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Resistance to black root rot (*Chalara elegans* Nag. Raj and Kendrick) and some growth characteristics in doubled haploid derivatives of the F1 hybrid of tobacco (*Nicotiana tabacum* L.)

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Abstract. Black root rot (BRR), caused by *Chalara elegans*, is a widespread and severe disease of tobacco (*Nicotiana tabacum* L.). Anther culture and stem culture techniques were used to produce the haploids and doubled haploids (DHR_0) lines of F_1 hybrids of *N. tabacum* cv. 'K 326' susceptible to BRR and cv. 'Wentura', a male parent of the widely cultivated BRR-resistant hybrid 'VRG 2'. Twenty four mature DHR_0 plants were self-pollinated and their derivatives (DHR_1) were screened for BRR resistance in greenhouse tests. From a total of 24 DHR_1 lines, seven (29.2%) had no necrotic symptoms while only five of them (20.8%) were completely resistant. In a one-year study growth indicators and developmental characteristics of five resistant DHR_1 were compared to those of the parental cultivars in order to select BRR resistant lines with morphological traits of the reputable cv. 'K326'. The DHR_1 lines showed a considerable variation for basic growth parameters. Usually DHR_1 flowered later than cv. 'K 326' and 'Wentura'. Three DHR_1 lines had leaf number similar to cv. 'Wentura' while $DHR_1$28 line produced the number of leaves close to that of cv. 'K 326'. Most of the DHR_1 lines produced the tallest plants compared to the parental cultivars. Significant variation was observed among DHR_1 for mid-stalk leaf parameters. Two lines DHR_1 168 and $DHR_1$28 had leaves of length similar to that in 'K 326', however line DHR_1 168 had wider leaves than cv. 'K 326'. Finally one line $DHR_1$28 was selected as initial material for further breeding.

key words: *Nicotiana tabacum, Nicotiana debneyi,* doubled haploids, black root rot, resistance

INTRODUCTION

Black root rot (BRR) caused by *Chalara elegans* (Nag Raj and Kendrick) syn. *Thielaviopsis basicola* (Berk. and Broome) Ferraris causes significant damage in many tobacco (*Nicotiana tabacum* L.) growing regions of the world. Several Virginia type tobacco cultivars as: Canadian 'AC Gayed' and 'CT 681' (Haji, Brandle, 2001), 'ITB 3304' or other French cultivars (Julio et al., 2006), American 'KY 14' (Wilkinson et al., 1991) and the Polish breeding cultivar 'Wentura' (Berbeć, Trojak-Goluch, 2004) are resistant to black root rot. Resistance is controlled by a single dominant gene derived from *N. debneyi* (Wilkinson et al., 1991) and is linked to several quality-compromising traits (Legg et al., 1981). Those linkages do not seem to be unbreakable as indicated by recent releases of cultivars that combine *N. debneyi*-type resistance with acceptable quality in Canada (Haji, Brandle, 2001) and in Europe. Also in Poland several flue-cured cultivars have been developed that combine resistance to black root rot with acceptable agronomic traits and suitability to the exigencies of climate in Poland (Berbeć, 2008).

The improvement of tobacco cultivars can be achieved by conventional intraspecific breeding. An alternative can be provided by regeneration of doubled haploids (DH) lines from F_1 tobacco hybrids via anther culture and stem culture techniques. The primary advantage of this methodology compared with conventional breeding is the shorter time to develop homozygous lines with a desired improved traits. Examples of effective DH regeneration with resistance to black shank (Walker, Aycock, 1994), Potato virus Y (Hamada et al., 2001; Czubacka, Doroszewska, 2004) have been reported. Apart from considerably shortened time required to develop homozygous lines this method is also known for its depressing effect on morphological and agronomic traits of anther-derived doubled haploid lines (Burk, Chaplin, 1980). On the other hand, the reports on the morphological and agronomic deterioration of anther derived DHs were not consistent (Walker, Aycock, 1994) and the method has never been used to develop black root rot resistant tobacco. The objective of this study were to develop doubled haploid lines of *N. tabacum* cv. 'K326' × cv. 'Wentura' possessing black root rot resistance and basic morphological characteristics cv. 'K326',

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highly regarded for its quality, in order to use them for further breeding.

MATERIAL AND METHODS

Development of doubled haploid lines

The initial plant material was the F_1 hybrid between two Virginia tobacco cultivars: 1) the black root rot susceptible cultivar 'K326' of recognized high quality of leaves, 2) the Polish newly developed cultivar 'Wentura' with high *Nicotiana debneyi*-type resistance to *Ch. elegans* and the male parent of the widely cultivated hybrid 'VRG 2' (Trojak-Goluch, Berbeć, 2008). The F_1 hybrids were grown to maturity in the greenhouse to generate anther derived haploids. Anthers excised from flowers and cultured on Nitsch medium (1969), supplemented with 0.1 mg/l of indole-3-acetic acid. Anther-derived plantlets were successfully transferred to LS medium (Linsmaier, Skoog, 1965) to promote root development and then transferred to greenhouse and grown to maturity to verify their haploidy by observing them for the absence of seed set and mitotic chromosome counts in somatic cells. Stem fragments from haploids were cultured on Lloyd (1975) medium to generate double haploid DHR_0 shoots from spontaneously diploidized tissue. Plants of 24 DHR_0 lines were grown to maturity, self-fertilized and the doubled haploids derivatives DHR_1 were greenhouse tested for BRR resistance.

Test for black root rot resistance

A pathogenic culture of *Ch. elegans* was isolated from the flue-cured tobacco plants as described earlier by Trojak-Goluch and Berbeć (2005). The test generally followed the procedure as described by Samek and Jankowski (1987) with small modifications. One week-old seedlings were transferred into polystyrene trays (240 cells, the surface area of tray 0.3 m^2) containing peat mix inoculated with *Ch. elegans* (10.000 spores per gram of peat) and placed in a floated multicell tray system at an air temperature of 17 to 24^0 C. After 30 days the plant roots were washed and rated for disease symptoms, first by visual observation and subsequently by microscopic evaluation. Twenty four entries were tested and parental cultivars: 'K326' and 'Wentura' were included in the test as checks. Each entry was represented by 40 tobacco seedlings, 1027 plants were tested altogether.

Field evaluation of doubled haploid lines

Based on BRR greenhouse test, five resistant DHR_1 lines were chosen for morphological assessment under field conditions. Plants were grown under agronomic regime for Virginia tobacco. The experiment was arranged in a randomized complete design with three replications. Each entry was planted in a single-row plot containing 10 plants spaced 45 cm apart with 90 cm distance between rows. Morphological assessment included plant height, number of leaves per plant, days from transplanting to full blossom, length and width of midstalk leaf and estimated leaf area. Leaf area was calculated as leaf length × width × 0.6403 (Suggs et al., 1960). Evaluation of plant growth was based on the number of days from transplanting to midstalk leaf (i.e. 10-th leaf) maturity. For each of the above parameters ten plants per plot were measured. The data were analyzed by an analysis of variance (ANOVA). The differences between means were examined for significance using Tukey's honestly significant difference (HSD) at $P = 0.05$.

RESULTS

Development of doubled haploid lines

Anthers of studied plants generally responded well to culture, though there was considerable variation among individual anthers from F_1 hybrids of cv. 'K 326' × 'Wentura' for the number of plantlets – from 1 to 26 per anther. All of 179 anther derived plants were haploids. It was confirmed by cytological examination and then through absence of seed set. Stem pith fragments from all haploids were cultured and from these cultures, 24 doubled haploids DHR_0 were produced.

Test for black root rot resistance

Twenty four doubled haploids of the DHR_1 generation produced as a result of the self-pollination of DHR_0 were evaluated for resistance to *Chalara elegans*. When visually observed for necrotic lesions 7 out of 24 tested DHR_1 lines (29.2%) were found to have no root injuries. Microscopic analysis showed the lack of *Ch. elegans* spores on the roots of 5 out of 24 lines (Table 1). The disease-free lines were regarded as resistant to *Ch. elegans* and accounted for 20.8% of the total number of DHR_1 lines.

Field evaluation of doubled haploid lines

Means for plant growth characteristics show morphological differences among five black root rot resistant DHR_1 lines and their parental cultivars (Table 2). There was an extensive line-to line variation in the values of the studied agronomic traits reflecting extensive gene segregation in the original F1 hybrids. Only one line $DHR_1$52 flowered on approximately the same day as cv. 'K326'. The rest flowered significantly earlier or later. The wide range of variation in days to flower was accompanied by variability in leaf number. Four DHR_1 lines had a lower leaf number than 'K326', only in line $DHR_1$28 the average leaf number was not significantly different from that in the parental cultivar. Relative to the parental cultivars DHR_1 lines were usually taller but only in the case of DHR_1 52, DHR_1 168 the differences were statistically significant. In the majority of DHR_1 lines midstalk leaf length was smaller than that in cv. 'K326' while leaf width was considerably larger. Only DHR_1 28 was similar to 'K326' in re-

spect of leaf size. Estimated leaf areas of DHR_1 in all cases were larger than that of parental cv. 'Wentura' whereas non statistically significant differences from the parameter in cv. 'K326' were reported. The number of days from transplanting to leaf maturity of DHR_1 were substantially varied when compared to parental cultivar 'Wentura' and 'K326'. Among the DHR_1 lines the latest in reaching leaf maturity was DHR_1168. From the range of phenotypes obtained it was possible to select one which was morphologically close to the renowned cultivar 'K 326' but unlike its parent showed resistance to black root rot, a combination that was the primary practical objective of the study.

Table 1. Resistance to *Chalara elegans* in the doubled haploid derivatives DHR_1 of *Nicotiana tabacum* cv: 'K326'×'Wentura' hybrids obtained by self-pollination of regenerated plants DHR_0.

DH R_1 or cultivar	Number of plants		
	tested	with root necrosis	with *Ch. elegans* spores
$DHR_1$28	39	0	0
$DHR_1$51	40	40	40
$DHR_1$52	38	0	0
$DHR_1$93	40	35	40
$DHR_1$138	40	33	40
$DHR_1$139	40	39	40
$DHR_1$123	39	0	0
$DHR_1$131	40	39	40
$DHR_1$164	40	37	40
$DHR_1$165	39	39	39
$DHR_1$166	37	35	37
$DHR_1$167	40	0	0
$DHR_1$168	40	0	0
$DHR_1$169	40	40	40
$DHR_1$173	39	35	39
$DHR_1$29	40	39	40
$DHR_1$20	40	0	39
$DHR_1$120	40	40	40
$DHR_1$125	39	36	39
$DHR_1$79	39	39	39
$DHR_1$93	40	40	40
$DHR_1$96	40	39	40
$DHR_1$115	40	40	40
$DHR_1$172	38	0	26
'K326'	40	40	40
'Wentura'	40	0	0

DISCUSSION

The development of black root rot resistant tobacco cultivars continues to be a major objective of tobacco breeders, especially where cool climate favours the disease. However the use of traditional inbreeding process is time-consuming and usually requires six or more selfed generations. Examples of regenerated doubled haploids with resistance to black shank, *Phytophthora parasitica* (Dastur) (Walker and Aycock, 1994) and Potato virus Y (Hamada et al., 2001) prompted the authors of this study to use androgenesis to develop homozygous tobacco lines resistant to black root rot. In this study, regeneration of stem fragments of haploids plants made it possible to obtain a population of doubled haploids (DHR_0).

The screening method used in this study to identify black root rot resistant individuals within DHR_1 population was simple, fast and weather-proof. Resistant genotypes were selected after 30 days while usually it takes ca. 4 months to screen for resistance in the field. However, to reduce substantially the number of doubled haploid plants cultured *in vitro* and to increase the success rate of generating resistant genotypes, testing for resistance should be done at the haploid stage. The evaluation of DHR_1 generation for black root rot resistance was highly successful, however, the level of expression of resistance was found to be a little low. The disease-free lines accounted for 20.8% of the total number of DHR_1 lines. Julio et al. (2006) found 65% susceptible per 35% resistant in 114 recombinant inbred lines derived by single seed descent from an F_1 involving a resistant line ('ITB 32', BRR resistance from *N. debneyi* origin) crossed as female to susceptible '4K' as male. In this study the distorted ratio for BRR resistance

Table 2. Plant growth and leaf characteristics of the *Chalara elegans* resistant doubled haploid derivatives DHR_1 of *Nicotiana tabacum* cv: 'K326'× 'Wentura' hybrids and parental cultivars.

DHR_1 or cultivar	Days to full blossom*	Plant height (cm)*	Leaf number per plant*	Midstalk leaf (10-th leaf)			
				length (cm)*	width (cm)*	estimated leaf area*	days to maturity*
$DHR_1$28	95.4d	165.5bc	36.1c	59.8d	26.0a	989.25ab	89.7c
$DHR_1$52	90.8c	177.1c	30.1ab	55.1bc	27.9abc	978.56ab	92.1c
$DHR_1$123	80.7b	156.6ab	31.3ab	54.3ab	28.3bc	976.84ab	82.9b
$DHR_1$167	79.5b	150.5a	30.0ab	51.6ab	28.2abc	927.07a	83.4b
$DHR_1$168	95.1d	172.4c	31.8b	59.4cd	28.5c	1076.37b	94.3c
'K326'	90.7c	157.0ab	35.0c	60.1d	26.2ab	996.16ab	92.4c
'Wentura'	69.5a	147.8a	29.5a	49.9a	28.1abc	891.54a	72.9a
HSD (0.05)	3.37	14.19	2.07	4.63	2.22	143.28	5.04

* Values within a column followed by the same letter are not significantly different at P = 0.05 according to HSD (Tukey) test.

from *N. debneyi* origin per susceptibility, instead of 50/50 to be expected, was probably the result of a small size of the studied population.

Growth and developmental characteristics such as time of flowering, plant height, number of leaves per plant varied among the population of the DHR_1 lines. The range of variation for morphological traits reflected gamete segregation in the F_1 generation and roughly followed the pattern that would be expected in a F_2 population. From the range of phenotypes obtained in the DHR_1 generation one ($DHR_1$28) was morphologically close to the renowned cultivar 'K326' with respect to plant height, leaf number per plant and size of leaves but unlike the 'K326' parent it showed resistance to black root rot. It seemed that DHR_1 28 made a combination that was the primary practical objective of the study, however it reached the flowering stage and leaf maturity considerably later as compared to the parental cultivars. The increase in days to flower observed with the doubled haploids in this study is a rather common occurrence because previous studies in flue-cured tobacco (Brown and Wernsman, 1982) and in isogenic lines of Burley tobacco (Legg et al., 1981) reported similar delays in maturity. It could be the effect of the adverse impact of anther culture or/and of the gene for black root rot resistance (Walker and Aycock, 1994).

The line DHR_1 28 generated in this study will be used to develop flue-cured tobacco cultivars, true-breeding or hybrid, that would combine recognized quality of cv. 'K326' with good adjustment to Poland's climatic conditions plus resistance to black root rot.

CONCLUSIONS

1. Anther culture followed by stem tissue culture of F_1 hybrids of *N. tabacum* cv. 'K 326' × 'Wentura' is a useful tool in black root rot resistant tobacco breeding.
2. The results of greenhouse test enabled good evaluation of genetic resistance to black root rot in DHR_1 population.
3. The range of phenotypes in the DHR_1 lines reflected gamete segregation in the F_1 generation and roughly followed the pattern that would be expected in a self-fertilized F_2 population.
4. The most noticeable differences between the doubled haploid R_1 generation of *N. tabacum* cv. 'K 326' × 'Wentura' and cultivar 'K 326' were duration of the period from transplanting to flowering and increased height of DHR_1 plants.
5. The best performing DHR_1 28 line might be used as component of black root rot resistant hybrid cultivars.

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