ROXANE DO CARMO LEMOS

PERFORMANCE OF DOUBLED HAPLOID LINES IN RELATION TO THOSE OBTAINED BY THE CONVENTIONAL BREEDING METHOD IN TOBACCO

LAVRAS-MG

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Tese apresentada a Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Genética e Melhoramento Plantas, área de concentração Genética e Melhoramento de Plantas, para a obtenção do título de Doutor.

Prof. Dr. Magno Antonio Patto Ramalho
Orientador

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ROXANE DO CARMO LEMOS

PERFORMANCE OF DOUBLED HAPLOID LINES IN RELATION TO THOSE OBTAINED BY THE CONVENTIONAL BREEDING METHOD IN TOBACCO

DESEMPENHO DE LINHAGENS DUPLO-HAPLOIDES EM RELAÇÃO ÀS OBTIDAS PELO MÉTODO CONVENCIONAL DE MELHORAMENTO NO TABACO

Tese apresentada a Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Genética e Melhoramento Plantas, área de concentração Genética e Melhoramento de Plantas, para a obtenção do título de Doutor.

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Aos meus pais, Dinélia e Fernando,

Dedico

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RESUMO

Em algumas espécies, a alternativa mais utilizada para acelerar o processo de obtenção de linhagens é a utilização de duplo haploides (DH). A dúvida existente é se o processo de indução e duplicação dos cromossomos ocorre de forma aleatória ou se há restrição na segregação. Caso isso ocorra, seria uma limitação ao seu uso, uma vez que a variabilidade gerada nos cruzamentos não seria totalmente explorada. Este trabalho foi realizado com o objetivo de comparar o desempenho de linhagens de tabaco (*Nicotiana tabacum* L.) obtidas pelo método convencional de melhoramento (CM), com as obtidas pela metodologia DH por meio de cultura de anteras. Além disso, verificar a viabilidade do emprego rotineiro de DH em um programa de seleção recorrente (RS). Para isso, três populações de tabaco do grupo varietal Virginia foram obtidas por cruzamento biparental. Considerando todas as populações, o número de linhagens avaliadas foi de 190 DH e 194 CM. Elas foram avaliadas simultaneamente, em um experimento por população em dois locais. As características avaliadas foram produtividade de massa verde (YLD), teor de alcaloides totais (ALK) e teor de açúcar (SU). Os parâmetros genéticos e fenotípicos foram estimados por população e considerando todas as linhagens independente do cruzamento. Constatou-se que o desempenho do DH em comparação ao CM diferiu entre as características. Para aqueles relacionados à qualidade, ALK e SU, o desempenho foi semelhante. Para YLD, as estimativas de variação genética e h^2 entre os DH foram maiores que as de CM. No entanto, o oposto ocorreu para a média. Considerando todas as linhagens, independente do cruzamento, a média do CM foi 13,3% maior do que a média do DH, para YLD. Além disso, o ganho anual com a seleção foi maior para CM para esta característica. O uso de DH em um programa de SR será eficiente em comparação ao MC se for obtido um grande número de DH que deve passar por uma seleção preliminar antes da avaliação mais intensiva para identificar as linhagens a serem recombinadas.

Palavras-chave: *Nicotiana tabacum* L., melhoramento de plantas, seleção recorrente.

ABSTRACT

In some species, the most used alternative to accelerate the process of obtaining lines is by using doubled haploids (DH). The existing question is whether the process of induction and duplication of chromosomes occurs randomly or there is a restriction in the segregation. If this fact occurs, it would be a restriction on its use since the variability generated in the crossings would not be fully explored. This work was carried out with the purpose to compare the performance of tobacco lines (*Nicotiana tabacum L.*) obtained by conventional breeding method (CM), with the obtained by the DH methodology, using anther culture. Additionally, to verify the feasibility of routine employment of DH in a recurrent selection program (RS). For this purpose, three populations of tobacco from the Virginia varietal group were obtained by biparental crossing. Considering all populations, the number of lines evaluated was 190 DH and 194 CM. They were evaluated simultaneously, in one experiment per population at two locations. The traits assessed were green leaf yield (YLD), total alkaloid content (ALK), and sugar content (SU). The genetic and the phenotypic parameters were estimated by population. It was found that: the performance of the DH in comparison to CM differed between the traits. For those related to quality, ALK and SU, the performance was similar. For YLD, the genetic variation estimates and h^2, among the DH was higher than those of CM. However, the opposite occurred for the mean parameter. Considering all lines, regardless of the crossing, the CM mean was 13.3% higher than the DH mean, for YLD. Also, the annual gain with the selection was greater for CM to this trait. The use of DH in a SR program will be efficient in comparison to CM if a large number of DH are obtained which must undergo a preliminary selection before the most intensive evaluation to identify the lines to be recombined.

Key words: *Nicotiana tabacum* L., plant breeding, recurrent selection.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS

∑Z: Sum of standardized variables index

ALK: Total alkaloid content

BLUP: Best Linear Unbiased Estimator

CM: Conventional breeding method

DH: Doubled haploid

GS: Genetic gain in percentage of the general mean

GSA: Annual genetic gain in percentage of the general mean

h2: heritability

L1: Location 1

L2: Location 2

MS: Murashige- Skoog medium

REML: Restricted Maximum Likelihood

r_{gg}: Selective accuracy

RIL: Recombinant Inbred Lines

RS: Recurrent Selection

SSD: Single Seed Descent Method

SU: Total Sugar content

YLD: Green leaf yield

SUMÁRIO

CAPÍTULO 1

1 INTRODUÇÃO GERAL

No melhoramento genético da maioria das espécies cultivadas, a etapa mais demorada é a obtenção de indivíduos homozigotos, ou seja, linhagens. Isso porque a partir de uma geração F_2 ou S_0, é necessário realizar autofecundações sucessivas até que a maioria dos locos estejam em homozigose. Algumas estratégias para acelerar esse processo têm sido buscadas ao longo dos últimos anos, dentre elas o emprego de duplo-haploides (DH) tem sido amplamente pesquisado. De maneira simplificada, essa estratégia consiste na geração de indivíduos haploides, que após terem seus cromossomos duplicados irão originar as linhagens DH (CHAIKAM *et al.*, 2019; LENAERTS; COLLARD; DEMONT, 2019; YAN *et al.*, 2017).

Assim, a indução de haploides é o primeiro passo para a obtenção do DH. Para tanto, algumas alternativas são factíveis, como a produção espontânea de haploides, a indução *in vitro* e o uso de plantas indutoras de haploidia. No caso específico do tabaco (*Nicotiana tabacum* L.), onde há facilidade da cultura de tecidos, a geração de plantas haploides a partir da cultura de anteras já é possível há algum tempo (BELOGRADOVA *et al.*, 2009; BOURGIN; NITSCH, 1967; NITSCH; NITSCH, 1969). Nessa espécie, também se verificou a possibilidade de obtenção de haploides *in vivo* por meio do cruzamento da *N. tabacum* com a espécie selvagem *Nicotiana africana*, ou outra espécie da seção *Suaveolentes* (BURK *et al.*, 1979; HANCOCK *et al.*, 2015). Neste cruzamento, alguns descendentes são obtidos a partir de células gaméticas não fertilizada da *N. tabacum*, ou seja, indivíduos haploides.

Uma vez obtido o haploide, a segunda etapa é a duplicação do número de cromossomos para obter o DH. Para tanto, várias metodologias são possíveis (HÄNTZSCHEL; WEBER, 2010; KLÍMA, VYVADILOVÁ; KUCERA, 2008; KITAMURA; AKUTSU; OKAZAKI, 2009; WU *et al.*, 2017).

Embora os procedimentos de indução haploide e duplicação cromossômica tenham sido aprimorados, sua frequência ainda é baixa. Nesta condição, questiona-se se há uma segregação preferencial durante o processo de obtenção de DH, ou seja, se apenas determinadas combinações genotípicas podem gerar o DH. Caso isso ocorra, seria uma restrição ao seu uso, uma vez que a variabilidade gerada nos cruzamentos não seria totalmente explorada. No milho, há evidências de que no processo de indução de haploides e duplicação dos cromossomos

podem existir genes envolvidos (BECKERT 1994; CHAIKAM *et al.*, 2019; KALINOWSKA *et al.*, 2019).

Na literatura, as informações da existência ou não de alteração na segregação das linhagens DH em relação às linhagens obtidas pelo método convencional são contraditórias. Existem relatos na cultura do milho, utilizando marcadores SNP, em que o DH não apresentou restrição na variabilidade (MELCHINGER *et al.* 2017). Porém, utilizando os mesmos dados genômicos, Zeitler, Ross-Ibarra, Stetter (2020) obtiveram resultados contrários, ou seja, houve restrição na variabilidade quando o DH foi utilizado. Em outros estudos, também com milho, observou-se desempenho semelhante entre o DH e os obtidos pelo método de melhoramento convencional (MC) (BORDES *et al.*, 2006, 2007; LASHERMES; GAILLARD; BECKERT, 1988). No entanto, em algumas espécies autógamas, o desempenho dos dois tipos de DH e CM não foi semelhante (BJORNSTAD; SKINNES; THORESEN, 1993; CHARMET; BRANLARD, 1985; MA *et al.*, 1999). Infelizmente, especificamente para a cultura do tabaco no Brasil, não foram encontrados relatos comparando o uso de DH e MC. Esta informação é fundamental, pois o uso da seleção recorrente (SR) no melhoramento desta cultura tem sido incentivado e, obviamente, espera-se maior eficiência da SR com o uso da metodologia DH.

Diante do exposto, o objetivo deste trabalho foi verificar se a partir de um mesmo "pool gênico" de tabaco, as linhagens obtidas pelo método de melhoramento convencional diferem das obtidas pela metodologia DH, por meio da cultura de anteras. Além disso, verificar a viabilidade do emprego rotineiro de DH em um programa de SR.

2 REFERENCIAL TEÓRICO

2.1 Tecnologia de Duplo Haploides (DH)

O principal objetivo da maioria dos programas de melhoramento, seja de plantas autógamas ou alógamas, é a obtenção de linhagens, para serem utilizadas como cultivares ou na produção de híbridos. Normalmente esta etapa consiste em um processo demorado e de alto custo, pois para atingir um elevado nível de homozigose são necessárias pelo menos seis gerações de autofecundações (BERNARDO, 2020; RAMALHO *et al.*, 2012). Considerando uma espécie em que é possível apenas uma safra ao ano, como é o caso do tabaco, seriam necessários de seis a oito anos após a F_1 para a obtenção das linhagens. Assim, a busca por alternativas para reduzir o tempo de obtenção de linhagens é um anseio antigo dos melhoristas (LENAERTS; COLLARD; DEMONT, 2019). Para atingir esse objetivo várias estratégias podem ser utilizadas, como o emprego do "speed breeding" (HICKEY *et al.*, 2019; WATSON *et al.*, 2018), a condução de várias gerações em um mesmo ano (COLLARD *et al.*, 2017; LIU *et al.*, 2016; SAXENA *et al.*, 2017), a condução da população segregante pelo método SSD e o emprego de linhagens duplo-haploides (DH) (CHAIKAM *et al.*, 2019; MAQBOOL; NESHIR; KHOKHAR, 2020). O foco desse trabalho será voltado para o uso do DH, que é a estratégia que vem sendo mais utilizada nos últimos anos.

De maneira simplificada, a tecnologia de DH consiste no desenvolvimento de linhagens com 100% dos locos em homozigose, por meio da duplicação cromossômica de indivíduos haploides. A ocorrência de uma planta haploide induzida de forma espontânea foi observada pela primeira vez em 1920, na cultura do algodão (HARLAND, 1936; MAQBOOL; NESHIR; KHOKHAR, 2020). Alguns anos depois foram encontrados haploides em outras culturas de importância econômica, como o tabaco (CLAUSEN; MANN, 1924), o trigo (GAINES; AASE, 1926) e o milho (CHASE; 1949). No entanto, a indução natural de haploides é um fenômeno raro e o seu uso no melhoramento somente seria possível com a elaboração de protocolos para a produção de haploides em laboratório em larga escala. Na década de 1960, houve o desenvolvimento da tecnologia da cultura de anteras inicialmente para a indução de haploides em *Datura stramonium* (GUHA; MAHESHWARI, 1964, 1966). Contudo, foi somente nos anos 90 que surgiram protocolos e alternativas mais eficientes para a indução dos haploides e a sua duplicação cromossômica, viabilizando assim o uso do DH no melhoramento de outras culturas. Atualmente, é conhecida a possibilidade de obtenção de DH para cerca de duzentas espécies

vegetais, sendo a tecnologia amplamente empregada em brássicas e cereais, incluindo trigo, cevada, arroz e milho (REN *et al.*, 2017; YAN *et al.*, 2017).

Como já salientado, uma das principais vantagens do emprego do DH é a redução expressiva no tempo gasto na obtenção das linhagens em relação ao método convencional de melhoramento, ou seja, por meio de sucessivas gerações de autofecundação. Além disso, o melhorista tem a segurança de que as linhagens DH são completamente homozigotas, ou seja, não possuem heterozigosidade residual. Essa homogeneidade do DH permite também maior agilidade no registro/proteção da cultivar, pois a presença de locos em heterozigose, como ocorre nas linhagens convencionais, pode atrasar o atendimento aos critérios de homogeneidade e estabilidade exigidos para o registro de cultivares (CHAIKAM *et al.*, 2019). Dessa forma, o DH pode reduzir mais ainda o tempo necessário até a comercialização das cultivares desenvolvidas. Atlin, Cairns, Biswanath (2017) comentaram ocorrer uma diminuição de no mínimo dois anos no ciclo completo de melhoramento, em comparação aos métodos SSD e Bulk, quando foi utilizado o DH na cultura do milho.

No método convencional de melhoramento inicialmente são geradas muitas progênies que serão conduzidas em sucessivas gerações de endogamia, avaliadas e selecionadas até a obtenção do número desejável de linhagens. Quando a tecnologia DH é utilizada as linhagens finalizadas podem ser obtidas de uma vez, simplificando assim a logística relacionada ao manejo e condução das progênies nas diferentes gerações (CHAIKAM *et al.*, 2019). Dessa forma, algumas atividades, como registro dos cruzamentos, obtenção das mudas nos viveiros, condução de experimentos em campo, autopolinização e manutenção das linhagens, tornam-se mais simples com o uso do DH. Assim, a longo prazo além da redução do tempo de obtenção das linhagens, o uso da metodologia DH pode levar a uma redução significativa dos custos relacionados ao manejo e condução das progênies nas sucessivas gerações.

Além das vantagens mencionadas acima, em teoria o uso do DH pode levar ao maior ganho genético com a seleção. Devido a completa homozigosidade das linhagens DH, quando são avaliadas em campo, em vários locais e anos, espera-se que a precisão obtida nos experimentos seja maior em comparação a avaliação de progênies nas gerações iniciais pelo método convencional (YAN *et al.*, 2017). Vale ressaltar também que a maior variância entre as linhagens DH pode levar a melhor resposta a seleção, quando estiver associada a médias altas para os caracteres de interesse. Ademais, o ganho genético com a seleção por ano é esperado que seja maior com o uso do DH, devido a redução da variável tempo na equação do ganho genético (CHAIKAM *et al.*, 2019; COBB *et al.*, 2019).

Do exposto, nota-se que são inúmeras as vantagens do emprego do DH para acelerar a obtenção das linhagens. Vale ressaltar também que além desse objetivo, nos últimos anos buscou-se ampliar o uso do DH para aumentar a eficiência de alguns processos do melhoramento, tais como: a obtenção de linhagens parentais de híbridos comerciais, a introdução de macho esterilidade em linhagens mais rapidamente, o estaqueamento de genes e em estudos genômicos (REN *et al.*, 2017). Quando da obtenção de linhagens em híbridos comerciais, se for obtido DH a partir da geração F_1 do híbrido, a frequência de permuta é ainda pequena. Isso porque, no processo tradicional com sucessivas autofecundações até a homozigose, ocorre a recombinação entre vários genes ligados. Depreende-se que o uso do DH na geração F_1 aumenta a chance de obter linhagens mais próximas daquelas que originaram o referido híbrido. Após a obtenção das linhagens DH realiza-se o cruzamento entre elas para tentar regenerar o híbrido e, com isso, obter a combinação dos parentais (DIRKS *et al.*, 2009). No entanto, essa estratégia ainda não tem sido amplamente adotada.

A tecnologia DH pode auxiliar também a produção de linhagens com macho-esterilidade citoplasmática (ME) em menor período de tempo, o que seria muito vantajoso em programas de melhoramento que utilizam essa estratégia. Esse procedimento normalmente é realizado por retrocruzamentos sucessivos, o que além de demandar muito tempo não permite a completa recuperação do genótipo macho-fértil (MF) com o citoplasma ME. Em milho essa possibilidade foi vislumbrada pela primeira vez por Goodsel (1961). Nessa cultura, a indução de haploides paternos é controlada pelo gene *ig1*, onde genótipos *ig1ig1* mutantes possuem uma série de anormalidades nos gametas incluindo células do óvulo sem um núcleo gamético (ANDORF *et al.*, 2019; KERMICLE, 1969). Assim, após a fertilização desses óvulos por um gameta masculino normal, poderá ser gerado um embrião haploide com o citoplasma materno e apenas os cromossomos paternos. Dessa forma, linhagens *ig1ig1* com citoplasma ME podem ser utilizadas na conversão de linhagens MF em ME em apenas uma geração, resultando em uma redução substancial no tempo e recursos dispendidos na obtenção de linhagens ME (ANDORF *et al.*, 2019).

Outra aplicação viável do DH é no processo de introgressão de genes. Tradicionalmente, a introgressão de um ou mais genes de interesse em linhagens elites é realizada por meio de retrocruzamentos e ao final dessa etapa uma planta heterozigota é autofecundada para gerar uma linhagem com o(s) alelo(s) de interesse fixados. O uso do DH nessa etapa reduz o número de genótipos a serem avaliados para a seleção de um indivíduo homozigoto para o gene inserido. Isso porque, se considerarmos apenas um gene, a probabilidade de ocorrência do genótipo

desejado por meio da autofecundação do heterozitoto será de ¼, enquanto a obtenção do haploide com o alelo desejado será maior, de ½ (REN *et al.*, 2017).

2.2 Metodologias de obtenção de DH

Como já comentado as etapas de obtenção do DH são: a indução de indivíduos haploides, identificação dos haploides, duplicação cromossômica dos selecionados e multiplicação das sementes DH. A etapa de indução de haploidia pode ser realizada de duas formas, *in vitro* ou *in vivo.* A seguir serão discutidas mais detalhadamente essas metodologias.

2.2.1 Indução de haploidia *in vitro*

O sistema de indução *in vitro* nada mais é do que a cultura de gametas, masculinos ou femininos, utilizando técnicas da cultura de tecidos. Nesse método, a indução pode ser realizada pelo gameta feminino por partenogênese induzida (haploides ginogenéticos), via cultura de óvulos/ovários, ou pelo gameta masculino (haploides androgenéticos), por meio de cultura de anteras ou cultura de micrósporos isolados (KALINOWSKA *et al.*, 2018; NIAZIAN; SHARIATPANAHI, 2020).

A metodologia *in vitro* mais utilizada para a obtenção de haploides é via androgênese. O procedimento para a realização da cultura de anteras é simples e pode ser aplicado com sucesso em muitas espécies (NIAZIAN; SHARIATPANAHI, 2020; YAN *et al.*, 2017). No entanto, algumas culturas de importância econômica como a cevada, milho, batata e o centeio ainda são recalcitrantes para esse procedimento (MALUSZYNSKI *et al.*, 2003) e, nesse caso, outras metodologias de indução devem ser utilizadas.

A cultura de anteras baseia-se na inoculação de anteras em meio de cultura apropriado para favorecer o desenvolvimento de calos ou embrioides (embriogênese somática direta) (PIERRE *et al.*, 2011). A partir dos calos obtidos pode-se regenerar plântulas haploides, que irão originar as linhagens DH após a duplicação dos seus cromossomos. As plântulas haploides nesse caso são resultantes de um redirecionamento dos micrósporos da via gametofítica normal para o desenvolvimento esporofítico através da embriogênese gamética (NIAZIAN; SHARIATPANAHI, 2020). Como já salientado, diversos protocolos dessa metodologia já foram estabelecidos para várias culturas. No tabaco, a obtenção de DH por meio da cultura de

anteras é viável há muitos anos e os protocolos para o seu emprego já estão bem definidos (BELOGRADOVA *et al.*, 2009; BOURGIN; NITSCH, 1967; NITSCH; NITSCH, 1969).

Existem vários fatores que podem influenciar a eficiência da indução de haploides pelo método *in vitro*, tais como: o genótipo das plantas doadoras, as condições em que são cultivadas, o estágio de desenvolvimento dos gametas imaturos os meios de cultura utilizados, as concentrações dos reguladores de crescimento aplicados e o tipo e intensidade dos tratamentos físicos de estresses, quando utilizados (NIAZAN; SHARIATPANAHI, 2020). Dentre esses, o estágio ideal de desenvolvimento dos gametas está muito relacionado ao sucesso da obtenção de haploides por essa estratégia e, assim, o melhorista deve considerar o momento ideal para realizar a coleta dos botões florais para a extração das anteras. Para isso, é levado em consideração o tamanho do botão floral que provavelmente possibilita anteras com micrósporos no estágio apropriado de desenvolvimento. Contudo, isso pode variar de acordo com a espécie (GU *et al.* 2014). Em tabaco, foi observado que grãos de pólen maduros não regeneram plântulas *in vitro*, mas apenas formam os tubos polínicos (NITSCH; NITSCH, 1969). Segundo Nitsch e Nitsch (1969) o estágio ideal para a regeneração do haploide seria quando o grão de pólen estiver completamente individualizado, com apenas um núcleo e desprovido de amido, o que acontece no que foi denominado pelos autores de estágio 2 de desenvolvimento. Wang *et al.* (2018) observaram resultados similares em cultura de anteras na cultura do kiwi.

Algumas vantagens da indução *in vitro* são: existência de protocolos bem estabelecidos para diversas culturas, não ter a necessidade de condução dos cruzamentos com o indutor, o que demandaria mais uma safra quando a cultura não permite o plantio fora de época ou em casa de vegetação e as maiores taxas de indução obtidas por esse método. Como desvantagens pode-se citar a dependência do genótipo ou espécie, sendo algumas recalcitrantes para o método, a ausência de duplicação cromossômica espontânea, a necessidade de uma estrutura laboratorial de cultura de tecidos e a possibilidade do surgimento de variação gametoclonal deletéria (YUNG; WERNSMAN, 1990).

No tabaco, existem relatos indicando que a obtenção de DH por meio de cultura de anteras pode introduzir variações genéticas além das esperadas via mutação (BURK; MATZINGER, 1976; WERNSMAN, 1992). Burk e Matzinger (1976) observaram variação significativa entre os DH derivados da linhagem de tabaco Coker 139 do grupo varietal Burley. Após a realização de dois ciclos de cultura de anteras e a avaliação dos caracteres agronômicos das linhagens DH e da Coker 139, observaram que a média da produtividade de massa verde do DH foi 50% inferior à linhagem original. Outros estudos com tabaco também obtiveram resultados semelhantes (ARCIA; WERNSMAN; BURK, 1978; DEATON *et al.*, 1986;

SCHNELL; WERNSMAN, 1986). Contudo, outros autores relatam também o surgimento de variação genética favorável quando a cultura de anteras foi utilizada na cultura do tabaco. Whiterspoon *et al.* 1991 observaram variabilidade para a tolerância/resistência ao patógeno PVY entre linhagens DH oriundas de uma linhagem comercial suscetível ao vírus.

2.2.2 Indução de haploidia *in vivo*

A indução de haploides *in vivo* pode ser realizada por meio da polinização com linhagens indutoras, como é amplamente utilizado sobretudo na cultura do milho (EDER; CHALYK, 2002; GEIGER; GORDILLO, 2009; ROBER; GORDILLO; GEIGER, 2005). A partir desse cruzamento podem-se formar haploides ginogenéticos, quando a linhagem indutora é a doadora do grão de pólen, ou são gerados haploides androgenéticos, nesse caso a linhagem indutora é utilizada como genitor feminino. A identificação e utilização da linhagem indutora Stock 6, na década de 1950, revolucionou a obtenção de DH e, consequentemente, a produção de híbridos na cultura do milho. Inicialmente, a taxa de indução de haploides usando essa metodologia era baixa, apenas 2,3%, contudo, nos últimos anos o incremento da indução utilizando os indutores modernos foi crescente, podendo chegar a valores próximos a 15% (ANDORF *et al.*, 2019).

Outra forma de se obter indivíduos haploides *in vivo* é o cruzamento interespecífico entre espécies relacionadas, metodologia que tem sido utilizada com sucesso em algumas culturas como a cevada (KASHA; KAO, 1970, o trigo (LAURIE; BENNET, 1986; INAGAKI, 2003) e a batata (DE MAINE, 2003). Os mecanismos envolvidos nessa forma de indução ainda não foram totalmente elucidados, no entanto, sabe-se que na maioria dos casos ocorre a eliminação dos cromossomos paternos durante a divisão celular do zigoto (FORSTER *et al.*, 2007). Assim, são gerados haploides que, após a duplicação cromossômica, irão originar linhagens DH com constituição genética apenas do lado materno. Durante esse processo a formação do endosperma pode ser também bloqueada devido a incompatibilidade dos genomas dos genitores. Nesse caso específico, para assegurar a sua viabilidade, o desenvolvimento do embrião deve ser feito em meio de cultura *in vitro*. As desvantagens desse método são a necessidade de se realizar o escalonamento do plantio, para a coincidência do florescimento das duas espécies, e a necessidade da aplicação de agentes químicos, como a colchicina, para a duplicação cromossômica.

Um exemplo de sucesso na obtenção de DH pelo cruzamento interespecífico é o caso do trigo polinizado com o milho, que foi amplamente estudado (KISANA *et al.*, 2006;

LAURIE; REYMONDIE, 1991; NIROULA; BIMB, 2009). As etapas da geração dos haploides por meio desse cruzamento são descritas por FRITSCHE-NETO, GARBUGLIO e BORÉM (2012), em resumo, deve ser realizado a emasculação das panículas, cruzamento com o pólen das anteras maduras do milho e o resgate do embrião em meio de cultura, 16 a 18 dias após a fertilização. As plântulas irão se desenvolver no meio de cultivo *in vitro* até o momento da duplicação dos cromossomos.

Em tabaco, a viabilidade da indução de indivíduos haploides maternos já havia sido descrita desde 1979, sugerindo-se a polinização de indivíduos de tabaco *Nicotiana tabacum* com pólen da espécie selvagem *Nicotiana africana*, ou outra espécie da seção *Suaveolentes* (BURK; GERSTEL; WERNSMAN, 1979). Foi observado que a partir desse cruzamento a maioria dos descendentes não sobrevivem além do estágio cotiledonar, uma pequena fração dos sobreviventes (menos de 1%) são haploides maternos, ou seja, originados do gameta feminino da *N. tabacum*, e os demais são híbridos interespecíficos F_1 (BURK; GERSTEL; WERNSMAN, 1979; KRAMER; REED, 1988). Apesar da baixa taxa de indução da haploidia, esse método é viável na cultura do tabaco devido ao grande número de sementes que podem ser obtidas a cada cruzamento.

Vale ressaltar que em cruzamentos ou autofecundação de *N. tabacum* também são observados descendentes haploides de origem materna e paterna, embora em uma proporção muito inferior ao cruzamento interespecífico. Lewis e Rose (2011) identificaram 2,7 haploides em cada 10.000 sementes das progênies obtidas no cruzamento de duas cultivares de *N. tabacum*, originadas espontaneamente por partenogênese. No entanto, eles também observaram que quando o genitor masculino utilizado foi a *N. africana*, a indução haploide foi sete vezes maior do que no cruzamento com a *N. tabacum* como doador de pólen. Embora a taxa de indução de haploides espontâneos seja de baixa magnitude, como já salientado, o elevado número de sementes obtidas por cruzamento nessa espécie permite a viabilidade da obtenção de haploides maternos e paternos também a partir do cruzamento dentro do germoplasma de *N. tabacum*.

Como mencionado anteriormente, na obtenção do haploide materno, por meio do cruzamento de *N. Africana*, a maioria dos descendentes não sobrevive além do estágio cotiledonar e os sobreviventes são uma mistura de híbridos interespecíficos F_1 e haploides maternos. A maioria dos indivíduos não sobrevive devido a uma reação de semi-letalidade em híbridos interespecíficos, também chamada de "letalidade híbrida". Essa reação é um dos muitos sistemas que contribuem para o isolamento reprodutivo das espécies e pode ocorrer devido ao aborto do zigoto após a fecundação ou morte celular das mudas após a germinação

(HANCOCK *et al.*, 2015; MA *et al.* 2020). Tal mecanismo tem sido observado em várias espécies e está relacionado à evolução e especiação de plantas.

Hancock *et al.* (2015) determinaram que a letalidade interespecífica entre o cruzamento de *N. tabacum* x *N. africana* ocorre devido a um sistema genético complementar de letalidade. Nesse sistema, a morte das plântulas irá ocorrer quando os fatores de *N. tabacum* e *N. africana* estiverem presentes. No entanto, nesse cruzamento, muitos aneuploides interespecíficos ainda sobrevivem, provavelmente porque em algum ponto os indivíduos perderam cromossomos ou parte dos cromossomos envolvidos na reação de letalidade. Dessa forma, a distinção entre os indivíduos haploides de interesse e os híbridos aneuploides sobreviventes é mais difícil, podendo ser realizada por marcadores morfológicos, o que requer grande experiência dos avaliadores, ou outros marcadores fenotípicos. Ma *et al.* (2020) identificaram o gene *NtHL1* envolvido no controle da reação de letalidade entre cruzamentos entre *N. tabacum* x *N. aficana*. Embora ainda não tenha sido identificado o gene responsável por essa reação na *N. africana*, os autores argumentam que é provável que o gene *NtHL1* interaja com um único produto de um gene correspondente presente no genótipo da *N. africana* para ativar uma resposta de defesa resultante na reação da letalidade do cruzamento (Ma *et al.* 2020).

2.2.3 Identificação dos haploides e duplicação cromossômica

Após a indução da haploidia as próximas etapas consistem na identificação e seleção dos indivíduos haploides e posterior duplicação do seu número de cromossomos, para a obtenção das linhagens DH. A identificação precoce dos indivíduos haploides é uma das etapas mais importante e constitui um gargalo no processo de obtenção de DH em algumas espécies. Ela pode ser realizada principalmente por marcadores fenotípicos, por contagem cromossômica, pela citometria de fluxo ou pelo uso de marcadores moleculares (FRITSCHE-NETO; GARBUGLIO; BORÉM, 2012).

Em milho, um exemplo de marcador morfológico ainda utilizado e que facilitou a viabilidade da obtenção de DH na cultura é o R-navajo. O gene R1-navajo (*R1-nj*) é de herança dominante e está relacionado à produção de antocianina, se expressando na aleurona e embrião do grão do milho. Assim, quando a linhagem indutora possui esse gene, as sementes obtidas no cruzamento que possuem embrião com coloração arroxeada indicam que esses indivíduos contêm alelos do indutor, ou seja, são diploides, enquanto a ausência de coloração do embrião indica o indivíduo haploide. Embora muito utilizado no passado, essa metodologia pode

apresentar erros, uma vez que a expressão do marcador pode variar conforme o genótipo do indutor ou até mesmo por questões ambientais (LIU *et al.*, 2017). Assim, outras alternativas de marcadores baseados no alto teor de óleo, coloração, peso das sementes e espectroscopia de infravermelho estão sendo buscadas (LIU *et al.*, 2017).

Na cultura do tabaco ainda não foi encontrado um marcador morfológico eficiente para a identificação dos haploides. Embora alguns estudos indiquem que o formato das folhas, tamanho e densidade dos tricomas, e medidas dos estômatos podem auxiliar na distinção das plântulas haploides dos híbridos aneuploides, na indução via cruzamento *N. tabacum* x *N. africana* (BURK; GERSTEL; WERNSMAN, 1979; REED, 1993), somente esses caracteres não permitem a identificação dos haploides com alta precisão. Buscando um marcador fenotípico mais eficiente para a cultura, Hancock *et al.* (2015) desenvolveram um sistema de identificação de plântulas haploides pela expressão de uma proteína verde fluorescente (GFP) na planta, por meio da transgenia. Para isso, inicialmente foi inserido um gene com alelo dominante para a super expressão do GFP em um genótipo de *N. africana*. Assim, ao realizar o cruzamento entre genótipos de *N. tabacum* com a *N. africana* modificada, os descendentes obtidos que forem híbridos interespecíficos F_1 apresentarão plântulas com coloração fluorescente, enquanto os descendentes haploides, uma vez que não possuem cromossomos provenientes do parental *N. africana*, serão plântulas com coloração normal.

A duplicação cromossômica é outra importante etapa no processo de obtenção de DH. Essa duplicação na maioria das vezes é realizada pela aplicação de agentes antimitóticos, que impedem a formação das fibras do fuso durante a mitose (BARNABÁS *et al.*, 1999). A colchicina foi o principal agente utilizado no passado, no entanto, os programas de melhoramento atualmente estão buscando outras alternativas de duplicação. Alguns tratamentos químicos como trifluralina, amiprofos-metilo e gás óxido nitroso têm sido sugeridos para melhorar as taxas de duplicação (KATO; GEIGER, 2002; KLIMA; VYVADILOVÁ; KUCERA, 2008; GRZEBELUS; ADAMUS, 2004; PINTOS; MANZANERA; BUENO, 2007; KITAMURA; AKUTSU; OKAZAKI, 2009; HÄNTZSCHEL; WEBER, 2010). Em algumas culturas, pode ocorrer também a duplicação espontânea do haploide, mesmo em pequenas taxas. Wu *et al.* (2017) discutiram os mecanismos genéticos da macho fertilidade do haploide em milho e concluíram que é possível selecionar genótipos haploides com maior fertilidade e, consequentemente, maior restauração do diploide para posterior uso em programas de melhoramento visando a obter linhagens DH.

Uma complicação encontrada na obtenção de linhagens DH refere-se às baixas frequências obtidas na indução de haploides e na subsequente duplicação cromossômica. No

milho, as linhagens indutoras modernas apresentam taxas de indução relativamente altas, de 8 a 15% (ANDORF *et al.*, 2019). No entanto, em outras culturas como o tabaco, a frequência de haploides pode ser menor que 1% (HANCOCK *et al.*, 2015). Dessa forma, as baixas frequências de indução de haploidia e duplicação cromossômica fazem com que se questione a existência de uma segregação preferencial, ou seja, se em apenas determinadas combinações genotípicas é possível obter o DH. Nesse contexto, Li *et al.* (2013) investigaram por meio de simulação computacional o benefício de usar DH em comparação a estratégia convencional de melhoramento de trigo utilizada no Centro Internacional de Melhoramento de Milho e Trigo (CIMMYT). Neste caso, embora a estratégia DH tenha economizado um ano no ciclo de melhoramento, os ganhos genéticos por ano foram muito menores do que aqueles do melhoramento convencional. Em outro trabalho, Melchinger *et al.* (2017) compararam variedades de polinização aberta com linhagens DH de milho, a partir de análises moleculares com marcadores SNPs. Os autores afirmaram que a obtenção de linhagens DH não ocasionou seleção direcional sistemática em regiões genômicas específicas, assim, não existiria evidências de segregação preferencial (MELCHINGER *et al.*, 2017). No entanto, usando os mesmos dados genômicos, Zeitler, Ross-Ibarra, Stetter (2020) obtiveram resultados contraditórios, ou seja, nesse caso foi observada restrição na variabilidade quando o DH foi utilizado. Entretanto, em nenhum dos dois trabalhos as linhagens DH e as obtidas pelo método convencional de melhoramento foram avaliadas simultaneamente em condições de campo.

Na cultura do milho, existem alguns trabalhos comparando o desempenho de linhagens DH e as obtidas por meio de sucessivas autofecundações, em avaliações em campo (BORDES *et al.*, 2006; BORDES *et al.*, 2007). Contudo, o método utilizado para a avaliação do DH nesses casos foi por meio de “topcrosses”, ou seja, pela avaliação do híbrido obtido pelo cruzamento com uma linhagem testadora, e não pelo desempenho da linhagem *per se*. Vale ressaltar que nesse caso, a linhagem testadora utilizada pode influenciar no desempenho do híbrido (BERNARDO *et al.*, 2020), podendo assim esconder o real comportamento do DH. No caso do tabaco, na literatura não foram encontrados trabalhos de comparação de linhagens DH e obtidas pelo método convencional de melhoramento em experimentos em condições de campo, o que foi proposto neste trabalho.

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CAPÍTULO 2 - PERFORMANCE OF DOUBLED HAPLOID LINES IN RELATION TO THOSE OBTAINED BY THE CONVENTIONAL BREEDING METHOD IN TOBACCO

Artigo redigido conforme a NBR 6022 (ABNT, 2018) e formatado de acordo com o Manual da UFLA de apresentação de teses e dissertações

RESUMO

Em algumas espécies, a alternativa mais utilizada para acelerar o processo de obtenção de linhagens é a utilização de duplo haploides (DH). A dúvida existente é se o processo de indução e duplicação dos cromossomos ocorre de forma aleatória ou se há restrição na segregação. Caso isso ocorra, seria uma limitação ao seu uso, uma vez que a variabilidade gerada nos cruzamentos não seria totalmente explorada. Este trabalho foi realizado com o objetivo de comparar o desempenho de linhagens de tabaco (*Nicotiana tabacum* L.) obtidas pelo método convencional de melhoramento (MC), com as obtidas pela metodologia DH por meio de cultura de anteras. Além disso, verificar a viabilidade do emprego rotineiro de DH em um programa de seleção recorrente (SR). Para isso, três populações de tabaco do grupo varietal Virginia foram obtidas por cruzamento biparental. Considerando todas as populações, o número de linhagens avaliadas foi de 190 DH e 194 CM. Elas foram avaliadas simultaneamente, em um experimento por população em dois locais. As características avaliadas foram produtividade de massa verde (YLD), teor de alcaloides totais (ALK) e teor de açúcar (SU). Os parâmetros genéticos e fenotípicos foram estimados por população. Constatou-se que o desempenho do DH em comparação ao MC diferiu entre os caracteres. Para aqueles relacionados à qualidade, ALK e SU, o desempenho dos dois tipos de linhagens foi semelhante. Para YLD, as estimativas de variância genética e h^2 entre as DH foram maiores que as de CM. No entanto, o oposto ocorreu para a média. Considerando todas as linhagens, independente do cruzamento, a média do CM foi 13,3% maior do que a média do DH, para YLD. Além disso, o ganho anual com a seleção foi maior para CM para esta característica. O uso de DH em um programa de SR será eficiente em comparação ao MC se for obtido um grande número de DH, que deve passar por uma seleção preliminar antes da avaliação mais intensiva para identificar as linhagens a serem recombinadas.

Palavras-chave: *Nicotiana Tabacum* L., Melhoramento de Plantas, Seleção recorrente

ABSTRACT

In some species, the most used alternative to accelerate the process of obtaining lines is by using doubled haploids (DH). The existing question is whether the process of induction and duplication of chromosomes occurs randomly or there is restriction in the segregation. If this fact occurs, it would be a restriction on its use since the variability generated in the crossings would not be fully explored. This work was carried out with the purpose to compare the performance of tobacco lines (*Nicotiana tabacum L.*) obtained by conventional breeding method (CM), with the obtained by the DH methodology, using anther culture. Additionally, to verify the feasibility of routine employment of DH in a recurrent selection program. For this purpose, three populations of tobacco from the Virginia varietal group were obtained by biparental crossing. Considering all populations, the number of lines evaluated was 190 DH and 194 CM. They were evaluated simultaneously, in one experiment per population at two locations. The traits assessed were green leaf yield (YLD), total alkaloid content (ALK), and sugar content (SU). The genetic and the phenotypic parameters were estimated by population. It was found that: the performance of the DH in comparison to CM differed between the traits. For those related to quality, ALK and SU, the performance was similar. For YLD, the genetic variation estimates and h^2, among the DH was higher than those of CM. However, the opposite occurred for the mean parameter. Considering all lines, regardless of the crossing, the CM mean was 13.3% higher than the DH mean, for YLD. Also, the annual gain with the selection was greater for CM to this trait. The use of DH in a recurrent selection program will be efficient in comparison to CM if a large number of DH are obtained which must undergo a preliminary selection before the most intensive evaluation to identify the lines to be recombined.

Keywords: *Nicotiana tabacum* L., Plant Breeding, Recurrent selection

1 INTRODUCTION

In the genetic improvement of most cultivated species, the most time-consuming step is the obtention of homozygous individuals - lines. This is because from an F_2 or S_0 generation, it is necessary to carry out successive self-fertilizations until most of the loci are homozygous. Alternatives to accelerate this process have been sought over time. One of the most researched alternatives is the use of doubled-haploid (DH), that is the generation of haploid individuals that have their chromosomes duplicated to obtain DH lines (CHAIKAM *et al.*, 2019; LENAERTS; COLLARD; DEMONT, 2019; YAN *et al.*, 2017).

Thus, the induction of haploids is the first step to obtain DH. For this purpose, some alternatives are possible, such as the spontaneous production of haploids, the *in vitro* induction, and the use of haploid inducers plants. In the specific case of tobacco (*Nicotiana tabacum L.*), where there is facility of tissue culture, the generation of haploid plants from the anther culture has been made possible for some time (BELOGRADOVA *et al.*, 2009; BOURGIN; NITSCH, 1967; NITSCH; NITSCH, 1969). Subsequently, it was found the possibility to obtain haploids *in vivo* by crossing *N. tabacum* with the wild specie *Nicotiana africana*, or with other species from the *Suaveolentes* section, used as male parent (BURK *et al.*, 1979; HANCOCK *et al.*, 2015). In this case, some descendants are obtained from the non-fertilized gametic cell of *N. tabacum*, i.e., haploid.

Once the haploid individual is obtained, the second step is to double the number of chromosomes to obtain the DH. For this purpose, several methodologies are possible (HÄNTZSCHEL; WEBER, 2010; KITAMURA *et al.*, 2009; KLIMA *et al.*, 2008; WU *et al.*, 2017).

Although the procedures for haploid induction and chromosome duplication have been improved, their frequency still is low. In this condition, it is questioned whether there is a preferential segregation during the DH obtaining process, i.e., whether only given genotypic combinations can generate the DH. If this fact occurs, it would be a restriction on its use since the variability generated in the crossings would not be fully explored. In corn, there is evidence that in the process of induction of haploids and haploid duplication there are genes involved (BECKERT, 1994; CHAIKAM *et al.*, 2019; KALINOWSKA *et al.*, 2019).

The existence or not of alteration in the segregation of DH in relation to CM lines available in the literature is contradictory. There are reports in corn crop, by using SNP markers, that the DH did not show restriction in variability (MELCHINGER *et al.*, 2017). However,

using the same genomic data Zeitler, Ross-Ibarra, Stetter (2020) obtained contradictory results, that is, there was restriction in the variability when the DH was used. In other studies, also with corn, they observed similar performance between the DH and those obtained by the conventional breeding method (CM) (BORDES *et al.* 2006, 2007; LASHERMES; GAILLARD; BECKERT, 1988, SEITZ 2005). However, in some autogamous species, the performance of the two types of DH and CM was not similar (BJORNSTAD *et al.* 1993; CHARMET; BRANLARD, 1985; MA *et al.* 1999). In Brazil, still there was no report comparing the use of DH and CM lines of tobacco. This information is fundamental because the use of recurrent selection (RS) in the breeding of this crop has been encouraged, and greater RS efficiency is obviously expected from the use of the DH methodology.

From the above, the purpose of this study was to verify if from the same tobacco "gene pool", the lines obtained by the conventional breeding method differ from those of the doubled haploid methodology, derived from the anther culture. Additionally, to verify the feasibility of routine employment of DH in a RS program.

2 MATERIAL AND METHODS

The data used in this work was kindly provided by the company BAT Brazil, a subsidiary of the British American Tobacco (BAT) group.

The work was carried out in two steps, the first was the obtaining of the lines by the conventional breeding method (CM) and the doubled haploid methodology (DH), and then, the comparison of the lines obtained in each method in field experiments.

2.3 Description of the treatments

Three populations of tobacco from the Virginia varietal group, called A, B and C, were used. These populations were obtained by biparental crossing of the best lines available in the company. From the plants of generation F_2, two strategies were used to obtain the recombinant inbred lines (RIL), one by the conventional breeding method (CM), through successive self-fertilization up to generation F_6, and the other, by generating doubled haploid lines (DH), from the anther culture.

2.4 Obtaining lines by the conventional method (CM)

The seeds of generation F_1 were sown, and subsequently, the obtained descendants, now generation F_2, were harvested individually. A sample of equal number of seeds from each plant was taken to start the next generation. This procedure was repeated until generation F_4, i.e., the method used to conduct the population in this work was Bulk (FEHR, 1987), except that the contribution of each plant to the next generation in terms of number of descendants was similar. In this way, we tried to avoid the action of the natural selection.

From the F_4 generation, the plants were picked up individually to originate the progenies. These progenies were planted in 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 the seeds of the progenies $F_{6:7}$. All individual plant selections were made by a visual evaluation. As the plants of this generation have only 1/64 of the loci in heterozygosity average, they are considered virtually homozygous lines. It is worth pointing out that after F_1, six years were necessary to obtain the CM.

2.5 Obtaining doubled haploid lines (DH)

This step of the work was carried out in the Tissue Culture Laboratory of the company, located in the city of Rio Negro, in the state of Paraná. The DH were obtained by *in vitro* induction with plants of generation F_2, using anther culture. The methodology adopted was the recommended to tobacco crop (KASPERBAUER; COLLINS, 1974; MA, 2017). The steps are basically the following: 1- haploid induction; 2- identification of the haploid seedlings; 3- duplication of the chromosomes; 4- identification of the duplicated individuals; and 5- multiplication of the DH seeds.

The haploid induction was done by picking up flowers from fully blooming plants of generation F_2, from each population separately. For this purpose, five flowers from each plant were selected in the initial stage of development and sent to the laboratory. The flowers were disinfected with alcohol and hypochlorite 70%. Then, the anthers were extracted without the fillets and inoculated in Petri dishes in androgenesis induction culture medium (A-medium) (KASPERBAUER; COLLINS, 1974). The dishes were incubated for 24 h in BOD at 35ºC ± 1ºC, then, they were placed in a growth chamber at 25ºC ± 1ºC with a photoperiod of 16 h. Approximately three weeks later, the shoots containing at least two primary leaves were excised from the anthers and transplanted to MS medium (MURASHIGE; 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 walls tissue, which are diploid, it was necessary to confirm the ploidy of the obtained individuals. The haploid individuals were identified with the quantification of the seedling DNA, using flow cytometry. Flow cytometer BD Accuri C6 was used, and the procedure was performed according to the methodology recommended by the manufacturer. The samples for the analysis were composed of nuclear suspensions isolated from leaf segments, which were stained and analyzed. To determine the ploidy level of the obtained individuals, the amount of DNA in each sample was compared to that of a diploid control plant.

Upon confirmation of the ploidy, the chromosomes of the haploid individuals were duplicated using the company's protocol. After this procedure, the ploidy verification stage was carried out again and only the individuals which were effectively duplicated were selected. The selected plants went through a stage of acclimatization and production of seedlings in a greenhouse. Then, the seedlings were transplanted to the field to their development and to obtain the DH seeds.

2.6 Experiments for comparison of CM and DH lines

The experiments for evaluation of the doubled haploid lines (DH) and the lines obtained by the conventional method (CM) were conducted in the 2019/2020 crop season, in two locations. In the experimental farm of the company (L1) and in a farm of a tobacco producer (L2), both situated in the municipality of Mafra, in the northern region of the state of Santa Catarina. The L1 has clay soil and it is situated at 824 m altitude, 26º09'58.2" S and 049º48'08.1" W. The soil in L2 is clay loam and it is located at 864 m altitude, 26°10'13.3" S and 049°56'20.4" W.

Initially, the obtained seeds of CM and DH were sown in a polyethylene tray to obtain the seedlings in a float system in the greenhouse. Approximately 60 days after sowing, the seedlings were transplanted in the experimental area.

Three contiguous experiments were carried out in each location, one for each population. From populations A and B, 70 lines of each origin and four other controls were evaluated. The experimental design adopted was the 12 x 12 triple lattice. At population C, 58 lines of each type and five controls were evaluated in a triple lattice 11 x 11. The experimental plot consisted of a 10-meter line, spacing 0.50 m between plants and 1.20 m between lines. The crop management of the experiments was the same as that adopted by the company. The topping of the plants, which consists of removing the inflorescence, was carried out by plot according to the productive potential of the plants. From 15 to 30 days after topping, the leaves harvest started, which was carried out successively according to the point of physiological maturity.

The evaluated traits were green leaf yield (YLD) in Kg/plot, total alkaloids content (ALK) and total sugar content (SU). The last two traits were measured in the company's laboratory, from a sample of leaf dry mass, and given in percentage of the total sample (%). All data was obtained by plot.

2.7 Statistical data analyses

Statistical analyses were performed in the R environment (R CORE TEAM, 2020). Initially the data of green leaf yield (YLD), total alkaloid content (ALK) and total sugar content (SU) were analyzed considering each population separately, and then, all lines were analyzed regardless of the population. A joint analysis of the data obtained in the two locations was performed using the following model:

$$Y = X\beta + Z_1 l + Z_2 b + Z_3 g + e$$

where:

Y: vector of mean phenotypic data;

β: vector of the fixed effects, mean, location and repetition;

l: random line effect, in which $l \sim N(0, I\,\sigma_l^2)$;

b: random block within repetition effect, in which $b \sim N(0, I\sigma_b^2)$;

g: random interaction lines x environments effect, in which $g \sim N(0, I\,\sigma_g^2)$;

X, Z_1, Z_2 e Z_3: incidence matrices for *β*, *l*, *b*, and *g*, respectively; and

e: vector of the residual effects (random), in which $e \sim N(0, I\sigma_e^2)$.

To obtain the variance components estimates of the CM and DH lines effects separately, another analysis was made using a model similar to that presented above:

$$Y = X\beta + Z_1 l_{MC} + Z_2 l_{DH} + Z_3 b + Z_4 g + e,$$

where:

Y: vector of mean of phenotypic data;

β: vector of the fixed effects, mean, location and repetition;

l_{CM}: random CM line effect, in which $l_{CM} \sim N(0, I\,\sigma_{l_{MC}}^2)$;

l_{DH}: random DH line effect, in which $l_{DH} \sim N(0, I\,\sigma_{l_{DH}}^2)$;

b: random block within repetition effect, in which $b \sim N(0, I\sigma_b^2)$;

g: random interaction lines x environments effect, in which $g \sim N(0, I\,\sigma_g^2)$;

X, Z_1, Z_2, Z_3 e Z_4 incidence matrices for *β*, l_{CM}, l_{DH}, *b* and *g*, respectively; and

e: vector of the residual effects (random), in which $e \sim N(0, I\sigma_e^2)$.

Estimates of the variance components of the random effects were obtained by the Maximum Restricted Likelihood (REML) method. The Likelihood Ratio Test (LRT) was used to test the significance of these variance components estimated.

Subsequently, analyses were made considering all lines of the three populations simultaneously. The adjustment was made by the controls common to the three experiments and the models used were the same as the previously adopted ones.

The analysis was also made using an index involving traits YLD and ALK. The index used was the sum of standardized variables (∑Z), by means of the following estimator: $\sum Z_i = 0.7Z_{vi} + 0.3Z_{ai} + 3$, where $Z_{vi} = \frac{y_{iv} - \bar{y}_{.v}}{s_{.v}}$ is the standardized variable for the YLD trait in line

i and $Z_{ai} = \frac{y_{ia} - \bar{y}_{.a}}{s_{.a}}$, the standardized variable or the ALK trait in line *i*, where y_{iv} and y_{ia} are the means of line *i* for traits YLD and ALK, respectively, in each location; $\bar{y}_{.v}$ is the general mean in each location of trait YLD and $\bar{y}_{.a}$, of ALK; $s_{.v}$ standard deviation between the means of the lines for YLD and $s_{.a}$, for ALK. Weights of 0.7 for YLD and 0.3 for ALK were attributed according to the economic importance of the traits. In addition, as the standardized variables can have positive and negative values, constant 3 was added to make all values positive. After obtaining the index estimates for each plot, the analyses were made using the same models described above.

From the estimated variance components, the heritability estimates (h^2) were obtained for the selection of the means 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: $h^2 = \frac{\sigma_L^2}{(\sigma_L^2 + {\sigma_{LE}^2}/{k} + {\sigma_E^2}/{rk})}$, where σ_L^2 is the genetic variance between the lines; σ_{LE}^2 is the variance of the lines x environments interaction; σ_E^2 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 the ones estimated in the specific model for each situation, i.e., considering all lines or DH and CM separately. The confidence intervals of the h^2 estimates were obtained according to the method described by Knapp *et al.* (1985).

The expected gain with selection of the best ten lines in percentage of the general mean (GS) was obtained by the estimator: GS (%)= $\frac{\overline{BLUPs}}{\bar{Y}} * 100$, where $\overline{BLUPs}$: BLUP means of the selected lines; $\bar{Y}$: BLUP mean of all lines. All estimates were made considering the CM and DH of each population and all lines regardless of the population. For all situations, the expected annual genetic gain with selection (GSA) in percentage was also estimated, considering the time to obtain the CM of six years, and three years for DH. Additionally, GS and GSA were obtained for ∑Z.

3 RESULTS

The selective accuracy, considering the mean of the estimates obtained in each location, were high, above 0.88 for green leaf yield (YLD), and above 0.77 for the total alkaloid content (ALK), regardless of the population (TABLE 1). These results show that the experimental precision in the evaluation of YLD and ALK was good. For the total sugar content (SU) trait, the estimates were of low magnitude, lower than 0.45. It appears that this trait was more influenced by the environmental variations. This fact can be confirmed by the error variance estimations (σ_E^2), which, for SU, were much higher than the genetic variances between lines ($\sigma_{G_L}^2$) (TABLE 1).

Table 1 – Estimates of the genetic and phenotype parameters of CM and DH lines. Obtained in the joint analyses, for the traits green leaf yield (Kg/plot) (YLD), total sugar content (%) (SU) and total alkaloid content (%) (ALK). Data obtained from the evaluation of CM and DH lines from the populations A, B, and C of tobacco from the Virginia varietal group.

	YLD			**SU**			**ALK**		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
$\boldsymbol{\sigma_{G_L}^2}$ [1]	2.93**	4.42**	3.44**	0.39^{ns}	0.31^{ns}	0.28^{ns}	0,029**	0,017**	0,027**
$\boldsymbol{\sigma_{G_{CM}}^2}$	0.82**	1.48**	0.39**	0.38^{ns}	0.08^{ns}	0.12^{ns}	0,016**	0,019**	0,012**
$\boldsymbol{\sigma_{G_{DH}}^2}$	3.74**	2.67**	1.96**	0.41^{ns}	0.49**	0.13^{ns}	0.041**	0.014**	0.029**
$\boldsymbol{\sigma_{LE}^2}$	0.20^{ns}	0.00^{ns}	0.24^{ns}	0.00^{ns}	0.05^{ns}	0.06^{ns}	0,007**	0,000^{ns}	0,004**
$\boldsymbol{\sigma_E^2}$	2.47	3.56	2.49	10.91	5.50	3.91	0.037	0.035	0.031
r_{gg} [2]	0.89	0.88	0.89	0.32	0.41	0.44	0.86	0.77	0.86

[1] $\sigma_{G_L}^2$: genetic variance between all lines; $\sigma_{G_{CM}}^2$: genetic variance between CM lines; $\sigma_{G_{DH}}^2$: genetic variance between DH, σ_{LE}^2: variance of the lines x environments interaction; and σ_E^2: residual variance.
[2] Average selective accuracy of the two locations.

Source: from author (2021)

The estimated $\sigma_{G_L}^2$ for traits YLD and ALK were significant in all populations (TABLE 1). The same happened for the genetic variance between lines from the CM ($\sigma_{G_{CM}}^2$) or the DH ($\sigma_{G_{DH}}^2$). For both traits, there was a significant difference between the lines (TABLE 1). However, for SU, it did not happen in most situations. It shall be pointed out that the estimates of the DH genetic variances were of higher magnitude than for CM in most cases. For example, for trait YLD, $\sigma_{G_{DH}}^2$ was up to 5 times $\sigma_{G_{CM}}^2$.

The lines x environments interaction variance (σ^2_{LE}) was not significant in most situations (TABLE 1), except for trait ALK in populations A and C, but even in these two cases, the estimates of σ^2_{LE} were lower than $\sigma^2_{G_L}$. Thus, it can be inferred that the line performance was coincident between the two locations. From the above, the comments on the results will be focused on what happens in the mean of the two locations.

The mean YLD of all lines was 17.75 Kg/plot, i.e., 9.2% higher than the means of the controls, which are commercial cultivars (TABLE 2). When ALK was considered, the mean of the lines was 2.28%, value slightly lower than that obtained by the commercial controls. For SU, the general mean of the lines was 12.85%, estimation similar to that for the controls.

The variation range of the line means for YLD considering DH was 45% of the average in population A, 39% in B, and 37% in C; therefore, very similar values among the populations (TABLE 2). The CM showed slightly lower range of variation than those reported for DH, i.e., 30% for A, 35% for B. and 21% for C. It shall be pointed out that although $\sigma^2_{G_{DH}}$ of population B has been higher than $\sigma^2_{G_{CM}}$, for the trait ALK, the variation range did not reflect this estimation (FIGURE 1). For this trait, the range between the means of the lines of the three populations for DH was 27% and for CM, 25% in relation to the mean of all lines.

Table 2 – Estimation of BLUP mean of all lines, only CM and DH, and the controls, in each population and considering all populations, for the green leaf yield (YLD) (Kg/plot), total sugar content (SU) (%) and total alkaloid content (ALK) (%).

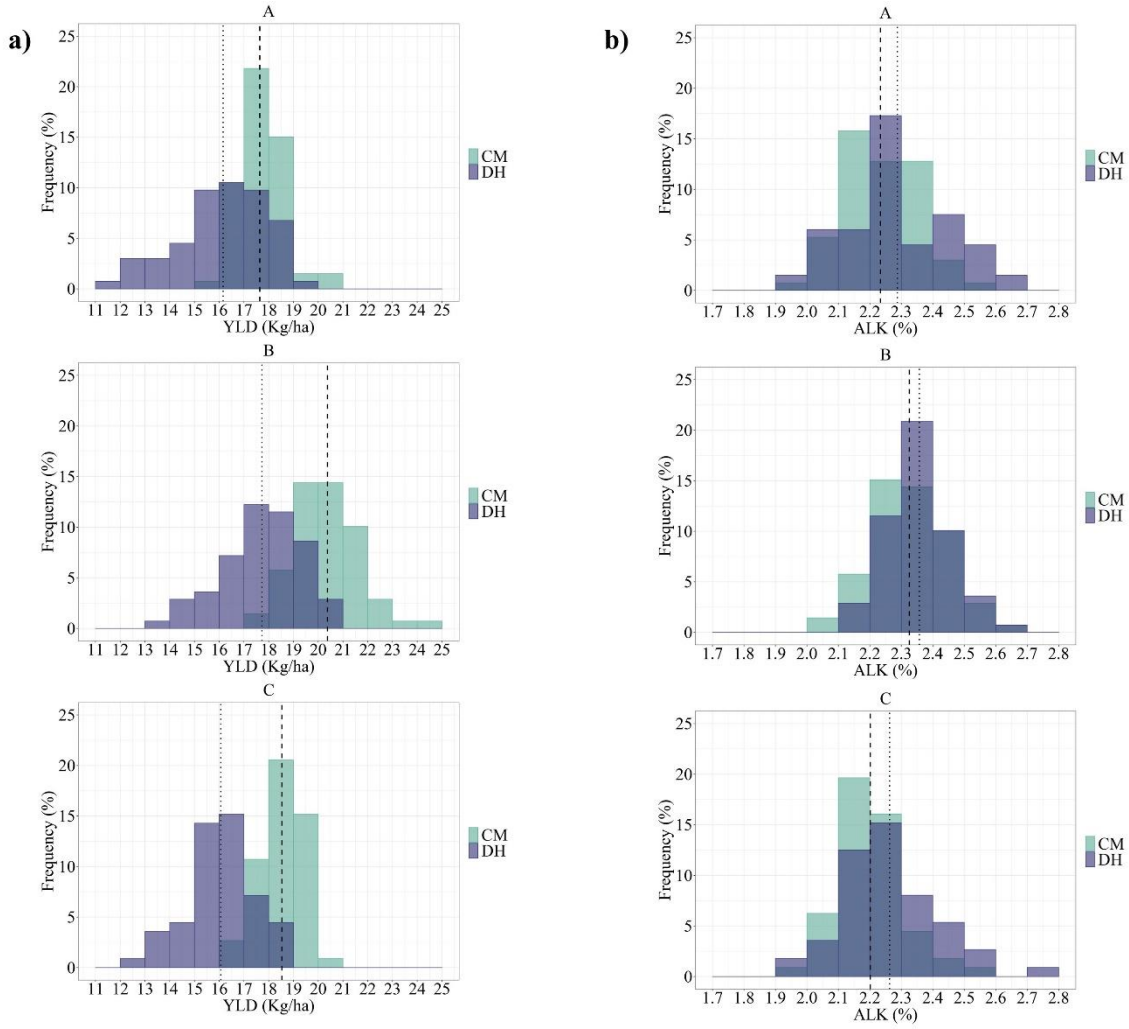
Origin	Population	YLD	SU	ALK
CM	**A**	17.64	14.23	2.23
	B	20.37	11.92	2.33
	C	18.53	12.37	2.20
	All	18.85	12.84	2.25
DH	**A**	16.16	14.14	2.29
	B	17.73	11.99	2.36
	C	16.06	12.45	2.26
	All	16.65	12.86	2.30
All	**A**	16.90	14.19	2.26
	B	19.05	11.96	2.34
	C	17.30	12.41	2.23
	All	17.75	12.85	2.28
Controls		16.26	13.11	2.33

Source: from author (2021)

At the moment, most important is to compare the estimated means according to the origin of the lines. It is observed that for YLD, regardless of the population, the mean of DH was 11.7% lower than CM (TABLE 2). For ALK, the opposite happened, the mean of the DH was 2.2% higher than CM. It is also interesting to comment whether the population means were different, regardless of the way of how the lines were obtained. See that the estimate in absolute values for population B was 11% higher than the mean of A and C for YLD (TABLE 2). For ALK, the same fact occurred, i.e.., the mean of the B was higher than for other populations.

The difference in the higher release of genetic variance of DH in relation to CM can be observed also by the means of the frequency distribution of the BLUP means (FIGURE 1), confirming the previous observations related to the performance of DH in relation to CM. Note that for YLD, there was a big overlap in the frequency distributions of the means; however, some CM showed higher means regardless of the population. For ALK, the frequency distributions of the means for DH and CM were virtually the same variation range, although the DH mean was higher in all situations.

Figure 1 – Frequency distribution of the means for CM and DH, for populations A, B and C. a) green leaf yield (YLD) (Kg/plot); b) Total alkaloid content (ALK) (%). Means of lines of tobacco from the Virginia varietal group evaluated in two locations.



Source: from author (2021)

The variation between the different types of lines can also be observed by estimation of heritability (h^2) (TABLE 3). As expected, the results are coincident with the estimates of selective accuracy. The h^2 were high for traits YLD and ALK and practically null for SU (TABLE 3). When all lines within the same population were considered, there was no major difference between the values of h^2 in each population for YLD, varying from 0.85 to 0.88 (TABLE 3). A similar result was observed for ALK. However, when the lines of different origins were compared, it was observed that in all populations the estimated h^2 for DH were of higher magnitude compared to CM, except for ALK in population B (TABLE 3).

Table 3 – Estimates of heritability (h^2) for all lines (h_L^2), CM (h_{CM}^2) and DH (h_{DH}^2) for the traits green leaf yield (YLD), total sugar content (SU) and total alkaloid content (ALK), in each population.

Population	Origin	YLD	SU	ALK
A	h_L^2	0.85 (0.84 – 0.91)[1]	0.18 (-0.07 – 0.38)	0.83 (0.80 – 0.88)
	h_{CM}^2	0.62 (0.57 – 0.79)	0.17 (-0.16 – 0.44)	0.73 (0.67 – 0.84)
	h_{DH}^2	0.88 (0.86 – 0.93)	0.18 (-0.15 – 0.45)	0.87 (0.83 – 0.92)
B	h_L^2	0.88 (0.85 – 0.91)	0.25 (0.05 – 0.44)	0.73 (0.67 – 0.81)
	h_{CM}^2	0.71 (0.60 – 0.80)	0.07 (-0.26 – 0.38)	0.76 (0.68 – 0.84)
	h_{DH}^2	0.82 (0.75 – 0.88)	0.34 (0.11 – 0.57)	0.70 (0.61 – 0.81)
C	h_L^2	0.87 (0.86 – 0.92)	0.29 (0.10 – 0.50)	0.79 (0.80 – 0.89)
	h_{CM}^2	0.42 (0.35 – 0.71)	0.14 (-0.18 – 0.47)	0.62 (0.61 – 0.83)
	h_{DH}^2	0.79 (0.76 – 0.89)	0.16 (-0.15 – 0.48)	0.80 (0.79 – 0.91)

Source: from author (2021)

Considering that the DH methodology is expected to be used in a recurrent selection program, focus will be given to what happened considering all lines regardless of the population of origin. In addition, SU was disregarded in these analyses, once the source of variation of lines for this trait had no significant difference in any population, as already commented. Estimation of the selection index Z ($\sum Z$) were also obtained for traits YLD and ALK simultaneously.

As expected, the variance between lines was significant for all traits considered (TABLE 4). Estimation $\sigma_{G_L}^2$ for trait YLD was higher compared to the evaluation of lines in each population (TABLES 1 and 4). For trait ALK, the estimation obtained by evaluation of all lines corresponded to the mean of the genetic variances of the three populations. As observed in the evaluation of lines in each population, the DH variances were of higher magnitude in relation to CM in all cases (TABLE 4). This difference was proportionally more expressive for trait ALK, where $\sigma_{G_{DH}}^2$ was 1.7 times greater than $\sigma_{G_{CM}}^2$.

Table 4 – Estimation of genetic and phenotype parameters between lines, considering all populations simultaneously, obtained in the joint analyses of traits green leaf yield (YLD) (Kg/plot), total alkaloids content (ALK) (%) and standardized selection index (∑Z). Data from 194 conventional and 190 DH tobacco lines from the Virginia varietal group, evaluated in two locations.

	YLD	**ALK**	**∑Z**
$\boldsymbol{\sigma^2_{G_L}}$ [1]	4.454**	0.025**	0.210**
$\boldsymbol{\sigma^2_{G_{CM}}}$	2.357**	0.017**	0.119**
$\boldsymbol{\sigma^2_{G_{DH}}}$	3.364**	0.029**	0.163**
$\boldsymbol{\sigma^2_{LE}}$	0.000^{ns}	0.005**	0.021^{ns}
$\boldsymbol{\sigma^2_{E}}$	3.816	0.044	0.274

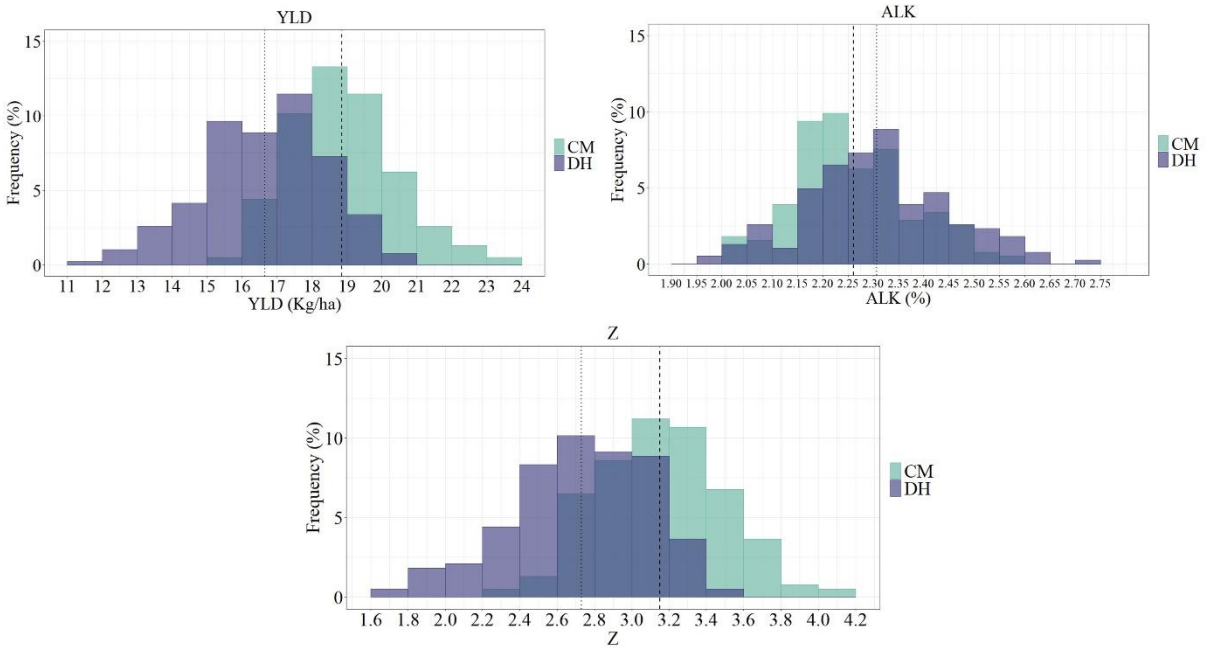
[1]Genetic variance among all lines ($\sigma^2_{G_L}$), only conventional ($\sigma^2_{G_{CM}}$) and DH ($\sigma^2_{G_{DH}}$), lines x environments interaction variance (σ^2_{LE}) and residual variance (σ^2_E).

Source: from author (2021)

The lines x environments interaction variance (σ^2_{LE}) was not significant for YLD and ∑Z (TABLE 4). For ALK, estimation σ^2_{LE} was significant, although the magnitude was lower in relation to the genetic variance between lines, as observed when the populations were considered separately.

The frequency distributions of BLUP means of the variables YLD, ALK and ∑Z involving all lines are shown in Figure 2. For YLD and ∑Z, the range of variation in relation to the general mean were high, 73 and 85%, respectively; however. for ALK it was lower, only 37% (FIGURE 2). When the origin of the lines was considered, it could be observed that the variation range for YLD in relation to the mean for all lines was virtually equal, in which, 47% in CM and 49% in DH. Similar results were observed for trait ALK, 25% among the CM ones and 31% in the mean of DH. For ∑Z, the estimation was 59% for CM and 62% for DH.

Figure 2 - Distribution of the mean frequencies considering all conventional (CM) and doubled haploid (DH) lines, regardless of the population of origin, for traits green leaf yield (YLD) (Kg/plot), total alkaloid content (ALK) (%) and ∑Z.



Source: from author (2021)

The mean of CM was higher for YLD (18.86 kg/plot) when compared to that of DH (16.65 kg/plot) (TABLE 5). When considering ALK, the mean of the DH lines (2.30%) was slightly higher than that of the CM lines (2.26%). Considering the best ten lines, the mean for the YLD trait for CM was 12.5% higher than for DH. For ALK, the mean of the best ten DH lines was 3.2% higher than that obtained by the best CM. For ∑Z, the mean of the CM lines with the best performance was 14% higher than the mean of the best DH.

Table 5 – Estimation of the general mean and of the best ten lines and controls, considering all, only CM or DH, for traits green leaf yield (YLD) (kg/plot), total alkaloid content (ALK) (%) and selection index (∑Z).

Origin	YLD	ALK	∑Z
All	17.76	2.28	3.94
CM	18.86	2.26	3.15
DH	16.65	2.31	2.73
Controls	15.79	2.46	2.72
MS_{CM}[1]	22.30	2.52	3.82
MS_{DH}	19.82	2.60	3.35

[1] Mean of the best ten lines selected within CM (MS_{CM}) and DH (MS_{DH}).
Source: from author (2021)

The estimated h^2 obtained considering all lines and the different origins, CM or DH, are shown in Table 6. It can be observed that h^2 involving all lines can be considered of medium to high magnitude for all traits, varying from 0.72 for ALK, to 0.88 for YLD. The estimated h^2 involving only DH in relation to CM were higher for all traits (TABLE 6). However, these differences were of lower magnitude when compared to the analyses of the populations separately.

Table 6 – Estimated heritability (h^2) for the selection in the mean of the lines involving all (h_L^2), only CM (h_{CM}^2) or DH (h_{DH}^2), for traits green leaf yield (YLD), total alkaloid content (ALK) and selection index (∑Z).

h^2	YLD	ALK	∑Z
$\boldsymbol{h_L^2}$	0.88 (0.85–0.89)[1]	0.78 (0.75–0.82)	0.82 (0.80–0.85)
$\boldsymbol{h_{CM}^2}$	0.79 (0.74–0.83)	0.70 (0.67–0.78)	0.72 (0.68–0.79)
$\boldsymbol{h_{DH}^2}$	0.84 (0.81–0.87)	0.80 (0.77–0.85)	0.78 (0.74–0.83)

[1]Values between parentheses correspond to the confidence intervals.
Source: from author (2021)

The estimated total gain (GS) or annual gain (GS_A) with the selection of the best ten CM or DH lines are presented in Table 7. It is worth pointing out that for YLD, the GS estimate considering all lines was equal to the gain with the selection considering only CM (data not presented), indicating that the ten lines with the best performance were obtained by the conventional breeding method. The GS for YLD trait was 14% higher for CM compared to DH. The same pattern for this trait was also observed for GS_A, although with a difference of lower magnitude than that observed in each population. For the ALK, the estimated gain obtained was higher with the selection of DH, with GS_A 2.7 times higher than the selection of the CM. The gains in YLD and ALK were also estimated with the selection of the best ten lines by ∑Z. A

similar result to that previously mentioned for YLD was observed. For ALK, the difference in GS between CM and DH lines was of lower magnitude.

Table 7 – Estimate of total (GS*) (%) or annual (GS_A) (%) gain with selection of the best ten lines for the traits green leaf yield (YLD) (kg/plot), total alkaloid content (ALK) (%), and selection index (∑Z); and the total gain (RC%) or annual gain (RC_A%) for the traits YLD and ALK, with the selection of the best ten lines by ∑Z. Data obtained in the evaluation of 194 CM and 190 DH in two locations.

Trait	Origin	GS	GS_A**	RC	RC_A**
YLD	**CM**	25.64	4.27	21.54	3.59
	DH	11.67	3.89	6.28	2.09
ALK	**CM**	10.24	1.71	6.28	1.05
	DH	13.97	4.66	6.35	2.12
∑Z	**CM**	29.97	5.00		
	DH	13.87	4.62		

* GS in the percentage in relation to the overall mean considering all lines.
**CM obtaining cycle: six years; DH: three years.

Source: from author (2021)

4 DISCUSSION

Although the locations of the experiments are situated in the same region, they differ in some aspects, such as type of soil, altitude, and geographical position. The difference in the mean for green leaf yield (YLD) between the locations was 14.2%, showing the environmental differences in the two locations (data not shown).

In the experiments, it was observed that some lines were lost after transplantation; however, this number was very low. Regardless of the crossing, there were only four lost treatments in the CM and eight in DH, considering both locations. The accuracy estimates (r_{gg}) were of high magnitude for the traits green leaf yield (YLD) and total alkaloid content (ALK) (TABLE 1), indicating high experimental precision in the evaluation of these two traits (RESENDE; DUARTE, 2007). On the contrary, the total sugar content (SU) showed r_{gg} estimates with lower magnitude. When r_{gg} magnitude is low, it cannot be argued a priori that the experimental precision has not been good. Accuracy depends on the experimental error and the existence of genetic variation between the genotypes. The sugar content in tobacco is influenced by numberless environmental factors, such as: crop management, types of soil and nutrients, weather conditions, occurrence of pathogens, leaf position on the stem, harvest time and curing process (LEFFINGWELL, 1999; LEWIS, 2015; BANOŽI´C *et al.*, 2020). Due to the probable difference in the manifestation of these factors, the experimental error was most likely expressive. This fact was proven because the estimated variance error was much higher than the genetic variance estimates obtained for this trait (TABLE 1). Thus, it can be inferred that the experimental precision for this trait was low, making it difficult to obtain significant genetic variances estimates between lines, as observed. Similar results were found by Carvalho (2016) in the evaluation of tobacco lines and hybrids from the Virginia varietal group in the same region.

The variance of the lines x environments interaction was not significant in most cases, or the magnitude was low in relation to the genetic variance between lines (TABLE 1). It appears that, although there was a difference between the locations, this fact did not significantly contribute to change the classification of the lines and/or the response to the environmental effects. However, the occurrence of genotypes x environments interaction with the tobacco crop in the region is often described in literature (CARVALHO, 2016; PULCINELLI *et al.*, 2014, PSCHEIDT, 2020). As in the present situation no lines x environments interaction has been detected, the discussions will be focused on the mean of the two locations.

The tobacco yield is one of the most important traits for the breeder of this crop because it obviously affects the profit of the producers, who will choose whether to accept or not the cultivars. In tobacco, cured leaves are the commercialized product. However, obtaining cured leaves in experiments involving hundreds of lines is very difficult. The most feasible option is to use the green leaf yield data, which is more easily obtained and probably with lower error. It is worth pointing out that the correlation between the YLD and the commercialized product must be high.

Another evaluated trait was the total alkaloids content (ALK), which is of high importance from an industrial point of view. The biggest challenge the tobacco breeders face is the need to adjust the concentration of alkaloids to the requirements of the industry. The estimation of genetic correlation between YLD and ALK was of low magnitude, r_g = -0.09, indicating that the genes that control traits YLD and ALK are not closely linked and/or that are not pleiotropic. This is a favorable condition for the breeders once there should be no restriction on the obtaining of cultivars with high YLD and any ALK content. However, there are reports where phenotype correlation between the traits related to productivity and the total alkaloid content were different than zero (CHAPLIN; WEEKS 1976; LEWIS, 2006). This correlation has probably been observed due to the effect of dilution of the alkaloid content in relation to the tobacco productivity, i.e., the concentration of alkaloids in percentage would remain diluted by the increase of the tobacco productivity (MATZINGER; MANN, 1964, WOLF; BATES, 1964). Some studies with tobacco from the Dark and Virginia varietal group carried out in Brazil also obtained negative phenotype correlation between these two traits; however, of small magnitude (CARVALHO, 2016; PSCHEIDT, 2020).

Obviously, the genetic control of YLD is polygenic and it seems that there is a predominance of additive effects (MATHER, 1949; MATZINGER; MANN; COCKERHAM, 1962; ROBINSON; MANN; COMSTOCK, 1954), although heterosis may occur; however, of low magnitude (CARVALHO, 2016; DEXTER-BOONE; LEWIS, 2019). For alkaloids content in tobacco, the genetic control shall be oligogenic. Collins et al (1974) showed that in the genetic control of the alkaloids content in tobacco, two duplicated genes (A and B) would be involved, with independent distribution and allele interaction of dominance, with segregation in F_2 of: 9 (A_B_) high content, 6 (A_bb or aaB_) intermediary, and 1 (aabb) low. However, the environment also influences the expression of the trait (HENRY *et al.* 2019; LEWIS, 2019), contributing to the continuous frequency distribution of the means.

In the tobacco crop, as already mentioned, several traits shall be considered in the development of new cultivars. To meet the demands of the farmers, the industry and the

consumers, the breeder shall obtain lines/hybrids that combine good productivity and good quality attributes. For this purpose, the use of selection indexes has been widely used in different crops (BAKER, 1986; BERNARDO, 2020; LIMA *et al.*, 2015), including tobacco more recently (CARVALHO, 2016; PSCHEIDT, 2020). From what said above, in this work, it was also decided to make the simultaneous selection for the traits YLD and ALK. There are numberless indexes in literature that can be used for the simultaneous selection of traits (BERNARDO, 2020; RAMALHO *et al.* 2012; RESENDE; SILVA; AZEVEDO, 2014). However, it was decided to use the mean of the standardized variables as it is easy to interpret. In the standardized selection index (∑Z), weights were attributed to the variables according to their economic importance, in which, 70% for YLD and 30% for ALK, as used by Pscheidt (2020). The ALK means of the evaluated lines were close to 2.5%, which corresponds to the lower limit of the perfect range of ALK of the Virginia group. Thus, in the index, it was decided to make the selection in a way to increase the mean of this trait.

In this work, it can be seen that for YLD, when all lines were considered, the mean was 17.76 kg/plot. This estimate is higher than that obtained in relation to the mean of the controls, which are commercial hybrids (TABLE 5). This evidences the good potential of these populations to obtain superior lines for YLD. The same happened when ∑Z was considered, as expected. For ALK, the opposite was observed, the lines showed a lower performance than the controls.

In the conduction of a breeding program, whether for autogamous or allogamous plants, the intention is to obtain the lines as quickly as possible. In this context, some alternatives have been proposed. Among them, conduction of more generations per year (COLLARD *et al.*, 2017; LIU *et al.*, 2016; SAXENA *et al.*, 2017). However, for tobacco, in the region where it is cultivated in Brazil, the obtaining of more than one generation per year has not been feasible.

Another option highly recommended is the use of strategies to speed the growth and the development of plants up under artificial conditions, called “speed breeding” (WATSON *et al.*, 2018; HICKEY *et al.*, 2019). Nevertheless, it appears that this procedure has not been used routinely to improve tobacco yet. Another proposed alternative is the use of SSD method, using greenhouses. However, no report was found of its use in tobacco.

The most desired strategy is the obtaining of doubled haploid (DH). The use of DH has been very much researched and used in some species (DUNWEL, 2010; LI *et al.*, 2013; MAQBOOL; NESHIR; KHOKHAR, 2020; MISHRA; RAO, 2016), including tobacco (HANCOCK *et al.* 2015; MA *et al.* 2020). The reason for this interest is due to some advantages, among them: a) reduction in the time spent for the line obtaining. Atlin, Cairns,

Biswanath (2017), comparing the total time spent in a corn breeding program using DH technology in relation to SSD method and pedigree, showed a reduction of up to 5 years when DH was used; b) the assurance that the obtained lines have 100% of the loci in homozygosity. When lines are obtained by successive cycles of self-fertilization and selection, as in the conventional breeding methods, even in more advanced generations, they do not achieve complete homozygosity, i.e., there is still residual heterozygosity; c) due to the fact that the lines have 100% of the loci under homozygosity, the advantage of DH is that they meet the distinguishing criteria required for registration of new cultivars more easily (CHAIKAM *et al.*, 2019; RÖBER; GORDILLO; GEIGER, 2005). Thus, the use of DH can also speed the cultivar registration/protection process up.

Additionally, the DH methodology also 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 self-fertilization generations, as it is in the conventional method. This way, some activities, such as the crossing record, obtaining of seedlings in the nurseries, conduction of experiments in the field, self-pollination and maintenance of the lines, become simpler upon use of DH, especially since the operations are carried out once only (CHAIKAM *et al.,* 2019). All these mentioned factors contribute to the reduction of the expenses for the long-term improvement program.

DH can be obtained *in vivo* or *in vitro* (MAQBOOL; BESHIR; KHOKHAR, 2019). Specifically, for tobacco, the two alternatives can be used: obtaining androgenetic haploids by using anther culture *in vitro*, or development of gynogenic haploids through the interspecific crossings *in vivo*. The obtaining of haploids from seed, i.e., maternal haploids, is done by interspecific crossing between *N. tabacum* and pollen from *N. africana*, or other member of the *Suaveolentes* section. This strategy was also carried out in the present work; however, due to the little success in the obtaining of haploid individuals, the results were not included. However, there are reports of its successful use in other conditions (MA *et al.,* 2020; HANCOCK *et al.,* 2015; OLIVEIRA, 2016).

The obtaining of haploid individuals from anther culture has been studied for a long time and the technology is already provided with well-established protocols (BOURGIN; NITSCH, 1967; NITSCH; NITSCH, 1969; BELOGRADOVA *et al.*, 2009; MA., 2017). To obtain DH via anther culture some steps are necessary: 1- induction of haploids; 2- identification of the haploid seedlings; 3- duplication of the chromosomes; 4- identification of the duplicated individuals and 5- multiplication of DH seeds. In these steps, there are some difficulties to be overcome. One of them is the identification of the haploid individuals, because in this case, there is still no efficient morphological marker to enable differentiating the haploid

seedlings, originating from the gametes of the possible diploid individuals, which are regenerated from the anther wall tissue. Therefore, it is necessary to quantify the DNA concentration of the plant tissue samples of each individual using a flow cytometry. Although it is efficient, this strategy contributes to making the process more time-consuming and costly. Furthermore, certification of the ploidy level shall be made again after duplication of the number of chromosomes in the haploids, increasing the time spent in obtaining DH by this methodology. However, despite the difficulties, a significant number of DH lines were obtained to be used in this research.

The focus of this work was to verify whether the efficiency in obtaining good lines by the DH methodology is the same as the conventional breeding method (CM). The main difficulty found was how to test the hypothesis that the efficiency of the two methods of obtaining lines is the same. One of them would be by using genomics, as it was done by Melchinger *et al.* (2017), who compared open pollination varieties of corn with DH lines deriving from these varieties, based on molecular analyses with SNP markers. The authors stated that obtaining of DH lines did not cause systematic directional selection in specific genomic regions. However, using the same genomic data Zeitler, Ross-Ibarra, Stetter (2020) obtained contradictory results, that is, there was restriction in the variability when the DH was used. Nevertheless, it is worth pointing out that genomics alone would not make it possible to verify whether the performance of the lines obtained by the two procedures would be the same.

Another option would be to compare the DH lines obtained by the conventional method in field experiments. For the corn crop, there are some reports comparing the performance of the lines obtained by means of DH and SSD (BORDES *et al.* 2006, 2007; LASHERMES; GAILLARD; BECKERT, 1988; SEITZ, 2005). In these works, maternal DH were obtained, and the performance of the lines was evaluated by their performance in "topcrosses". In other species, such as wheat (MA *et al.,* 1999), barley (BJORNSTAD *et al.,* 1993) and triticale (CHARMET; BRANLARD, 1985), DH and SSD lines were also compared for some agronomic traits and have presented different performances.

The last described alternative was the one adopted in this work. Although three crossings have been used, highest emphasis in the discussion will be given to what occurred regardless of the population, i.e., using all lines. With the purpose to obtain information applicable to the use of DH routinely in the recurrent selection program in tobacco carried out by the company.

From the performance data of DH or CM, the question is how to evaluate the possible difference in the efficiency of the methods. Initially, there are two options, the first would be to

use the estimates of genetic and/or phenotype variances. The other option would be to compare the means.

Initially, the estimates of genetic/phenotype variances were considered, because, as already mentioned, there was wide genetic variation for YLD, ALK and ∑Z traits in all populations and when the different origins, DH or CM, were considered. For all traits, the highest estimates of genetic variance and heritability (h^2) for the selection in the line mean were observed within the DH (TABLES 3 and 6). In some studies carried out with corn, no significant differences were found between the genetic variances and the means of the lines obtained by the DH and SSD methodology, but evaluated by topcrosses with a testing line (BORDES *et al.* 2006, 2007; LASHERMES; GAILLARD; BECKERT, 1988; SEITZ, 2005). However, in other autogamous species, results that match those obtained in the present work were observed (MA *et al.*, 1999; BJORNSTAD *et al.*, 1993; CHARMET; BRANLARD, 1985).

Because of the results obtained with the estimation of variances and heritability, at first, DH showed to be more efficient, since it released higher variability. It shall be pointed out that for all estimates of genetic and phenotype parameters, there is an associated error. Thus, in some situations, although the estimates per point are different, it is possible this inference may not be correct, because the confidence intervals of the two estimates overlap, there is a possibility for them to be equal.

Another consideration is necessary. There is some variation beyond the expected exclusively due to the genetic recombination. There is evidence that the process of induction of DH by the anther culture may generate unexpected variability (HOFFMAN; THOMAS; WENZEL, 1982; NICHOLS; RUFTY, 1992). The methylation of the pollen grains has been suggested as the reason for this variation has been found (DEVAUX; KILIAN.; KLEINHOFS,1993; OAKELEY; JOAST, 1996; OAKELEY; PODESTÀ; JOST, 1997). This variability can produce both deleterious and favorable effects to selection (WERNSMAN, 1992; WITHERSPOON *et al.*, 1991; NICHOLS; RUFTY, 1992, MA, 2017).

Another alternative to evaluate the difference between the lines of different origins would be by comparing the means of CM and DH. In this case, CM exceeded the DH mean for YLD and ∑Z by 13%. For ALK, the opposite occurred, DH showed higher mean; however, the difference was small, 2.2% (TABLE 6). For YLD, it was verified that although there was overlap between the means of DH and CM, the DH means were concentrated at the lower end of the distribution, and the CM means, at the upper end (FIGURE 2).

At first, the reason why CM presented better performance than DH in terms of line means in relation to YLD can be questioned. A probable explanation would be the possible

effect of the sampling, i.e., the difference in favor of CM was due to the chance. However, considering that 190 DH lines and 194 CM lines were evaluated, the probability of sample error is small. Another explanation is the residue of heterozygosity in the CM lines. In generation $F_{6:7}$, the CM lines have 98.44% average of the loci in homozygosity and 1.56% heterozygosity. It shall be pointed out that the loci in heterozygosity contribute only to the higher estimate of the mean when there is dominance (RAMALHO *et al.,* 2012). There is evidence that the dominance in tobacco may occur; however, its contribution to the phenotype expression is way lower due to additive allele interaction, which is common in autogamous plants (BERNARDO, 2020; MATHER, 1949).

Another explanation is that the actions of the natural selection during the progress of the bulk generations would contribute to the increase of the general mean of the CM. There is evidence that the natural selections acts on the Bulk method, contributing to the increase in grain yield for some autogamous species (ALLARD, 1988; GONÇALVES; RAMALHO; ABREU, 2001). For tobacco, the leaf yield is the trait of interest, and in this case, the action of the natural selection can be questioned. Additionally, when conducting Bulk, the intention was to collect seeds from all plants, and then, to constitute the population of the next generation with the same number of seeds from each plant. Therefore, in this case, there is any effect of the natural selection of CM, or it is very low. It shall be pointed out that the occurrence of the natural selection effect in DH should not be unconsidered. This natural selection may occur during the growth and the development of the haploid and duplicated individuals. Although the environment in these stages is completely controlled, the most fragile plants hardly survive. The same happens upon transplantation of DH to the field, when many plants do not survive, or are eliminated by the artificial selection for being very fragile.

Finally, the difference between the performance of the lines from different origins may be related to the existence of preferential segregation in the process of obtaining DH lines, i.e., whether there would be restriction in the occurrence of some genotypes because of the process. For corn, there is information that in the genetic control of the inductor, some QTLs are involved, situated in different chromosomes (BARRET; BRINKMANN; BECKERT, 2008; PRIGGE *et al.* 2012). It appears that linkage and/or pleiotropy may occur between these genes, and consequently, restrict the occurrence of some genotype combinations. Although, in the use of anther culture, i.e., *in vitro*, there is no presence of inducer, the presence of genes involved in the induction of haploids individuals has been identified in this condition, which should also affect the growth and the development or not of the seedlings and further duplication. Thus, it is to be expected that there will be some difference in the segregation due to the linkage and/or

the pleiotropy between the genes involved in the capacity of the gametic cell to origin the haploid individual and/or in its duplication, with other genes of the plant, especially those controlling the traits of the highest agronomic or industrial interest.

Another way to assess the efficiency of DH and CM, which information is of most practical interest and obviously involves the variances and the means, is the estimation of the gain with selection. From the comments above, the estimates of the gain with selection for YLD and ∑Z was higher when involving the CM. Furthermore, among the selected best ten lines considering all lines regardless of the population, all of them came from CM. See that GS was 2.18 times bigger with CM in relation to the mean of all lines. For the estimated GS, using the ∑Z, the results showed the same YLD pattern, which was expected because the weight of this trait in the index was bigger. The opposite happened for ALK, but the difference in the GS estimates with DH in relation to CM was smaller.

It is worth pointing out that the advantages to use DH is the acceleration of the improvement process (ATLIN; CAIRNS; BISWANATH, 2017; FORSTER *et al.*, 2007), because it enables obtaining lines in a shorter period of time. For tobacco, where it is possible to have only one harvest per year, six years were necessary since the obtaining of F_1 to generate the evaluated conventional lines. On the other hand, only three years were necessary for the development and evaluation of the DH. Thus, the tobacco DH lines can be obtained by reducing the time twice in relation to CM. It was expected the annual gain to be higher with DH, since it presented higher estimates of V_G and h^2. However, this fact did not happen. The annual gain (GS_A) for YLD, with the selection of CM was 9.76% greater than that obtained for DH (TABLE 7).

From the exposed above, what are the conditions to use DH routinely in the RS programs? a) The conduction of experiments with higher number of DH. Question is, what would be that number? To answer this question, the number of DH line necessary to obtain one with equal performance to the best CM line can be estimated. To obtain this number, the properties of normal distribution can be used (STEEL; TORRIE; DICKEY, 1997). For this purpose, the data obtained in the experiments will be considered. The estimate of the genetic variance between DH ($\sigma^2_{G_H}$) was 3.42 for YLD (TABLE 5), i.e., 1.85 genetic standard deviation ($\sigma_{g_{DH}}$) and the mean was 16.65 kg/plot (TABLE 6). Assuming that it is desired to obtain at least one DH with the highest mean obtained by one of the CM, i.e., 23.71 Kg/plot, it is possible to estimate how many standard deviations above the average of the DH population would be necessary to achieve the performance of best CM. Using the expression: $z = \frac{(X_i - \bar{X})}{\sigma}$, where

X_i: mean of the best CM, 23.71 Kg/plot; $\bar{X}$: mean of the DH, 16.65 Kg/plot; and σ: standard deviation of the DH population, 1.85. The obtained value is z = 3.81 $\sigma_{g_{DH}}$ above the average. This way the range of variation of the distribution is then 7.62 (3.81x2). In this condition, the prediction of the number of lines to be evaluated should be approximately 10000 to have YLD similar to the best CM. Obviously, this number is prohibitive in most situations.

b) Promote the elimination of the lines with low vegetative development or other problems by using genomics before the field evaluations. Considering that the number of DH involved in the process shall be high, the cost for genotyping would be prohibitive. Additionally, it is necessary to prove the efficiency of using genomic selection in this case. Preliminary evaluation of DH lines could also be made in field conditions. At first, there would be no need to use experiments with repetition. It would be only visual selection. The DH lines selected would be more intensively evaluated in the next season, in experiments with repetitions in some locations with the greatest possible precision. The problem with this strategy is that another crop season would be necessary. However, it seems that the time spent can be compensated by the increasing of gain in RS.

5 CONCLUSIONS

The performance of DH in comparison to CM differed between the traits. For those related to chemical quality, ALK and SU, performance was similar. For YLD, the genetic variation estimates and h^2 among the DH were higher than those of CM.

For the mean estimate, the opposite occurred for YLD. Considering all lines, regardless of the population, the CM mean was 13.3% higher than those for DH. The annual gain with selection was greater for CM.

The use of DH in a recurrent selection program will be efficient in comparison to CM 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.

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