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Evaluation of Tobacco Cultivars for Resistance to *Rhizoctonia solani* AG-3, Causal Agent of Target Spot Disease

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Target spot disease, caused by *Rhizoctonia solani* (Kühn) (teleomorph *Thanatephorus cucumeris* (Frank) Donk), significantly reduces tobacco yield and quality. In recent years, the spread of target spot has become a threat to tobacco production in China. However, researches on the isolation of highly resistant or immune tobacco cultivars to target spot are limited. In this study, 600 tobacco cultivars representing diverse genetic sources from different countries were evaluated for their resistance to tobacco target spot under greenhouse conditions. During screening at a temperature range of 19–26 °C, two immune cultivars and 11 resistant cultivars were found. The remaining cultivars produced symptoms that designated them as susceptible or highly susceptible at these same temperatures. Since a relatively higher temperature is conducive to *R. solani* AG-3 infection, which indicates a severe target spot symptom, the response of two immune cultivars and 11 resistant cultivars was further tested at a temperature range of 23–32 °C. Results indicated that two immune cultivars isolated in a relatively lower temperature range exhibited characteristics that could designate them as resistant, while three resistant cultivars were maintained phenotype among 11 lines tested. Taken together, our analyses tested a large number of tobacco cultivars with different ranges of temperature for response to *R. solani* AG-3 infection, and resistant lines Reams 51, DF 485, and KY 171 were identified as valuable sources to defend against *R. solani* AG-3 within a wide range of temperatures.

Key Words: resistance, *Rhizoctonia solani* AG-3, target spot, tobacco cultivars

Abbreviations: DI – disease index, PDA – potato dextrose agar, TTS – Tobacco target spot

INTRODUCTION

Tobacco target spot (TTS) disease caused by *Rhizoctonia solani* (Kühn) (teleomorph *Thanatephorus cucumeris* (Frank) Donk) has given rise to serious problems in tobacco production. In the most severe cases, death of the plant occurs when the fungus grows from a leaf tissue into the stem (Elliott et al. 2008). TTS was first reported in North Carolina (USA) in 1984, causing significant economic losses in tobacco production (Shew and Main 1985). The disease was subsequently reported in the neighboring states of South Carolina, Tennessee, Kentucky, Massachusetts, Connecticut (Shew and Main 1990; LaMondia and Vossbrinck 2010; LaMondia 2012), as well as in Canada (Reeleder 1996) and Argentina (Mercado Cárdenas et al. 2012). In August 2006, the disease first appeared in Kuandian and Fengcheng Counties of Dandong City, Kaiyuan and Xifeng Counties of Tieling City of Liaoning Province in Northeast China, and was reported to have caused severe losses in tobacco yields (Wu et al. 2012). More recently, the disease has been detected in other areas of Liaoning, Heilongjiang, and Jilin Provinces in Northeast China, Guangxi Province in Southwest China (Su et al. 2016). In July 2016, an outbreak of TTS severely affected production in Yunnan Province in Southwest China, the largest tobacco-growing regions in China where the temperature was stably maintained at 20–30 °C for a year. These conditions have made target spot disease a major concern in the Yunnan area.

The majority of researches on TTS have focused on etiology of the disease (Shew and Main 1990; Shew and Melton 1995; Wu et al. 2013; Zhao et al. 2013; Zhao et al. 2014a, b). However, a few recent reports on the evaluation of tobacco-resistant germplasm to target spot have begun looking into ways to combat this pathogen. Csinos and Stephenson (1999) tested 66 tobacco cultivars for their resistance to target spot, and nine of these showed a relatively low level of disease incidence. Elliott et al. (2008) evaluated 97 tobacco genotypes resistant to target spot and showed that a few burley cultivars exhibited only slightly lower disease incidence, while cultivar TI 1605 exhibited a lower disease incidence than all other accessions. In China, Su et al. (2016) evaluated 21 major domestic cultivating lines for testing resistance to target

spot, and none among the 21 lines showed resistance or immunity to the pathogen. TTS has severely affected the economy in China, but currently there are no resistant cultivars being grown domestically; therefore, screening of highly resistant or immune lines against target spot has become urgent.

The objectives of this study were (1) to survey cultivars that are resistant to target spot by using 600 tobacco cultivars which are germplasm resources or commercial cultivars belonging to different tobacco types, and (2) to identify useful sources for tobacco production and breeding for resistant cultivars.

MATERIALS AND METHODS

Plant Materials and Growth

The 600 tobacco cultivars were obtained from the Tobacco Germplasm Collection of China maintained in Yuxi City of Yunnan Province. The accessions included 542 cultivars of *Nicotiana tabacum*, 34 cultivars of *Nicotiana rustica*, and 24 cultivars of *Nicotiana hybrid*. *Nicotiana tabacum* cultivars consisted of 84 cultivars of burley, 17 of cigar binder, 55 of cigar filler, 73 of cigar wrapper, 8 of dark air-cured, 197 of flue-cured, 32 of fire-cured, two of Hungary, 39 of Oriental, 20 of Maryland, and 15 other cultivars.

Seeds were sown in breeding trays half-filled with soil nutrient medium in a greenhouse at the Shenyang Agricultural University. Subsequently 2-wk-old seedlings were transplanted into plastic pots (5 cm diameter × 10 cm tall) and covered with pasteurized soil mixture (85% soil and 15% sand). Plants were grown for a 12-h photoperiod at 19–26 ℃ for 8 wk in the same greenhouse. Water was added to the plastic pots as needed.

Fungal Isolates and Culture Conditions

The highly pathogenic *R. solani* AG-3 strain LN-9 was used in this study. It was domestically isolated from flue-cured tobacco in Tieling City of Liaoning Province in Northeast China (Zhao et al. 2013).

The cultures were grown in Petri dishes on potato dextrose agar (PDA), containing 200 g potato infusion, 20 g dextrose and 18 g agar per liter. Petri dishes were placed in a growth chamber at 25 ± 2 °C for 5 d without light, until the mycelium covered the entire Petri plate.

Experimental Design

Inoculation of *R. solani* AG-3 Strain

Artificial inoculation was performed according to the methods of Mpofu with modifications (Mpofu and Julian 1994). The tobacco leaves were penetrated by using a needle and inoculated by depositing a PDA plug (0.5 mm diameter) colonized with *R. solani* AG-3 strain LN-9. Non-infested PDA plugs served as control. Each inoculation spot was covered by a thin layer of wet cotton to maintain humidity (Fig. 1). The cotton was removed at 48 h post inoculation (hpi) when symptoms began to appear at the inoculation spot (Shew and Melton 1995; Cardenas et al. 2012). For the detached leaf test, the leaf petiole was also wrapped up by wet cotton and inoculated in a similar way. Inoculated leaves and plants were kept in the greenhouse where daily temperatures were 30 °C (daytime) and 20 °C (night).

Crude Screen of the 600 Tobacco Cultivars

Middle and lower leaves were collected when the tobacco seedlings were at the 7–8th leaf stage by using detached leaves as testing materials. Ten leaves from 10 different plants at the same developmental stage and height were tested for each variety as a replicate and all tests were repeated three times. Each tobacco leaf was washed with distilled water, sterilized with 0.5% NaOCl for 30 s, rinsed with sterilized distilled water, and inoculated at three different locations according to the above-mentioned methods. In this detached-leaf screen assay, 10 inoculated leaves of each variety were placed in a plastic tray with the bottom lined with wet cotton and filter paper, mist sprayed and covered with a plastic film to maintain high humidity. Flats were kept at temperatures between 19 °C and 26 °C. Distilled water was added to the trays as needed. Symptoms began to appear on inoculated locations at 48 hpi. Diameters of leaf lesions were measured at 5 d post inoculation (dpi) when the inoculated leaves showed typical disease symptoms (Fig. 2).

Further Tests of 13 Selected Cultivars

Based on the crude screen, 13 cultivars (2 immune, 11 resistant) comprising all the resistant cultivars identified from the crude screen, were selected for further testing. In order to verify stability of target spot resistance, two new treatments were explored. Tests were performed at a range of temperatures between 23 °C and 32 °C and monitored for maintenance of the resistance phenotype. Tobacco plants were monitored for symptoms on detached leaves as before and also on leaves of intact plants. The detached leaf test was performed similarly as in the crude screen. For the intact plant leaf test, middle and lower leaves were inoculated when seedlings were at the 7–8th leaf stage. Ten plants at equal heights of each variety were used, and tests for all cultivars were repeated three times. All the inoculated plants were covered with a plastic film after mist spraying to retain

Fig. 1. Moisturizing the leaves with wet cotton after inoculation.

Fig. 2. Symptoms after inoculation for 5 d.

moisture (Fig. 3). Every 4 h, the plastic film was uncovered to reduce the temperature and mist spraying to maintain humidity.

Disease Assessment and Statistical Analysis

After 5 d, all leaf lesion diameters were measured to calculate disease index (DI), and graded on a 0–9 scale (Zhao et al. 2013): 0: 0 mm; 1: 0–1 mm, 3: 1.1–2 mm, 5: 2.0–5.0 mm, 7: 5.01–9 mm, 9: > 9.01 mm. DI was calculated based on the formula:

$$DI = [\sum (\text{Number of lesions per grade} \times \text{Grade}) / (\text{Total lesions} \times \text{The highest grade})] \times 100$$

Based on the DI, the resistance evaluation was classified as: immune (0), highly resistant (1–5), resistant (6–34), susceptible (35–65), and highly susceptible (> 65) (Fig. 4).

Analysis of variance was performed, and data were analyzed using the software Stata (version 12.0, Stata Corp LP).

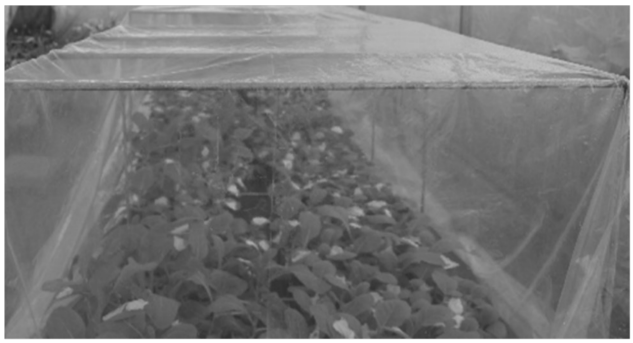

Fig. 3. Covering with plastic film after mist spraying to retain moisture in intact plant leaf test.

Table 1. Ratio of different reactions of 600 tobacco cultivars in crude screen.

Reaction Type	Quantity	Ratio (%)
Immune	2	0.3
Highly resistant	0	-
Resistant	11	1.8
Susceptible	145	24.2
Highly susceptible	442	73.7

RESULTS

Crude Screen

Based on the DI analysis of 600 tobacco cultivars (Annex Table S1, available upon request from the authors), two cultivars (Reams 51 and DF 485) exhibited immunity to *R. solani* AG-3 strain LN-9 during the crude screen. Eleven cultivars exhibited resistance to the pathogen. These include KY 171, Vesta 30, Chibuo Grande Correntino, Ward, GR14, Kavala No15A, Speight G-5, Coker 51, Tabaco Blanco, Tom Rosson (TR) Madole, and Kentucky 151. Other cultivars were susceptible or highly susceptible. DI can be found in Tables 1 and 2. The mock inoculated control leaves did not show target spot lesions. The above results indicate that the majority of the tested cultivars were susceptible to *R. solani* AG-3 strain LN-9.

Further Tests of 13 Selected Cultivars

The results showed that the resistance of the 13 further tested cultivars in both detached leaf and intact plant leaf assays performed at higher temperatures declined compared with the crude screen at lower temperatures (Table 2). At 23–32 °C, none of the 13 tested cultivars showed immunity to the pathogen. The cultivars Reams 51, DF 485 and KY 171 still showed higher resistance compared with the other cultivars while the remaining 10 cultivars became susceptible in both detached leaf tests and intact plant leaf tests.

Further testing of the 13 selected cultivars showed that the results of variance analysis indicated no significant differences between detached leaf and intact plant leaf tests within cultivars DI ($F = 0.41$, Prob. = 0.5265 > 0.05; Table 3). Bartlett's test confirmed that the variance of DI between the two tests were equal [chi^2 (1) = 0.0484

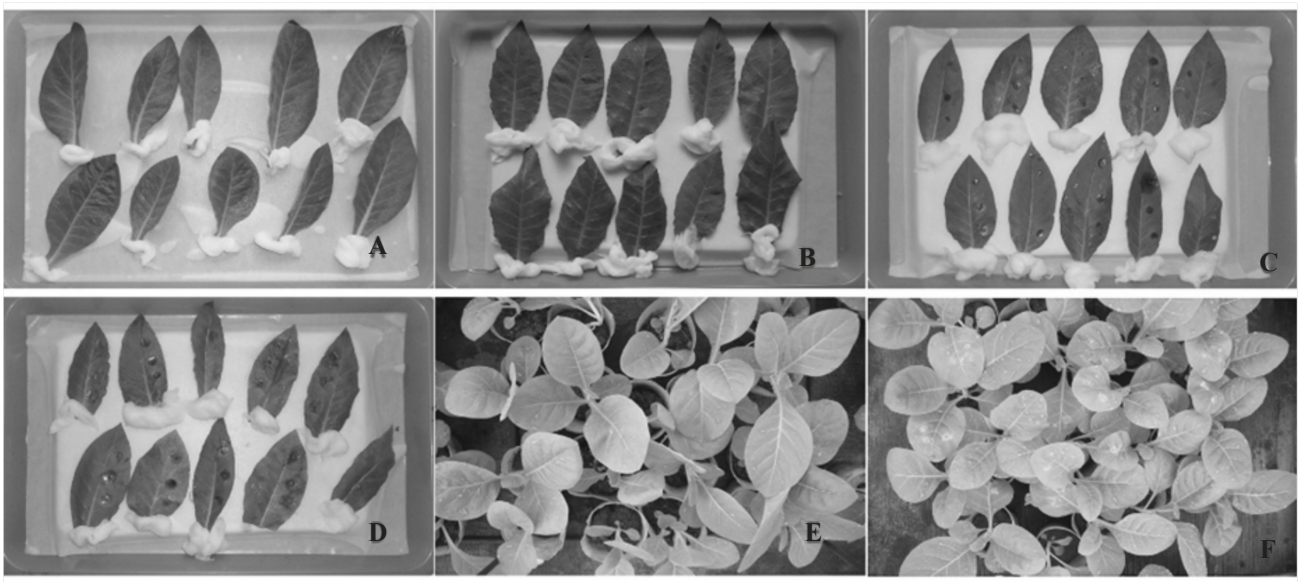

Fig. 4. Disease symptoms of different resistance evaluations in detached leaf test and intact plant leaf test: A, Immune to pathogen of detached leaf test; B, Resistant to pathogen of detached leaf test; C, Susceptible to pathogen of detached leaf test; D, Highly susceptible to pathogen of detached leaf test. E, Resistant to pathogen of intact plant leaf test; F, Susceptible to pathogen of intact plant leaf test.

Table 2. Resistance evaluation of 13 selected tobacco cultivars to *R. solani* AG-3 strain LN-9 in all the tests.

PI Number[a]	Selected Cultivar	Type[b]	Crude Screen (19–26 °C)		Further Tests (23–32 °C)			
					Detached Leaf Test		Intact Plant Leaf Test	
			DI[c]	Resistance Evaluation[d]	DI	Resistance Evaluation	DI	Resistance Evaluation
PI 552424	Reams 51	FC	0	I	13.65	R	18.8	R
PI 509533	DF 485	FRC	0	I	19.47	R	14.86	R
PI 551326	KY 171	FRC	8.25	R	17.61	R	22.82	R
PI 552769	Vesta 30	FC	9.15	R	36.98	S	43.67	S
PI 404943	Chibuo Grande Correntino	CF	15.24	R	35.56	S	36.48	S
PI 552775	Ward	MD	15.45	R	40.83	S	38.89	S
PI 551259	GR14	BU	19.59	R	38.33	S	43.32	S
PI 552416	Kavala No 15A	OR	21.50	R	36.06	S	38.78	S
PI 552457	Speight G-5	FC	21.87	R	40.47	S	45.76	S
PI 552503	Coker 51	FC	23.57	R	36.40	S	42.89	S
PI 114019	Tabaco Blanco	CW	27.41	R	48.07	S	53.07	S
PI 552764	Tom Rosson (TR) Madole	FRC	29.45	R	47.93	S	43.22	S
PI 552370	Kentucky 151	FRC	31.00	R	44.90	S	51.81	S

[a]Plant introduction number
[b]Tobacco types: BU, burley; CF, cigar filler; CW, cigar wrapper; FC, flue-cured; FRC, fire-cured; OR, Oriental; MD, Maryland
[c]DI = disease index; all experiments were repeated three times; the DI of cultivars was the mean of three tests, respectively.
[d]Resistance evaluation classification: I, Immune; R, Resistant; S, Susceptible; HS, Highly susceptible

Table 3. Analysis of variance for the disease index (DI) of detached leaf test and intact plant leaf test.

Source	SS	df	MS	F	Prob. > F
Between groups	55.860	1.000	55.860	0.41	0.5265
Within groups	3245.512	24.000	135.230		
Total	3301.373	25.000	132.055		

Bartlett's test for equal variances: chi^2 (1) = 0.0484 Prob. > chi^2 = 0.826

Prob. > chi^2 = 0.826], indicating that homogeneity of variance across methods of detached leaf inoculation and intact plant leaf inoculation can be compared. The correlation analysis of DI showed that the DI was highly correlated among the crude screen, the detached leaf test and the intact plant leaf test (Table 4). The correlation coefficient was 0.9373, indicating that the DI of the detached leaf test and that of the intact plant leaf test was highly correlated. For further regression analysis, the 13 selected varieties were ranked according to DI of the detached leaf test, the intact plant leaf test and the crude screen, respectively. The regression of rank between the detached leaf test and the intact plant leaf test sorted by DI (Fig. 5A) and the regression of DI between the

Table 4. Correlation of disease index (DI) among detached leaf test, intact plant leaf test and crude screen.

	Pearson's		
Spearman's	Crude Screen	Detached Leaf Screen	Intact Plant Leaf Screen
Crude screen		0,8903	0,8839
Detached leaf screen	0.8297		0,9373
Intact plant leaf screen	0,7637	0,8516	

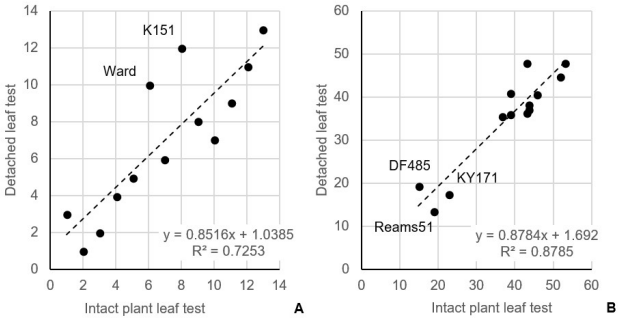

Fig. 5. Scatter diagram, fitted line and regression model between detached leaf test and intact plant leaf test. A) Scatter diagram, fitted line and regression model between sequence of detached leaf test and sequence of intact plant leaf test sorted by disease index (DI); B) Scatter diagram, fitted line and regression model of DI between detached leaf test and intact plant leaf test.

detached leaf test and the intact plant leaf test (Fig. 5B) showed that the result of detached leaf test and intact plant leaf test was highly consistent. The result supported the conclusion of the variance analysis and showed that the method with detached leaf for mass screening was convincing. From the regression analysis, K151 and Ward were slightly less correlated with each other, but the variance analysis showed no difference, which could be further studied in the future on their singularity.

The variance analysis showed that when the test performed at 19–26 °C was compared with the test performed at 23–32 °C, there was always a significant difference in the DI of the cultivars [F = 18.07, Prob. = 0.0003 < 0.05 (Table 5); F = 22.73, Prob. = 0.0001 < 0.05 (Table 6)]. Bartlett's test showed that the variance of DI between the two sets of data were equal [chi² (1) = 0.0875 Prob. > chi² = 0.767; chi² (1) = 0.2654 Prob. > chi² = 0.606]. The regression analysis showed the results of the detached leaf test and the crude screen were consistent, while the intact plant leaf test and the crude screen were also consistent, but the crude screen overvalued the resistance of the cultivars (Fig. 6A, B, C, D). These results indicate that the temperature regimes had an important effect on the resistance of the cultivars to *R. solani* AG-3 strain LN-9. For all cultivars, higher temperatures lessened the resistance of phenotypes.

DISCUSSION

In recent years, target spot has become a threat to domestic tobacco production as reports have been documented in most of China's tobacco-producing regions. The use of host-resistant cultivars is an economical and eco-friendly way to control the disease. However, tobacco lines which are highly resistant to target spot have not been reported. Therefore, screening and breeding resistant cultivars have become important tasks. In this study, 600 cultivars were tested for their resistance to the target spot pathogen. Isolates of *R. solani* were separated into different groups based on anastomosis (AG). Group AG-3 is the causal agent for target spot on tobacco, while group AG 2-1 has also been identified as causing target spot disease although it has not been found in China (Stevens et al. 1993; Mercado Cárdenas et al. 2012; Wu et al. 2012; Wu et al. 2013; Su et al. 2016).

Three resistant cultivars including Reams 51, DF 485, and KY 171 were identified. Also, 10 cultivars [Vesta 30, Chibuo Grande Correntino, Ward, GR14, Kavala No 15A, Speight G-5, Coker 51, Tabaco Blanco, Tom Rosson (TR) Madole, and Kentucky 151] showed resistance when the temperature regimes were held at 19–26 °C, while all of them became susceptible when the temperature regimes

Table 5. Analysis of variance for the disease index (DI) of crude screen and detached leaf test of further tests.

Source	SS	df	MS	F	Prob. > F
Between groups	2102.042	1.000	2102.042	18.07	0.0003
Within groups	2792.357	24.000	116.348		
Total	4894.399	25.000	195.776		

Bartlett's test for equal variances: chi^2 (1) = 0.0875 Prob. > chi^2 = 0.767

Table 6. Analysis of variance for the disease index (DI) of crude screen and intact plant leaf test of further tests.

Source	SS	df	MS	F	Prob. > F
Between groups	2843.237	1.000	2843.237	22.73	0.0001
Within groups	3002.610	24.000	125.109		
Total	5845.847	25.000	233.834		

Bartlett's test for equal variances: chi^2 (1) = 0.2654 Prob. > chi^2 = 0.606

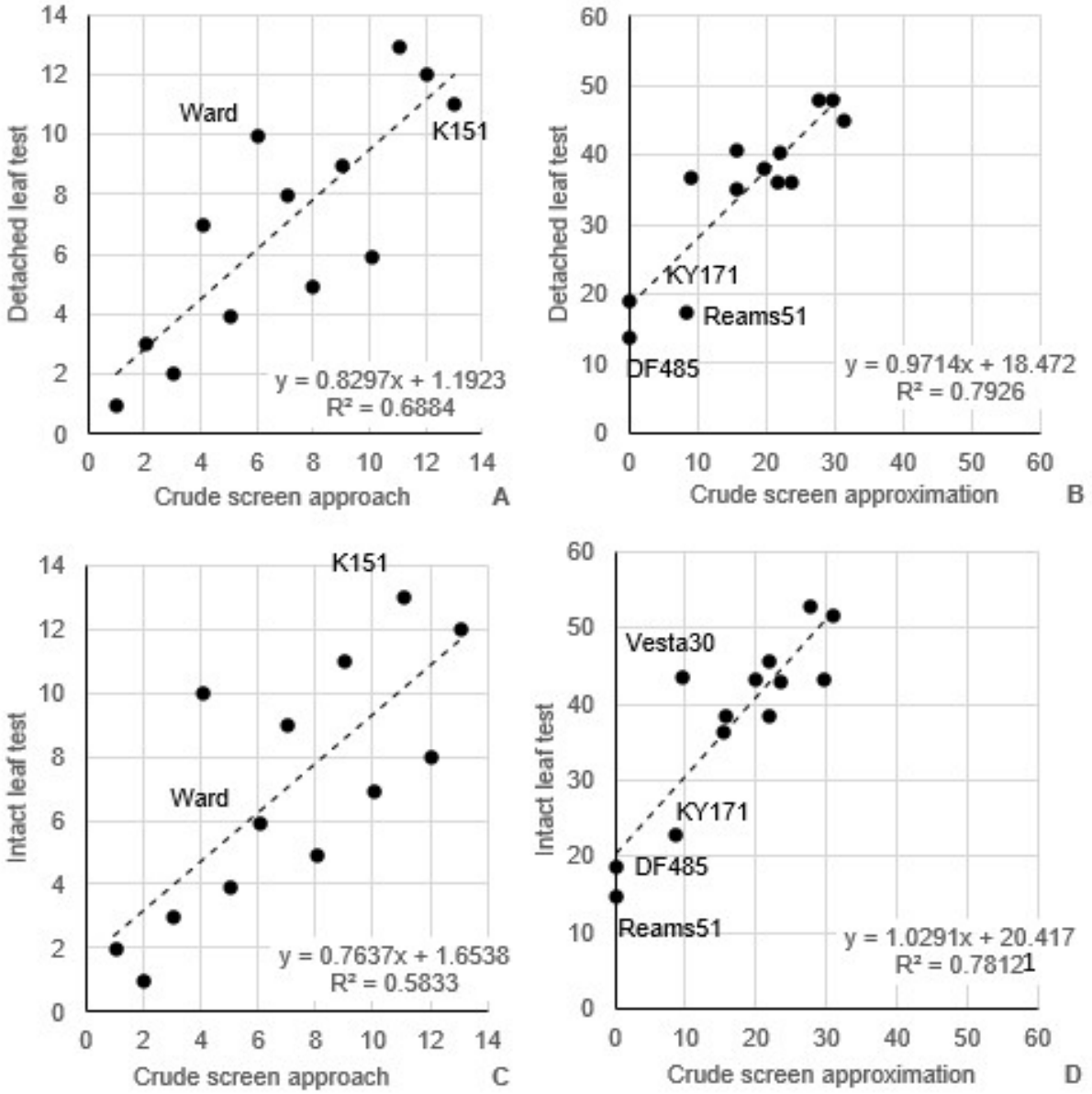

Fig. 6. Scatter diagram, fitted line and regression model between detached leaf test and crude screen, intact plant leaf test and crude screen. A) Scatter diagram, fitted line and regression model between sequence of detached leaf test and sequence of crude screen sorted by disease index (DI); B) Scatter diagram, fitted line and regression model of DI between detached leaf test and crude screen; C) Scatter diagram, fitted line and regression model between sequence of intact plant leaf test and sequence of crude screen sorted by DI; and D) Scatter diagram, fitted line and regression model of DI between intact leaf test and crude screen.

increased to 23–32 °C. Other cultivars showed susceptibility or high susceptibility in the crude screen. The cultivars K-326, Coker 371-Gold, Speight G-70, NC 95, and NC 2326 had relatively low levels of disease incidence, but these cultivars did not appear to have resistance to target spot (Elliott et al. 2008). In our tests, Speight G-70, NC 95, Coker 371-Gold, and NC 2326 were designated susceptible or highly susceptible to the disease in the crude screen.

The most important environmental factor in the development of target spot is humidity. In our study, all tests were executed under high humidity conditions, which were very suitable for disease development. However, the temperature variation in our tests was controlled only enough to maintain a narrow temperature range. Temperature affects fungal hymenium production, infection, and subsequent plant tissue lesion development caused by most plant pathogenic fungi (Shew and Main

1990). Some of the tobacco cultivars tested here showed high resistance in the first screening test (at a relatively low temperature condition), but became susceptible or highly susceptible in the further tests at higher temperatures. It has been shown in the literature that the percent of target spot primary lesions expands significantly at 24–30 °C, while the rate of lesion expansion is greatest between 20 °C and 30 °C (Shew and Main 1990). Wu et al. (2012) reported that the target spot pathogen could infect tobacco at temperatures between 15 °C and 35 °C, with an optimum temperature between 25 °C and 32 °C. In our crude screen, temperature regimes were between 19 °C and 26 °C, below the previously reported optimum temperature for *R. solani* AG-3 infection and development. Further tests carried out at 23–32 °C were within the optimum temperature regimes, which could explain how resistant and immune cultivars from lower temperature regimes now showed greater DI. Importantly, even at optimum temperatures for tobacco target spot, the cultivars Reams 51, DF 485, and KY 171 were identified as resistant.

Jian et al. (2003) reported that temperature and resistance of varieties to cotton *Verticillium* Vilt had obvious interactions. Liu et al. (2011) reported that with the rise in temperature, powdery mildew caused aggravated damage to zucchini plants. He et al. (2017) reported that the latent period of tobacco bacterial wilt decreased with the rise in temperature, and that the latent period in varieties with a higher disease resistance was longer. Our conclusions are consistent with these findings. Previous research had shown artificial inoculations inducing very similar disease reactions under suitable environmental conditions (Franke et al. 1999). Our study demonstrated that under artificial inoculation conditions, results of detached leaf tests and intact plant leaf tests in cultivars inoculated with *R. solani* AG-3 strain LN-9 had similar results. These results indicate that artificial inoculations on detached leaves will allow a high-throughput screening method that could be used as a quick assay to screen many cultivars for resistance to target spot.

CONCLUSION

This is the first study to incorporate a large number of cultivars with a wide range of genetic diversity to screen resistance of tobacco to *R. solani*, causal agent of target spot. The connection between disease development and growing temperature conditions was revealed, with the increase in temperature lessening cultivar resistance. The usefulness of artificial inoculations on detached leaves was demonstrated as a high-throughput screening method to evaluate tobacco cultivar resistance to target spot. Resistant genotypes that can be grown at the temperatures optimal for the pathogen were successfully uncovered. These are valuable resources for selection of cultivars that are resistant to target spot. The information generated provides a tool for the integrated management of the disease.

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