).

Despite the better performance of the RIL compared to the DHL for GLY in this study, the use of DH in tobacco should not be discouraged. This is because of other advantages of the DH method, such as a) the reduced time spent in obtaining the lines, b) the assurance that the obtained lines have 100% of the loci in homozygosity, and c) the fact that the DH easily meets the distinguishing criteria required for the registration of new cultivars and, consequently, the registration and protection process is accelerated (Chaikam et al. 2019).

Finally, the DH methodology enables the desired number of lines to be obtained in a short cycle, with no need to deal with the conduction of progeny of different generations. This way, some activities in the breeding program are simplified with the use of DH, especially because the operations are only carried out once (Chaikam et al. 2019). All these factors contribute to the reduction in expenses for long-term improvement programs. Some studies comparing different methods to obtain lines have shown that reducing the cycle time is the most efficient alternative for increasing genetic gain associated with a more cost-effective strategy (Atlin and Econopouly 2021, Marques et al. 2022).

The question that remains is under what conditions should DH be routinely used in breeding programs. a) The first condition is to conduct experiments with a higher number of DH. However, to determine what this number could be, the number of DHL necessary to obtain one with equal performance to the best RIL must be estimated. The properties of a normal distribution can be used to obtain this number (Steel et al. 1997). For example, we consider the data obtained in this study. The estimate of the genetic variance between the DHL ($\sigma^2_{G_{DHL}}$) was 3.42 for GLY (Table 1); the genetic standard deviation ($\sigma_{G_{DHL}}$) was 1.85 and the mean was 16.65 kg plot^{-1} (Table 2). Assuming that it is desirable to obtain at least one DHL with the highest mean obtained by one of the RIL, i.e., 23.71 kg plot^{-1}, it is possible to estimate how many standard deviations above the average of the DHL population would be necessary to achieve the best performance of the RIL. Using the expression $z = \frac{(Y_i - \overline{Y})}{\sigma}$, where Y_i is the mean of the best RIL, 23.71 kg plot^{-1}, $\overline{Y}$ is the mean of the DHL, 16.65 kg plot $^{-1}$, and σ is the standard deviation of the DHL population, 1.85, the obtained value was $z = 3.81\ \sigma_{G_{DHL}}$, which was above average. Therefore, the range of variation of the distribution was 7.62 (3.81×2). Based on this, the prediction of the number of lines to be evaluated should be approximately 10000 to obtain a GLY similar to the best RIL; this number is unrealistic in most situations. b) The second condition is to promote the elimination of lines with low vegetative development or other problems by using genomics before the field evaluations. Considering that the number of DHL involved in the process should be high, the cost of genotyping is prohibitive. In addition, it is necessary to prove the efficiency of genomic selection. A preliminary evaluation of the DHL can also be performed under field conditions. First, there would be no need to use experiments with repetition; for example, using p-rep could be an alternative (Moehring et al. 2014). The DHL selected would be more intensively evaluated in the next season in repetition experiments in locations with the greatest possible precision. The problem with this strategy is that it requires

a different crop season. However, the time spent can be compensated for via an increase in the breeding program gain.

CONCLUSIONS

The performance of the DHL compared to that of the RIL differed between traits. For ALK, which is related to chemical quality, the performance was similar. For GLY, the genetic variation estimates and h^2 among the DHL were higher than those of the RIL. However, the mean estimate of the RIL was 13.3% higher than that of the DHL. Thus, the annual gain with selection was greater for the RIL.

The use of DHL in a breeding program will be more efficient than RIL if a large number of lines are obtained, which must undergo preliminary selection before the most intensive evaluation to identify the lines that should be recombined.

ACKNOWLEDGMENTS

The authors would like to thank British American Tobacco (BAT) for providing the data and for the partnership in this work, as well as the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarships granted.

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