

## Research Paper

# Morphological and chemical characteristics of doubled haploids of flue-cured tobacco combining resistance to *Thielaviopsis basicola* and TSWV

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*Thielaviopsis basicola* and *Tomato spotted wilt virus* (TSWV) are the most important problems in a moderate climate zone. Previously obtained doubled haploids (DH) of F<sub>1</sub> hybrids of the flue-cured line WGL3 resistant to *Th. basicola* and the dark-cured line PW-834 carrying *R<sub>TSW</sub>-al* gene provided the research material. Biological tests and SCAR markers linked with TSWV were applied to confirm resistance of DH. Lines combining resistance to TSWV and *Th. basicola* were evaluated for morphological and chemical characteristics. Most of DH were significantly shorter than parents but two lines, 31/A/2 and 31/B/3, were close to the flue-cured WGL3. Usually DH possessed fewer leaves while one of them 31/B/3, exceeded parental forms. The doubled haploids flowered later than their parents. The most negative effect was reduced area of mid-position leaves of DH. It might be explained by a recombination during microsporogenesis in F<sub>1</sub>, however the influence of ‘Polalta’-derived *R<sub>TSW</sub>-al* gene cannot be excluded. Extensive line to line variation for nicotine and sugars content was not associated with the genes for TSWV and *Th. basicola* resistance. Biological tests and field performance of DH revealed potential to overcome the negative effect of coupling between the *R<sub>TSW</sub>-al* gene and genes responsible for the morphological deformations.

**Key Words:** *Nicotiana tabacum*, doubled haploid, *Thielaviopsis basicola*, *Tomato spotted wilt virus*, SCAR markers, morphological characteristic.

## Introduction

*Tomato spotted wilt virus* (TSWV) is one of the most pathogenic and aggressive plant viruses. It attacks more than 900 species of plants belonging to 85 botanical families. The annual losses caused to crops worldwide by TSWV amount to a billion dollars (Adkins 2000). In Central Europe, Balkans, South Africa and some regions of North America, TSWV is the cause of one of the most severe diseases of tobacco. It causes chlorosis, necrosis of leaves, and dwarfing of plants. Fungal diseases, such as black root rot (BRR), are another very important problem of the tobacco cultivation in the countries with moderate climate. It is caused by the soil-borne fungal pathogen *Thielaviopsis basicola*, that inhabits the root system and contributes to a significant reduction in yields and in the quality of the raw material (Legg *et al.* 1981). The best way to control the presence of TSWV and *Th. basicola* is to introduce genetic resistance to cultivated tobacco. Many years of breeding allowed to obtain

only two cultivars resistant to TSWV, dark-cured type ‘Polalta’ and flue-cured type ‘Wiktoria’, showing *R-Avr* mediated defense response derived from *N. alata* (Gajos 1988, 1993). The resistance of ‘Wiktoria’ cultivar was unstable and shortly after being transferred into the tobacco genome it was broken by TSWV (Laskowska *et al.* 2013). Dark-cured type ‘Polalta’ was not cultivated because of poor agronomic performance, however it proved to be an excellent source of resistance to TSWV (Gajos 1993, Laskowska *et al.* 2013). Its hypersensitivity resistance (HR) is conditioned by a single dominant gene *R<sub>STV</sub>-al* (Laskowska *et al.* 2013, Yancheva 1990). The attempts to use ‘Polalta’ in classical breeding or to incorporate its resistance genes into flue-cured types of tobacco were unsuccessful. The F<sub>1</sub> hybrids of ‘Polalta’ and other tobacco genotypes had an irregular venation, tumors, malformations of leaves, and inflorescences, probably due to the impact of genetic material derived from *N. alata* (Moon and Nicholson 2007). Attempts were made to obtain lines which would be resistant to TSWV and free from morphological deformations by creating a homozygous forms by androgenesis of F<sub>1</sub> hybrids such as *N. tabacum* ‘K 326’ × ‘Polalta’ (Moon and Nicholson 2007) and ‘Wislica’ × ‘Polalta’ (Laskowska and Berbec

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2010). Moon and Nicholson (2007) observed a very depressed rate of haploid production. Furthermore, from the 23 haploids that they obtained, only 7 had TSWV resistance. Laskowska and Berbec (2010), obtained around 400 haploids but most of them, 98.8%, did not inherit resistance factor or showed numerous morphological abnormalities. The research of Laskowska and Berbec (2010) allowed three doubled haploids PW-833, PW-834, PW-900 to be selected which showed full resistance to TSWV and a reduced number of malformations. The disadvantage of these doubled haploids was a strong resemblance to the dark-cured cultivar 'Polalta'.

Trojak-Goluch *et al.* (2011) were ones of the authors who made attempts to extend the use of full genetic resistance to TSWV in breeding and to introduce it into the genome of flue-cured tobacco. The study also involved the breeding line WGL3 which carried the *N. glauca*-derived resistance to *Th. basicola* (Trojak-Goluch and Berbec 2009). Twenty four haploids which had the combined resistance to TSWV and *Th. basicola* were obtained from F<sub>1</sub> hybrids of the breeding lines PW-834 and WGL3. The plants showed some negative phenotypic changes. However, there were also haploids which did not show any deformations, which indicated their high suitability for commercial breeding. A study was undertaken to obtain unique, homozygous flue-cured lines with the resistance to TSWV and *Th. basicola* and which would answer the requirements of agricultural practice. There are many attempts of the induction of doubled haploids of tobacco. They are concerned mainly with the efficiency of regeneration of DH with resistance to different isolates of *Potato virus Y* (Czubacka and Doroszevska 2004, Šmalcelj and Čurković Perica 2000) and with resistance to *Th. basicola* (Trojak-Goluch and Berbec 2009). In the literature, there are only two reports on combining the resistance to *Tobacco mosaic virus* (TMV) and *Potato virus Y* (Burk and Chaplin 1980) as well as TMV, *Pseudomonas syringae* pv. *tabaci* and *Phytophthora parasitica* var. *nicotianae* (Walker and Aycock 1994). In most of these works, a prevailing view is that the technique of induction and regeneration of doubled haploids adversely affects the commercial traits of tobacco (Brown and Wersman 1982, Czubacka and Doroszevska 2004). However, the reports on the morphological and agronomic deterioration of anther derived DH are not consistent. There were reports of homozygous lines with improved morphological and biochemical traits (Trojak-Goluch and Berbec 2009) and with higher yields (Šmalcelj and Čurković Perica 2000, Walker and Aycock 1994) than their parental cultivars.

The aim of the first stage of the study was to evaluate the resistance of doubled haploids obtained from the hybrids of *N. tabacum* WGL3 × PW-834 to TSWV and *Th. basicola*. A further part of this work contains the analysis for selected commercial traits, the determination of the interaction of the resistance factors with commercial traits, and the selection of genotypes similar to the flue-cured form WGL3.

## Materials and Methods

### Plant material

The plant material was produced as a result of plant regeneration from stem pith fragments (data not published) of previously obtained haploids derived from PW-834 × WGL3 tobacco hybrids combining resistance to TSWV and *Th. basicola* (Trojak-Goluch *et al.* 2011). Regenerated plants verified as doubled haploids were grown in the greenhouse and then self-pollinated for seed production (data not published). A set of evaluated genotypes also comprised the parental forms: PW-834 an inbred line completely resistant to TSWV (Laskowska and Berbec 2010), and the line WGL3 with *glauca*-type resistance to *Th. basicola* (Trojak-Goluch and Berbec 2009). The research material also included the commercial Polish cultivar 'Wislica' susceptible to *Th. basicola* as well as to TSWV and 'Polalta', the standard of resistance to TSWV.

### Test for black root rot resistance

Fifteen doubled haploid lines, inbred lines PW-834, WGL3 as well as 'Wislica' were evaluated for black root rot resistance using the modified method of Samek and Jankowski (1987). Seeds were sown into steamed peat substrate and four-week-old seedlings were transferred into plastic pots (9 × 9 cm) filled with peat mix and vermiculite (3:1). Spores of *Th. basicola* in an amount of 10.000 spores per gram of growth substrate were added. The inoculum suspension was prepared from *Th. basicola* cultures as described by Trojak-Goluch and Berbec (2005). During the experiment plants were kept in a growth chamber at 20–22°C with a 16-h photoperiod at a light intensity of 65 μmol/m<sup>2</sup>/s. After 30 days plants were washed and then examined microscopically for the presence of *Th. basicola*. The DH lines, parental lines and 'Wislica' were represented by thirty plants each.

### Tests for Tomato spotted wilt virus resistance

Screening for TSWV resistance was conducted in the greenhouse conditions. Inoculum was prepared from plants that grew on a tobacco plantation (south-eastern part of Poland) and showed systemic infection of TSWV. The presence and purity of the TSWV isolate were assessed serologically. Then apical leaves (25 g) were ground in 50 ml phosphate buffer (9.078 g/l KH<sub>2</sub>PO<sub>4</sub> and 11.867 g/l Na<sub>2</sub>HPO<sub>4</sub>) with an addition of an anti-oxidant 0.5% mercapthoethanol and 1% carborundum (400 mesh) (Tsakiridis and Gooding 1972). Mechanical inoculations of plants were made at the six leaf stage. Plants were kept in a greenhouse at 20–25°C/day and 16–18°C/night temperature. After one to three weeks observations of disease symptoms were performed. Subsequently, the plants were subjected to DAS-ELISA using polyclonal antiserum (BIOREBA AG, Switzerland) (Clark and Adams 1977). Absorbance index of the samples was measured at 405 nm (Sunrise, Tecan, Austria). Cultivar 'Wislica' was used as a positive control while 'Polalta' was

a negative control. The susceptibility of thirty plants of each genotype was assessed.

### Molecular analysis using SCAR markers

For genomic DNA isolation, 100 mg of young leaves were collected and squeezed using CTAB-buffer, followed by the method described by Czubacka and Doroszewska (2010). DNA was amplified by polymerase chain reaction (PCR). The ACT/CTA268 AFLP SCAR marker previously determined to be closely associated in coupling with the *N. alata*-derived gene for resistance to TSWV present in cultivar 'Polalta' (Moon and Nicholson 2007) was used in this study. The primer pairs for ACT/CTA268 markers (F: 5'CTGATCGTTCCAGCAGGTTCTTAT3', R: 5'GGAGCTATTCCAGACACGAA3') generated two amplified products (161 and 200 bp) in genotypes carrying the TSWV resistance gene from 'Polalta' and one product (200 bp) in genotypes that do not possess that gene. The PCR reaction was carried out in 20 µl of mixture which contained 20 ng plant DNA, 1.9 units of Taq polymerase (Fermentas) and 4.5 pmol of primers, 1.5× PCR buffer, 2 mM MgCl<sub>2</sub>, 312.5 µM dNTPs. Amplification was carried out for 2 min at 94°C, followed by 30 cycles 30 s at 94°C, 30 s at 55°C, 40 s at 72°C and a final elongation step for 5 min at 72°C. Electrophoresis of the amplification products was conducted in 2% agarose gels in 1× TBE buffer (100 mM Tris, 90 mM H<sub>3</sub>BO<sub>3</sub>, 1 mM EDTA, pH 8.5) at 7–10 V/cm.

### Agronomic characteristics of doubled haploid lines

Fifteen DH lines that are TSWV resistant and *Th. basicola* were grown in 2013 in a one-year field experiment. The experiment was in a randomized complete block design with three replications. Each genotype was grown in a 40.5 m<sup>2</sup> plot containing four 25-plants rows per replication. Plants were spaced 90 × 45 cm between rows and plants. Cultural practices recommended for flue-cured tobacco were applied and inflorescences were left on the stems. First, plants were rated for morphological deformation using an ordinal scale as follows: 0 = no symptoms, 1 = thickened veins of leaves, 2 = non-parallel and thickened veins, 3 = wide, irregular and thickened veins, 4 = wide, irregular venation and narrow leaves, 5 = wide, irregular nerves and ribbon-shaped leaves, 6 = irregular venation and tumors on blossoms. Nine doubled haploid lines exhibiting none or slight phenotypic deformations <1 were assessed of functional characteristics. Plant height, number of leaves per plant, number of days from transplanting to beginning of blooming, length, width and area of midstalk leaves (i.e. the 8th to 10th leaf on the stalk), calculated according to the formula: length of the leaf × width × 0.6345 (Suggs *et al.* 1960), were recorded. Midstalk leaves were also analyzed for weight of 1 dm<sup>2</sup> leaf blade and chemical characteristics. The parental line WGL3, the male parent of the good quality cultivar 'Wigola' provided standard references of flue-cured type.

### Chemical compositions of midstalk leaves

Leaves were manually reaped at the phase of technological maturity and dried in automated curing barn. Six grams of dried leaves were ground in a grinder then extraction was performed in methyl-tetra-butyl ether (MTBE) and the content of nicotine was determined. A gas-chromatograph Agilent 5975C (Series MSD) with Triple-Axis Detector coupled with mass spectrometer Agilent 7890A was used. Helium was the carrier gas with a non-polar, capillary column HP-5MS Agilent Technologies 30 m × 0.25 mm × 0.25 micron. The quantity of the reducing sugars was analyzed by Bertrand's standard procedure (PB 53.1-ed. II 01.08.2013). The results obtained for DH, PW-834 and WGL3 were expressed as a percentage of the dry matter.

### Statistical analysis

For morphological characteristics ten randomly selected plants were measured and the mean was treated as plot value. Weight of 1 dm<sup>2</sup> leaf blade and chemical traits of leaves were analyzed in mixed samples which represented plants on a plot. Means and variances of doubled haploid lines and parental forms PW-834 and WGL3 were analyzed with the Statgraphics Centurion software, version XV, StatPoint, Inc. Herdon, Virginia, USA. Fisher's least significant difference at  $P \leq 0.05$  was used to compare parameters of morphology and chemicals characters of leaves.

## Results

In the greenhouse resistance test, all DH lines showed a hypersensitive reaction (HR) to inoculation with TSWV. After seven days, the infected leaves developed small, necrotic spots of 2–3 millimeter diameter, similar to those developed by the resistance donor PW-834. The WGL3 line showed systemic infection (S); severe leaf chlorosis, necrotic spots, distortions of the upper leaves as well as yellowing and bending of shoot apex. Plants with HR symptoms, parental lines PW-834 and WGL3, 'Polalta' as well as 'Wislica' were subjected to ELISA tests with antiserum against TSWV. The presence of the virus was detectable only in WGL3 and in the positive control 'Wislica' showing ELISA absorbance values  $A_{405}$  from 2.091 to 3.740. In the case of DH and PW-834 lines, lower absorbance values (from 0.092 to 0.137) at a level comparable to that in 'Polalta' and the negative result of the biological tests showed the absence of virus (Table 1). The PCR electrophoresis performed using two primers for the ACT/CTA268 SCAR marker amplified two products (161 and 200 bp) in all DH lines, resistant tobacco 'Polalta' and PW-834 line (Fig. 1). Gel analysis of PCR products synthesized with DNA from 'Wislica' and paternal line WGL3 revealed the presence of only one band (200 bp) indicating that there are no markers associated with TSWV resistance.

The first symptoms of BRR appeared among the susceptible genotypes 'Wislica', 'Polalta' and PW-834 two weeks after inoculation. These symptoms included wilting of

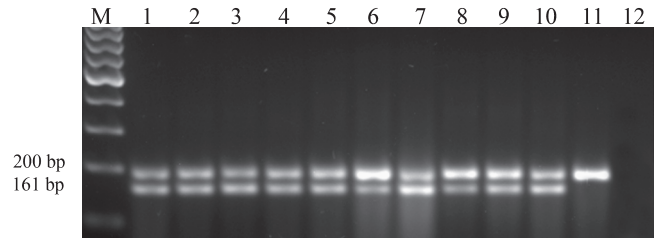


**Table 1.** Resistance to TSWV and *Th. basicola* and the degree of deformation in doubled haploids derived from *N. tabacum* L. WGL3 × PW-834

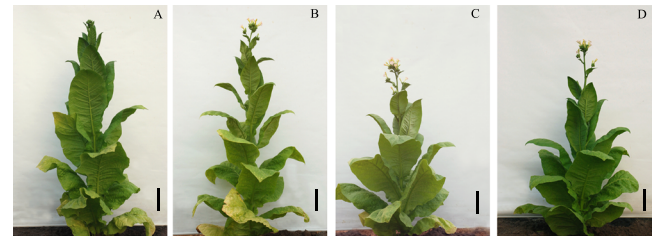
Line/cultivar	Number of plants		Degree of deformation*
	Resistant to <i>Th. basicola</i>	Resistant to TSWV	
31/A/2	30	30	0.08
31/A/6	30	30	0.01
31/A/8	30	30	1.18
31/B/3	30	30	0.18
31/B/5	30	30	1.1
31/B/6	30	30	1.5
31/C/14	30	30	0.07
31/C/33	30	30	0.14
33/9/1	30	30	1.3
33/9/8	30	30	1.2
33/9/9	30	30	0.11
34/B/2	30	30	1.2
34/8/29	30	30	0.02
34/10/10	30	30	0.16
34/10/23	30	30	0.61
WGL3	30	0	0.0
PW-834	0	30	2.1
Wislica	0	0	0.0
Polalta	0	30	4.3

\* - Average score on 0–6 scale.

leaves, chlorosis, and growth reduction. The roots developed necrotic lesions which were typical of the infection. Microscopic observations revealed the presence of hyphae and many chlamydo spores on the surface of the roots. DH lines and the parental form of WGL3 did not show any symptoms of the infection. There were no pathogen hyphae nor chlamydo spores during microscopic observation. All tested DH lines were regarded as resistant to *Th. basicola* (Table 1). Nine out of fifteen doubled haploids exhibiting no or slight phenotypic deformation <1 and the resistance to TSWV and *Th. basicola* were evaluated for agronomic performance. Some lines 31/A/2, 31/B/3 (Fig. 2B), 34/8/29 resembled the parental form WGL3 in terms of plant habit, structure and color of leaves. Several doubled haploids



**Fig. 1.** PCR analysis showing amplification products generated by SCAR marker S-ACT/CTA268 linked with TSWV resistance gene. Lane M 100-bp ladder; lane 1–9 resistant doubled haploids derived from *N. tabacum* WGL3 × PW-834 F<sub>1</sub> hybrids; lane 10 resistant cultivar ‘Polalta’; lane 11 susceptible WGL3; lane 12 negative control (water).



**Fig. 2.** Plant habit of doubled haploids derived from *N. tabacum* WGL3 × PW-834 F<sub>1</sub> hybrids; (A) maternal line WGL3, (B–C) DH lines and (D) paternal line PW-834, bar = 14 cm.

34/10/10 (Fig. 2C), 31/C/14, 33/9/9 showed similarity to PW-834 (Fig. 2D). These lines had a slow rate of growth, the leaves had flat surface and dark green color. Other DH showed, however, intermediate traits which deviated from the phenotype of the parental forms. There was an extensive line-to-line variation in terms of morphological characters (Table 2). The average height of plants ranged between 84.9 and 135.3 cm. The line 34/10/23 was the shortest of the studied, while 31/B/3 the tallest. There was a tendency to reduce the height among doubled haploids compared to parental forms. Seven out of nine studied DH were significantly shorter compared to the model parental line WGL3. In the field experiment, doubled haploids differed in terms of a

**Table 2.** Analysis of agronomic characters of the doubled haploids derived from *N. tabacum* L. WGL3 × PW-834 hybrids and their parental forms WGL3, PW-834

Line	Plant height (cm)	Leaf number	Midstalk leaf		
			Length (cm)	Width (cm)	Area (cm <sup>2</sup> )
31/A/2	134.9 ± 2.07 <sup>ef</sup>	17.6 ± 1.90 <sup>bcd</sup>	46.6 ± 1.25 <sup>d</sup>	29.1 ± 0.55 <sup>e</sup>	860.2 ± 39.23 <sup>de</sup>
31/A/6	117.4 ± 2.96 <sup>d</sup>	15.5 ± 2.29 <sup>a</sup>	43.7 ± 1.40 <sup>bcd</sup>	27.6 ± 1.01 <sup>de</sup>	764.4 ± 50.79 <sup>c</sup>
31/B/3	135.3 ± 2.72 <sup>ef</sup>	19.8 ± 0.96 <sup>f</sup>	46.1 ± 0.68 <sup>d</sup>	26.4 ± 0.51 <sup>cd</sup>	772.4 ± 19.59 <sup>cd</sup>
31/C/14	114.1 ± 7.61 <sup>cd</sup>	16.8 ± 1.17 <sup>abc</sup>	44.3 ± 5.60 <sup>cd</sup>	25.3 ± 0.83 <sup>bc</sup>	711.9 ± 111.17 <sup>bc</sup>
31/C/33	96.0 ± 3.39 <sup>b</sup>	17.5 ± 0.45 <sup>bcd</sup>	40.2 ± 1.85 <sup>ab</sup>	22.6 ± 0.11 <sup>a</sup>	576.1 ± 26.53 <sup>a</sup>
33/9/9	107.8 ± 2.31 <sup>c</sup>	18.2 ± 0.26 <sup>cde</sup>	39.9 ± 1.23 <sup>ab</sup>	28.0 ± 1.49 <sup>de</sup>	709.6 ± 60.23 <sup>bc</sup>
34/8/29	127.1 ± 4.69 <sup>e</sup>	16.6 ± 0.43 <sup>ab</sup>	41.2 ± 1.20 <sup>abc</sup>	23.7 ± 1.95 <sup>ab</sup>	619.4 ± 68.48 <sup>ab</sup>
34/10/10	105.8 ± 13.74 <sup>c</sup>	16.7 ± 0.75 <sup>ab</sup>	42.9 ± 1.75 <sup>bcd</sup>	28.2 ± 1.16 <sup>de</sup>	769.2 ± 63.11 <sup>cd</sup>
34/10/23	84.9 ± 2.90 <sup>a</sup>	17.0 ± 1.08 <sup>bcd</sup>	37.7 ± 1.92 <sup>a</sup>	22.4 ± 1.18 <sup>a</sup>	563.3 ± 52.48 <sup>a</sup>
WGL3	143.6 ± 2.40 <sup>f</sup>	19.2 ± 0.10 <sup>ef</sup>	46.6 ± 1.07 <sup>d</sup>	30.9 ± 1.10 <sup>f</sup>	913.8 ± 49.76 <sup>e</sup>
PW-834	116.7 ± 3.44 <sup>d</sup>	18.3 ± 0.92 <sup>de</sup>	46.1 ± 3.43 <sup>d</sup>	27.5 ± 1.06 <sup>de</sup>	802.4 ± 74.72 <sup>cd</sup>

Data represents mean ± SD values of three replicates.

<sup>a-f</sup> Values within a column, means followed by different letters differ significantly based on Fisher’s protected LSD test (P ≤ 0.05).

**Table 3.** Number of days to flower, weight of leaf blade and main chemical components of the doubled haploids derived from *N. tabacum* L. WGL3 × PW-834 hybrids and their parental forms WGL3, PW-834

Line	Days to flower	Weight of 1dm <sup>2</sup> leaf blade	Nicotine (%)	Sugars (%)
31/A/2	78.13 ± 2.25 <sup>ab</sup>	0.84 ± 0.03 <sup>f</sup>	2.09 ± 0.31 <sup>b</sup>	24.00 ± 2.30 <sup>d</sup>
31/A/6	75.93 ± 0.90 <sup>a</sup>	0.70 ± 0.02 <sup>d</sup>	3.07 ± 0.25 <sup>d</sup>	18.10 ± 0.26 <sup>c</sup>
31/B/3	82.36 ± 0.57 <sup>def</sup>	0.81 ± 0.04 <sup>ef</sup>	2.66 ± 0.21 <sup>c</sup>	13.83 ± 1.10 <sup>ab</sup>
31/C/14	80.24 ± 1.53 <sup>bcd</sup>	0.71 ± 0.05 <sup>d</sup>	1.96 ± 0.22 <sup>b</sup>	15.64 ± 0.53 <sup>b</sup>
31/C/33	85.53 ± 0.99 <sup>g</sup>	0.78 ± 0.02 <sup>e</sup>	1.85 ± 0.05 <sup>ab</sup>	15.82 ± 1.19 <sup>b</sup>
33/9/9	83.95 ± 0.58 <sup>fg</sup>	0.66 ± 0.01 <sup>cd</sup>	2.41 ± 0.09 <sup>c</sup>	14.80 ± 1.25 <sup>ab</sup>
34/8/29	80.93 ± 0.11 <sup>cde</sup>	0.61 ± 0.02 <sup>abc</sup>	1.90 ± 0.15 <sup>ab</sup>	15.73 ± 1.94 <sup>b</sup>
34/10/10	82.63 ± 0.16 <sup>ef</sup>	0.60 ± 0.02 <sup>ab</sup>	2.05 ± 0.13 <sup>b</sup>	14.13 ± 1.27 <sup>ab</sup>
34/10/23	76.43 ± 1.89 <sup>a</sup>	0.56 ± 0.04 <sup>a</sup>	1.99 ± 0.10 <sup>b</sup>	15.13 ± 1.21 <sup>ab</sup>
WGL3	80.15 ± 1.02 <sup>bc</sup>	0.85 ± 0.01 <sup>f</sup>	1.62 ± 0.03 <sup>a</sup>	24.03 ± 0.71 <sup>d</sup>
PW-834	76.06 ± 1.28 <sup>a</sup>	0.64 ± 0.03 <sup>bc</sup>	3.64 ± 0.25 <sup>e</sup>	13.27 ± 0.78 <sup>a</sup>

Data represents mean ± SD values of three replicates.

<sup>a-f</sup> Values within a column, means followed by different letters differ significantly based on Fisher's protected LSD test ( $P \leq 0.05$ ).

number of leaves per plant, which amounted from 15.5 to 19.8. The number of leaves in DH lines was lower in comparison with that in the parental forms, which was the most regularly occurring relationship in doubled haploids vs. their parents. Seven genotypes produced fewer leaves than the model line WGL3 with the exception of 31/B/3 line, which exceeded WGL3 for that trait. The obtained difference stayed within the statistical error. The analysis of variation showed statistically significant difference in the size of midstalk leaves among the DH lines. The average leaf length ranged from 37.7 to 46.6 cm. The shortest leaves were recorded for 34/10/23 line. Lines 31/C/14, 31/A/6, 34/10/10 had a satisfactory length of leaves. Of special interest are two lines, 31/A/2 and 31/B/3, whose mean value of the studied trait reached the level recorded for WGL3. Narrowing of leaves was a characteristic feature of the doubled haploids which combined resistance to TSWV and *Th. basicola*. All tested DH lines had significantly narrower leaves than WGL3 line. As a result, estimated leaf area of DH was smaller than that of parental forms. The exception was the 31/A/2 line which leaf area were similar to that of WGL3. The analysis of weight of 1 dm<sup>2</sup> of cured leaf blade showed significant differences between doubled haploid lines derived from haploids PW-834 × WGL3 (Table 3). The results also indicated that most of doubled haploids produced significantly lighter leaves than the reference line WGL3, but 31/A/2 and 31/B/3 were near to the parental form WGL3. The analyzed doubled haploids differed also in terms of the duration of the vegetative phase compared to WGL3. Four lines flowered significantly later than WGL3 while three lines 31/A/2, 31/C/14, 34/8/29 reached blooming in the period similar to the reference line.

Chemical analysis of cured leaves from DH revealed that nicotine content was diverse and there was a significant line to line variation (Table 3). The highest content of nicotine, which is characteristic for dark-cured tobacco, was recorded for line 31/A/6 (3.07%). The average nicotine level of the

abovementioned line was, however, significantly lower compared to PW-834 (3.64%). In comparison with WGL3 line, all the DH lines had a higher nicotine content, and only in two cases, 31/C/33 and 34/8/29, the differences were statistically insignificant. The content of carbohydrates in DH lines was generally intermediate between the parents. The analysis of differences between WGL3 line and the population of doubled haploids did not show a significant impact of the maternal form on the content of sugars. Eight genotypes showed significantly lower level of carbohydrates in leaves. Only one doubled haploid 31/A/2 showed statistically similar to WGL3 content of sugars.

## Discussion

Anther culture and stem tissue culture method proved to be extremely effective in obtaining doubled haploid of tobacco combining resistance to TSWV and *Th. basicola*. These techniques did not cause the loss of *RTSW-al* gene, or the modification in its expression, as the trait of resistance to TSWV was well expressed in doubled haploids of the R<sub>1</sub> generation. All the plants showed the hypersensitive response similar to that of 'Polalta'. There were no systemic hypersensitive reaction SHR that sometimes occurs among plants that possess R-resistance gene e.g. in *N. alata* var. *alba*, *Nicotiana* × *sanderae*, *N. forgetiana* (Laskowska *et al.* 2013). The results of biological tests and DAS ELISA corresponded with the results of SCAR marker identification ACT/CTA268, strictly connected with the gene of resistance to TSWV. The subject of these studies was the evaluation of the resistance of DH lines to *Th. basicola*. The molecular markers linked with *Th. basicola* resistance derived from *N. glauca* have not yet been developed so the disease assessment included artificial inoculation-based tests. Greenhouse tests revealed that all DH lines showed resistance equal to that observed in *N. glauca*-derived resistant line WGL3. The gene of resistance to *Th. basicola* proved to be highly effective, as it was expressed in homozygous DH generation.

The present study showed a significant variability of morphological characteristics among DH lines. This phenotypic diversity resulted from the segregation of gametes in the F<sub>1</sub> hybrids of PW-834 × WGL3. It could also be the result of amplification of selected DNA sequences, which occurs during chromosome doubling of haploids (Reed and Wernsman 1989). There was no phenotypic variability within individual lines. DH were uniform and genetically stable. According to Schnell and Wernsman (1986) anther culture as well as plant regeneration from stem-derived callus may significantly modify the morphological characteristics within individual doubled haploid lines. Intra-line somaclonal variation was observed in DH obtained from a single plant of cultivar 'NC 95'. As reported, significant variance was detected for six of seven characters examined in the 'NC 95' DH population.

The present study showed significant differences in the

height of plant between DH lines and the reference parental line. The majority of the studied genotypes were lower than WGL3. The reduction in plant height might be explained by heterochromatin changes, DNA amplification during anther derived DH production (Reed and Wernsman 1989) and heterozygosity of the anther donors (Brown *et al.* 1983), however the influence of 'Polalta'-derived *RTSW-al* gene cannot be excluded. According to Burk and Chaplin (1980) the gene for PVY resistance appeared to be associated with reduced plant height of doubled haploids obtained from *N. tabacum* 'Coker 86' × 'VY 32' F<sub>1</sub> hybrid. The negative impact of *glauca*-type resistance gene on plant height is rather unlikely. Trojak-Goluch and Berbeć (2009) analyzed the impact of the gene of resistance to BRR on the agronomic features of WGL3 line. The studies have shown that plant height was not affected by *glauca*-type resistance. In turn, Legg *et al.* (1981) reported that homozygous breeding lines BC<sub>7</sub>S<sub>2</sub> of dark-cured (Burley) tobacco with *debneyi*-type resistance for BRR exceeded parental cultivars and susceptible counterparts for plant height. In this study, all DH lines produced fewer leaves than their parental forms. Burk and Chaplin (1980) studied doubled haploids obtained from the hybrids of *N. tabacum* 'Coker 86' and 'VY 32'. DH lines with combined resistance to TMV and PVY produced significantly fewer leaves compared with their parental forms and with the lines susceptible to both viruses. Walker and Aycock (1994) encountered a completely different phenomenon. They described doubled haploids of F<sub>1</sub> 'MD 609' × 'MD 341' with the combined resistance to TMV, wildfire, and black shank which did not significantly differ in terms of a number of leaves from their parental forms. In view of the above results the number of leaves in doubled haploids did not appear to be strongly related to the resistance factors and was probably the effect of mutations during anther culture or chromosome doubling process.

Reducing the size of leaves is one of the common features of tobacco doubled haploids. The narrowing of leaf blades and the simultaneous decrease of yields of DH lines in relation the parental forms occurred both in air-cured, dark-cured as well as in flue-cured tobacco (Brown and Wernsman 1982, Burk and Chaplin 1980, Kasperbauer *et al.* 1983). In this study all doubled haploid lines had shorter and narrower leaves than the reference line WGL3. Smaller dimensions of leaves may be result of negative impact of PW-834 genotype, which normally developed smaller leaves than WGL3. However, there were five lines that produced smaller leaves than both parental forms. The adverse effect on the leaf dimensions can be attributed to additive, dominant, epistatic effect or to the presence of TSWV and *Th. basicola* resistance genes. This finding seems to be correct in the light of the results presented by Legg *et al.* 1981. They noticed that incorporation of BRR *debneyi*-type resistance into isogenic lines of eight (Burley) tobacco cultivars was associated with shorter leaves and fewer yields. Similarly, Burk and Chaplin (1980) concluded that doubled haploid lines combining resistance to TMV and PVY produced

narrow leaves and yielded less than parental cultivars or their susceptible counterparts.

Delayed flowering, as compared to the parental forms, is a frequently encountered phenomenon. In the present study one line 31/A/6 flowered earlier than parental forms. In two cases, the length of plant growing stage was a derivative of flowering term of lines PW-834 and WGL3, while six lines flowered later than the initial forms. Delayed flowering of DH is similar as in other reports. Walker and Aycock (1994) report that DH of Maryland tobacco combining resistance to virus and bacterial pathogens flowered later (6%) than parental 'MD 609' and 'MD 341'. Extended period from transplanting to flowering was also reported by Legg *et al.* (1981) in the case of homozygous isogenic lines of Burley tobacco carrying *debneyi*-type resistance for BRR.

Our results showed significant interlinear differences in terms of nicotine content (from 1.86 to 3.07%). The observed variability is probably connected with the use of parental lines belonging to different functional type. The group of flue-cured tobacco, which includes parental line WGL3, had the nicotine content of 1 to 2%, while dark-cured tobacco, which includes maternal line PW-834, had a high nicotine content of 2–3.5%. It seems that combining the resistance to TSWV and *Th. basicola* did not have a significant impact on nicotine content, as there are no records of any doubled haploids with chemical parameters different from their parents. Other authors, studying quality of raw material of DH showed that total alkaloids content was similar to that of mid-parent value (Walker and Aycock 1994). Similarly, Trojak-Goluch and Berbeć (2009) tested WGL3 breeding line carrying gene for BRR resistance and found that *glauca*-type resistance did not affect the level of nicotine. Sugar content is an important indicator of the quality of raw flue-cured tobacco. Burk and Chaplin (1980) report that the homozygous forms showing resistance to TMV and PVY had significantly lower contents of sugars than their susceptible counterparts or their parental cultivars. Low level of sugars was attributed to the TMV and PVY resistance genes. The doubled haploids which are presented in this work showed significant variability in terms of sugar content. The observed variability resulted from combining different commercial types of tobacco rather than from the impact of the genes of resistance to TSWV or *Th. basicola*.

The results of the research are doubled haploids 31/A/2 and 31/B/3, which are morphologically and chemically close to the flue-cured type, while at the same time being resistant to TSWV and *Th. basicola*. The results of this work also indicate the potential of breaking the so-far negative dependency between *RSTV-al* resistance gene and the gene/s responsible for the occurrence of malformations of tobacco. The probable cause of breaking of linkage drag effects associated with TSWV resistance was crossing over during microsporogenesis in Polalta × Wislica (Laskowska and Berbeć 2010), as well as in PW-834 × WGL3 hybrids. The authors do not exclude the possibility that in plants with the normal morphology, there was a mutation in these

genes. Izard (1957), Smith and Stevenson (1961) induced mutation in the genes responsible for the formation of morphological deformation to obtain interspecific hybrids of *Nicotiana* of nearly correct morphology.

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