Evaluation of Doubled Haploid Derivatives of the Flue-cured *Nicotiana tabacum* cv. Wiślica*

by

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SUMMARY

A total of 51 doubled haploid (DH) derivatives of the flue-cured true-breeding tobacco variety Wiślica were obtained *via* anther culture and colchicine-induced chromosome doubling. Of that number eight DH lines showed morphological aberrations and/or reduced vigour that made them unfit to be grown in the field. The remaining 43 DH lines were grown in 2001 under regular flue-cured tobacco management and evaluated for principal growth indicators, yield, curability and gross money returns from unit area. In 2002, the evaluation was scaled down to include 13 best performing DH lines of 2001. The DH lines showed a considerable variation for basic growth parameters. Usually, the DH lines had more leaves but were shorter than the parental genotype mostly due to shorter internodes. The majority of DH lines showed delayed flowering compared to the parental cultivar. Yields of cured leaves varied extensively, some of the DH lines yielding above and some below cv. Wiślica. Curability measured as percentage of light (1st to 3rd) grades was generally lower in DH lines compared to that in cv. Wiślica. However, because of the high leaf yield some of the DH lines were not inferior to the parental cultivar with respect to money returns from unit area. [Beitr. Tabakforsch. Int. 22 (2006) 107–113]

ZUSAMMENFASSUNG

Doppelhaploide (DH) Abkömmlinge der reingezüchteten Virgin-Tabak-Sorte Wiślica wurden über Antherenkultur und Chromosomenverdoppelung durch Colchicin-Behandlung erhalten. Von den 51 erhaltenen dihaploiden Linien zeigten acht morphologische Aberrationen und/oder verringerte Vitalität; sie waren deshalb für Feldversuche ungeeignet. Die verbleibenden 43 DH-Linien wurden 2001 unter normalen Virgin-Tabak-Pflanzbedingungen angebaut

und ihre wichtigsten Wachstumsindikatoren, Ertrag, Trocknungsfähigkeit und der monetäre Ertrag pro Fläche bestimmt. 2002 wurden die 13 besten DH-Linien von 2001 bewertet. Die DH-Linien zeigten eine beträchtliche Variation der wichtigsten Wachstumsparameter. Gewöhnlich hatten die DH-Linien mehr Blätter, waren aber wegen kürzerer Internodien niedriger als der Ausgangs-Genotyp. Die meisten der DH-Linien zeigten verzögerte Blütezeit im Vergleich zur Eltern-Sorte. Der Ertrag an getrockneten Blättern variierte beträchtlich, einige Linien lagen über, andere unter dem Ertrag der Sorte Wiślica. Die Trocknungsfähigkeit gemessen in Prozentanteilen heller Grade war bei DH-Linien in der Regel geringer als bei Wiślica. Wegen der größeren Blattausbeute waren allerdings einige der DH-Linien hinsichtlich des monetären Ertrags der Elternsorte nicht unterlegen. [Beitr. Tabakforsch. Int. 22 (2006) 107–113]

RESUME

Cinquante et un dérivés de lignées d'haploïdes doublés (DH) de la variété Wislica (tabac flue-cured) ont été obtenus par culture d'anthers et doublement des chromosomes par la colchicine. Huit de ces lignées montrent des aberrations morphologiques et/ou une vigueur réduite qui empêchent leur culture en plein champ. Les autres quarante-trois lignées DH ont été cultivées en 2001 selon le mode de culture pour tabac flue-cured et évaluées pour leurs indicateurs principaux de croissance, rendement, aptitude au séchage et rentabilité par unité de surface. En 2002, l'évaluation a été réduite aux treize lignées DH ayant eu la meilleure performance en 2001. Les lignées DH montrent une variation considérable des paramètres de croissance fondamentaux. En général, les lignées DH ont plus de feuilles mais sont plus petites que le génotype parental en raison d'entre-noeuds plus courts. La plupart des lignées DH montrent une floraison plus tardive

par rapport au cultivar parental. Les rendements des feuilles séchées varient fortement, quelques-unes ayant des valeurs au-dessus et d'autres au-dessous de la variété cv. Wislica. L'aptitude au séchage mesurée en fonction des grades foliaires légers (premier au troisième) est généralement plus faible pour les lignées DH que chez cv. Wislica. Cependant, en raison d'un rendement important en feuilles, quelques-unes des lignées DH ne sont pas inférieures au cultivar parental du point de vue de la valeur économique par unité de surface. [Beitr. Tabakforsch. Int. 22 (2006) 107–113]

INTRODUCTION

The first tobacco haploids were produced from anthers *in vitro* in the late 1960's (11). At first hailed enthusiastically as a breakthrough for fast development of true-breeding lines, the anther-based method was later put into question as a viable breeder's tool because of the reported depressing effect on agronomic performance of anther-derived doubled haploid (DH) lines. Explanations that have been suggested to describe reduced performance of anther-derived dihaploids are residual heterozygosity in parental cultivars (6), genetic differentiation in the dihaploids due to changes in nuclear factors (2), mutagenic effects of the dihaploid technique (1) and amplification of DNA sequences during haploid formation (7,12,13). An alternative method was developed that was based on maternal plants selected in the offspring from crossing female *N. tabacum* by *N. africana* pollen (4). Though reportedly not involving any impairment of agronomic value, the latter method is not practical in cool climate since it requires high ambient temperatures during pollination for any significant recovery of maternal haploids (5). On the other hand, *in vitro* anther derived haploids and DHs can be easily produced in large quantities. The reports on the agronomic deterioration of anther derived DHs were not consistent (3,15) and the method has never been investigated in Poland for its applicability to Polish bred varieties of tobacco. Therefore, an attempt was made to produce DHs from domestic cultivar Wislica. The objective was to assess usability of the method of DH induction *via* anther culture to generate useful variation within the cultivar and to select improved breeding lines with the basic characteristics of Wislica.

MATERIALS AND METHODS

The parental cv. Wislica was developed at the Tobacco Production and Improvement Experimental Station at Skroniów, Poland, by J. Biskup. It was entered in the National Variety List in 1991 (8). Cv. Wislica is a true-breeding standard variety of flue-cured tobacco (*N. tabacum* L.) maintained by inbreeding over many years and showing minimal, if any, residual heterozygosity. A total of 51 fertile DH plants were obtained by means of culturing the anthers of cultivar Wislica by the method of NITSCH (11) and chromosome doubling of the resultant androgenic haploid plants by treating plantlets emerging from anthers with 0.25% colchicine. The plants were selfed and 51 completely homozygous true-breeding DH lines were obtained. Eight of the DH lines were unfit to be grown

in the field because of major growth disturbances and extensively altered morphology including a chlorophyll deficiency, cytogenetic instability or poor seed germination. Forty three DH lines and their parent cultivar Wislica were grown in the field in 2001. Based on the results from 2001 thirteen dihaploid lines were evaluated in 2002. Plot size was 10 m², plants were spaced at 90 × 40 cm, 25 plants per plot. All standard flue-cured tobacco management practices were applied except topping. The entries were grown in two randomized blocks.

The following records were taken during plant growth:

- ▶ days to flowering – number of days from transplanting to the appearance of the first flower (in 2001 and 2002)
- ▶ plant height – from the base of the plant to the tip of the uppermost flower at full bloom (in 2002 only)
- ▶ width and length of the midposition leaf (eighth leaf from the bottom of the plant, in 2002 only)
- ▶ number of leaves on a plant (in 2001 and in 2002).

For each of the above parameters means based on the measurements of 10 plants of each plot were used as plot values. The results are the means of two replications of plot values (two blocks of plots). Leaf area was calculated according to the formula: length of the leaf × width × 0.6345 (14) and internode length according to the formula: plant height/ number of leaves.

Cured leaves were weighed. Classification of the cured leaf was based on the grading system used in Poland involving grades 1 to 6, superior grades 1 to 3 are referred to as light grades because of the prevalent light color (yellow to orange) as opposed to darker (brown) color of the inferior grades 3 to 6. Money value of the crop was calculated based on prices current in 2002.

The results from 2001 were subjected to Analysis of Variance (ANOVA). The differences between means were examined for significance using Tukey's honestly significantly different (HSD) at $p = 0.05$. The 2002 data were examined for significance using Duncan's multiple range test and lines departing significantly from Wislica were assigned black bars in Figures 6 to 12.

RESULTS

Variation among doubled haploid lines in 2001

The DH lines generally closely resembled the parental cultivar Wislica. The exception were two lines: one was very slow growing with chlorophyll deficiency, and the other was slow growing and developing severe leaf spotting resembling bacterial leaf spots or severe weather fleck. Five DH lines showed increased susceptibility to injury by lygus bugs (*Lygus* sp.) as compared to cv. Wislica and the other DHs. There was an extensive variation among the DH lines in the duration of the period from transplanting to flowering (Figure 1). Thirty one of DH lines flowered later than cv. Wislica and twelve DHs flowered earlier than Wislica.

The wide range of variation in days to flower was not accompanied by much variability in leaf number (Figure 2). Generally, the average leaf number of DHs ranged from 22.2 to 26.8 with Wislica taking the intermediary position (24.9). Nineteen DH lines of Wislica had the leaf number above that of Wislica.

Figure 1. Days to flower of DH lines derived from cv. Wiślica. Grey bars represent number of DH lines (y-axis) within each interval with a corresponding midpoint value (x-axis); the dashed vertical line represents the days to flower of cv. Wiślica (75.5 days). Experimentation conducted in Puławy in 2001.

Figure 4. Percentage of superior grades (grade 1 to 3) of DH lines derived from cv. Wiślica. Grey bars represent numbers of DH lines (y-axis) within each yield interval with a corresponding midpoint value (x-axis); the dashed vertical line represents the value for cv. Wiślica (23.2 %). Experimentation conducted in Puławy in 2001.

Figure 2. Leaf number of DH lines derived from cv. Wiślica. Grey bars represent numbers of DH lines (y-axis) within each interval with a corresponding midpoint value (x-axis); the dashed vertical line represents the leaf number of cv. Wiślica (mean 24.9 of leaves). Experimentation conducted in Puławy in 2001.

Figure 5. Money returns (EUR/ha) from 1 ha of DH lines derived from cv. Wiślica. Grey bars represent numbers of DH lines (y-axis) within each yield interval with a corresponding midpoint value (x-axis); the dashed vertical line represents the money returns from Wiślica (2606 EUR/ha). Experimentation conducted in Puławy in 2001.

Figure 3. Cured leaf yields (t/ha) of DH lines derived from cv. Wiślica. Grey bars represent numbers of DH lines (y-axis) within each yield interval with a corresponding midpoint value (x-axis); the dashed vertical line represents the yield of cv. Wiślica (1.85 t/ha). Experimentation conducted in Puławy in 2001.

There was an extensive variation in the yields of cured leaves among the DHs in 2001 (Figure 3). The yields ranged from 1.20 to 2.62 t/ha with cv. Wiślica producing 1.85 t/ha. The number of DH lines that outyielded Wiślica (24) was consistently higher than the number of DHs that gave cured leaf yield lower than that of Wiślica (19). Five DHs were significantly superior to Wiślica and three were significantly inferior.

The percentage of superior grades (1st to 3rd) in the crop of cured leaves in the majority of DHs was lower than that in cv. Wiślica. However, the data showed large inconsistencies from block to block. In block one two DH lines had a percentage of superior grades higher than Wiślica, in block two only one DH was superior to Wiślica. Averaged over two blocks all DH lines showed lower percentage of superior grades compared to the parental cultivar (Figure 4).

Money value of the cured crop from one hectare is the combined effect of yield and crop quality. The DHs varied extensively for that trait and the differences were not

Table 1. Evaluation of thirteen DH lines and the parental cultivar Wiślica (values that share the same superscript are not significantly different at $p = 0.05$)

Genotype	Days to flower	Leaf number	Plant height (cm)*	Internode length (cm)	Area of mid-position leaf (cm ²)	Yields of cured leaves (t/ha)	Money returns (PLN/ha)
WDH118	68.70 ^{cdefghi}	26.60 ^{abocdef}	185	6.96 ^{cdefgh}	1198.73 ^{bocdefghijk}	2.72 ^{abcd}	6640.5 ^{abc}
WDH133	71.55 ^{abcd}	26.75 ^{abcd}	185	6.94 ^{cdefghi}	1321.81 ^{bocdefgh}	2.83 ^{ab}	7497.0 ^a
WDH134	68.35 ^{defghijk}	25.00 ^{cdefghijkl}	186	7.06 ^{cdefg}	1346.17 ^{abocdef}	2.62 ^{bocdefg}	5266.5 ^{bocdefgh}
WDH135	70.40 ^{bocdefg}	25.90 ^{abocdefghi}	194	7.53 ^{abc}	1545.11 ^a	3.08 ^a	6626.5 ^{abcd}
WDH137	66.45 ^{hijklm}	23.85 ^{hijklm}	186	7.84 ^a	1390.52 ^{ab}	2.16 ^{ghijklm}	4816.0 ^{cdefghijk}
WDH138	68.70 ^{cdefghij}	26.70 ^{abcde}	183	6.92 ^{cdefghij}	1287.21 ^{bocdefghij}	2.10 ^{ijklm}	3828.0 ^{hijklmn}
WDH140	69.40 ^{bocdefgh}	25.80 ^{abocdefghij}	183	7.13 ^{bocdef}	1317.76 ^{bocdefghi}	2.66 ^{abocdef}	6882.5 ^{ab}
WDH143	67.50 ^{efghijkl}	25.35 ^{abocdefghijk}	180	7.37 ^{abcd}	1378.15 ^{abc}	2.58 ^{bocdefghi}	5391.0 ^{bocdefg}
WDH145	70.85 ^{bocde}	26.05 ^{abocdefgh}	179	6.89 ^{cdefghijk}	1341.54 ^{abocdefg}	2.67 ^{abcde}	5025.5 ^{bocdefghij}
WDH148	73.00 ^{ab}	27.25 ^{abc}	176	6.49 ^{efghijkl}	1161.06 ^{cdefghijkl}	2.60 ^{bocdefgh}	6480.0 ^{abcde}
WDH151	74.95 ^a	27.55 ^a	173	6.32 ^{hijklm}	1146.17 ^{efghijklm}	2.43 ^{bocdefghijk}	4154.0 ^{efghijklm}
WDH153	70.70 ^{bocdef}	27.45 ^{ab}	177	6.48 ^{efghijklm}	1117.75 ^{hijklm}	2.41 ^{bocdefghijkl}	4697.5 ^{efghijkl}
WDH154	72.10 ^{abc}	26.15 ^{abocdefg}	190	7.29 ^{abcde}	1363.91 ^{abcde}	2.74 ^{abc}	5876.5 ^{abocdef}
Wiślica	66.60 ^{hijklm}	24.85 ^{defghijklm}	190	7.78 ^{ab}	1377.48 ^{abcd}	2.52 ^{bocdefghij}	5100.0 ^{bocdefghi}

^a No significant differences within the column.

Figure 6. Average days to flower of 13 dihaploid derivatives of cv. Wiślica. Experimentation conducted in Puławy in 2002. Black bars represent DH lines which are significantly different from Wiślica at $p = 0.05$ according to Duncan's multiple range test.

Figure 7. Average leaf number of 13 dihaploid derivatives of cv. Wiślica. Experimentation conducted in Puławy in 2002. Black bars represent DH lines which are significantly different from Wiślica at $p = 0.05$ according to Duncan's multiple range test.

always consistent (Figure 5). On average the money value ranged from 128 to 2990 EUR/ha with Wiślica giving average money returns of 2606 EUR. The majority of DH lines produced a lower revenue from 1 ha than did Wiślica and for 15 DHs the difference was statistically significant. Some DHs (six when averaged over two blocks) gave higher money returns than did Wiślica but in no case could the difference be proved statistically.

Performance of selected thirteen doubled haploid lines in 2002

In 2002, the growth indicators measured and calculated in 13 selected DH lines included: days to flower, leaf number, plant height, internode's length and leaf measurements. Yield of cured leaves and money returns were also studied (Table 1).

As in 2001, the majority of DH lines took more days to flower than did cultivar Wiślica. A lengthened period from

transplanting to flowering was a characteristic feature of all but one DHs. In seven DH lines average days to flower were significantly different from days to flower in Wiślica. Only WDH 137 and WDH 143 flowered on approximately the same day as did Wiślica (Figure 6). The majority of 13 DH lines studied had a higher leaf number than cv. Wiślica and only three DHs were significantly different from the parent cultivar (Figure 7). The majority of DH lines except WDH 135 were shorter than cv. Wiślica but in no case could the difference be proved statistically (Figure 8). Very characteristic of virtually all DHs except one (DH 137) was a shortening of internodes relative to cv. Wiślica (Figure 9) resulting in generally shorter plants. In case of eight lines the differences from the parameter in Wiślica were statistically significant. In the majority of DHs the eighth leaf was either shorter (in some of the DHs much shorter, WDH 151) or of the same length than that in cv. Wiślica. The same was true for leaf width except that the depressing effect involved an even larger number of DHs. As a result

Figure 8. Average plant height of 13 dihaploid derivatives of cv. Wiślica. Experimentation conducted in Puławy in 2002. There are no significant differences from Wiślica at $p = 0.05$ according to Duncan's multiple range test.

Figure 9. Average internode length of 13 dihaploid derivatives of cv. Wiślica. Experimentation conducted in Puławy in 2002. Black bars represent DH lines which are significantly different from Wiślica at $p = 0.05$ according to Duncan's multiple range test.

Figure 10. Average area of mid-position leaf of 13 dihaploid derivatives of cv. Wiślica. Experimentation conducted in Puławy in 2002. Black bars represent DH lines which are significantly different from Wiślica at $p = 0.05$ according to Duncan's multiple range test.

Figure 11. Average yields of cured leaves of 13 dihaploid derivatives of cv. Wiślica. Experimentation conducted in Puławy in 2002. Black bars represent DH lines which are significantly different from Wiślica at $p = 0.05$ according to Duncan's multiple range test.

Figure 12. Average money returns from unit area of 13 dihaploid derivatives of cv. Wiślica. Experimentation conducted in Puławy in 2002. Black bars represent DH lines which are significantly different from Wiślica at $p = 0.05$ according to Duncan's multiple range test.

the area of the eighth leaf in the eleven of DHs was reduced relative to the original cv. Wiślica. In lines WDH 153 and WDH 151 the departures from Wiślica were significant (Figure 10). Lines WDH 154, WDH 143 and WDH 137 were similar to cv. Wiślica in respect of leaf size. WDH 135 line was superior in that respect to Wiślica, but the difference was not significant.

In 2002, the majority of the 13 DHs gave a cured leaf yield similar to that of cv. Wiślica (Figure 11). WDH 135 line was the highest yielder and the difference was statistically significant from the yield of Wiślica. WDH 137 and WDH 138 gave appreciably lower yields than did cv. Wiślica. Estimates of money returns to the grower from the unit area varied among the DHs with five of the dihaploids performing worse and eight performing better than cv. Wiślica. In case of WDH 133 money returns were significantly better than those of Wiślica (Figure 12).

DISCUSSION

The results confirmed the high degree of variation generated by anther culture in DH derivatives of a true-breeding cultivar. Indeed, out of 51 DH lines produced from cv. Wiślica, eight were unfit to be grown under regular field culture, because of severely depressed vigour resulting from chlorophyll deficiencies and/or extensive leaf spots. The remaining 43 lines showed sufficiently good growth in the field to be assessed for agronomic performance. The results of this assessment agreed most closely with those reported by CHUNG *et al.* (5) concerning the increased number of leaves, delay in flowering and leaf narrowing as the most frequent manifestations of the effect of anther culture. Those results and ours were partly in disagreement with those of MCCONNELL and AYCOCK (9) who reported fewer leaves in DHs compared to the parental genotype. Delayed flowering and decreased leaf size resulting mainly from narrowing of the leaf are the most consistently reported adverse effects of anther culture. It was also the case in our study, although the depressing effect on leaf size could not always be related to the narrowing of the leaf lamina but, more frequently, to both leaf length and leaf width being smaller in DHs than in the parental cultivar. The DHs of cv. Wiślica had shorter internodes but at the same time were generally taller than the parental cultivar due to higher leaf number. Shortening of the internodes was the most consistently displayed morphological characteristic of those DHs and in that respect they differed sharply from those described by MCCONNELL and AYCOCK (9). It must be noted that the two respective studies were performed on different tobacco types and the differences could have been the reflection of DH performance being related to parental genotype, an observation also made by MCCONNELL and AYCOCK (9).

Chlorophyll deficiencies and, more notably, sharply increased susceptibility to lygus bugs found in some of the DH lines are indicative on mutation-generating potential of anther culture. This mutagenic potential of anther culture is explained by heterochromatin changes and DNA amplification in the course of haploid plant formation (7,13). Although the observed mutations were of clearly negative value in this particular case it need not necessarily always be the case, since WITHERSPOON (16) managed to find resistance to potato virus Y in an anther-derived DH line and NICHOLS and RUFFY (10) obtained resistance to *Phytophthora parasitica* var. *nicotianae* among anther-derived haploids.

Although yield depression was observed in a part of DHs investigated in this study some of the DH lines were found to be superior to cv. Wiślica in this respect. Indeed, the number of DHs outyielding Wiślica was higher than the number of DH outyielded by Wiślica. However, the above did not hold true with respect to curability. There was not a single DH to outperform cv. Wiślica in that respect and only two of them equaled their parent. That notwithstanding, some of the DH lines gave greater gross money returns than cv. Wiślica as the lower money value per kg was made up for by higher yields.

The results from the study of DH lines derived from cv. Wiślica indicated that anther culture generated substantial degree of variation. The large portion of the variation was clearly deleterious, especially the tendency of the DHs to be

late maturing and that related to negative impact on curability. On the other hand, some of the DHs showed vegetative vigour and yield potential surpassing that of Wiślica. Similar findings were reported by WALKER and AYCOCK (15) who worked with DH lines developed from an F_1 hybrid. Thus it can be concluded that given a sufficiently large number of DH lines to be selected from and well-designed selection procedures anther-produced DHs have a potential in breeding flue-cured tobacco in Poland.

CONCLUSIONS

- a) The most regular morphological effects observed in anther-derived DHs of cv. Wiślica were: extended period from transplanting to flowering, shortened internodes and decreased leaf size. Shortened internodes resulted in DH plants being mostly shorter than cv. Wiślica and the effect was not fully compensated by the tendency of the DHs to produce more leaves on the plant than did cv. Wiślica.
- b) Even though the leaves of the DHs were smaller than those of cv. Wiślica the performance of DHs in terms of the cured leaf yields generally was not adversely affected mostly due to higher number of leaves per plant in the DHs.
- c) Only two lines, DH 143 selected in 2001 and DH 135 selected in 2002, outperformed Wiślica with regard to some agronomical properties but the value of those lines must be confirmed in regular variety trials.
- d) The kind of genetic variation for agronomic traits generated by anther culture in cv. Wiślica indicates that it may be a useful breeding tool, but due account must be taken of the fact that it does produce a very large amount of undesirable variability, especially with regard to the rate of development (earliness) and also curability.
- e) In view of what was stated above using anther culture as a short-cut method to produce fully homozygous lines must involve a production of a very large number of DHs to be screened in the field for relatively rare agronomical superior genotypes.
- f) The best performing DH lines might be either used directly as true-breeding cultivars or might be used as components of hybrid cultivars.

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