Reaction of Indicator Tobacco Cultivars to Races of *Pseudomonas syringae* pv. *tabaci* Tox+

by

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SUMMARY

Three races of *Pseudomonas syringae* pv. *tabaci* Tox+ (wildfire) (races 0, 1 and 2) and two races of Tox- (angular leaf spot) (races 1 and 2) have been confirmed on tobacco in Zimbabwe (Zim). Very few cultivars with no resistance to *Ps. syringae* pv. *tabaci* are grown commercially and race 0 has not been isolated since 1996. Because we no longer have a viable culture of race 0, we obtained an isolate of race 0 from Kentucky (0 KY), USA in January 2000. We included this isolate in race tests on standard indicator cultivars K E1 (susceptible to all races), KM 10 (resistance to race 0 derived from *Nicotiana longiflora*), WZ (resistance to races 0 and 1 derived from *N. rustica*) and a hybrid, K 35 (resistance to races 0 and 0 and 1 derived from *N. longiflora* and *N. rustica* respectively). Two leaves on 10-week-old seedlings were inoculated with a bacterial suspension (10^6 colony forming units [cfu] per mL) by spraying selected areas until just watersoaked and incubating the plants at 28 °C and 70% RH for 10 d. The reaction to race 0, measured as lesion diameter, was different from that previously obtained with race 0 (Zim). Races 0 and 1 (Zim) are avirulent on WZ but race 0 (KY) was virulent. Further isolates of race 0 were received from Maryland (MD) and Tennessee (TN). The TN isolates overcame resistance derived from *N. longiflora* and *N. rustica*, except where both sets of genes were present in the same cultivar. Reactions have been variable with the race 0 (MD) isolate suggesting it is a mixed culture. We conclude that there are at least four races of *Ps. syringae* pv. *tabaci* Tox+ worldwide and race 0 (KY) should be designated race 3. On all cultivars, race 2 consistently caused the largest lesions. [Beitr. Tabakforsch. Int. 19 (2001) 353–59]

ZUSAMMENFASSUNG

Drei Rassen des Bakteriums *Pseudomonas syringae* pv. *tabaci* Tox+ (Wildfeuererkrankung) (Rassen 0, 1 und 2) und zwei Rassen der Tox- (angular leaf spot) (Rassen 1 und 2) sind bisher bei Tabak in Simbabwe (Sim) bekannt. Sehr wenige Tabaksorten ohne Resistenz gegen *Ps. syringae* pv. *tabaci* werden kommerziell angebaut und seit 1996 hat man die Rasse 0 nicht mehr isolieren können. Weil wir keine lebensfähige Bakterienkultur der Rasse 0 mehr besitzen, haben wir im Januar 2000 eine Kultur der Rasse 0 aus Kentucky (0 KY), USA, erhalten. Wir haben dieses Isolat in unsere Rassen-Tests mit den Standard-Indikator-Sorten KE 1 (anfällig für alle Rassen), KM 10 (Resistenz gegen Rasse 0, von *Nicotiana longiflora* abstammend), WZ (Resistenz gegen die Rassen 0 und 1, von *N. rustica* abstammend) und K 35 (Resistenz gegen die Rassen 0 und 0 und 1, von *N. longiflora* und *N. rustica* stammend) einbezogen. Zwei Blätter 10-Wochen alter Setzlinge wurden mit einer Bakteriensuspension (10^6 Zellen/mL) infiziert. Dazu wurden die ausgewählten Blattflächen so lange besprüht, bis die Flächen gerade durchnässt waren. Die Pflanzen wurden dann bei 28 °C und 70% rel. Feuchtigkeit 10 Tage lang inkubiert. Die Reaktion auf Rasse 0, gemessen als Durchmesser der Läsionen, unterschied sich von der früher erhaltenen Reaktion mit der Rasse 0 (Sim). Die Rassen 0 und 1 (Sim) sind nicht virulent auf WZ, die Rasse 0 (KY) war aber virulent. Weitere Bakterienkulturen der Rasse 0 erhielten wir aus Maryland (MD) und Tennessee (TN). Die TN-Kultur überwand die aus *N. longiflora* und *N. rustica* stammenden Resistenzen, allerdings nicht, wenn beide Gensätze gleichzeitig in der Tabaksorte enthalten waren. Die Rasse 0 verhielt sich unterschiedlich was vermuten lässt,

Figure 1.
Proportion of the tobacco crop planted to cultivars resistant to race 0 and races 0 and 1 of *Pseudomonas syringae* pv. *tabaci* Tox+

dass es sich um eine Mischkultur handelt. Wir schließen aus den Ergebnissen, dass mindestens vier Rassen des Bakteriums *Ps. syringae* pv. *tabaci* Tox+ weltweit existieren. Die Rasse 0 (KY) sollte in Rasse 3 umbenannt werden. Auf allen Tabaksorten verursachte die Rasse 2 die größten Läsionen. [Beitr. Tabakforsch. Int. 19 (2001) 353–59]

RESUME

La présence de trois races de *Pseudomonas syringae* pv. *tabaci* (Tox+) (maladie du feu sauvage) (races 0, 1 et 2) et de deux races de Tox- (maladie des taches angulaires) (races 1 et 2) a été confirmée sur le tabac au Zimbabwe (Zim). Très peu de variétés sensibles à *Ps. syringae* pv. *tabaci* sont cultivées industriellement et la race 0 n'a pas été isolée depuis 1996. Puisque nous n'avons plus de culture viable de la race 0, nous avons obtenu en janvier 2000 un isolat de la race 0 en provenance du Kentucky (KY), USA. Nous avons inclus cet isolat dans des tests sur les races chez des variétés de référence : K E1 (sensibles à toutes les races), KM 10 (comportant une résistance à la race 0 provenant de *Nicotiana longiflora*), WZ (porteur de la résistance aux races 0 et 1 issue de *N. rustica*) et une variété hybride K 35 (ayant la résistance aux races 0 et 0 et 1 provenant de *N. longiflora* et *N. rustica*). Deux feuilles de jeunes plantes âgées de 10 semaines ont été inoculées avec une suspension bactérienne (10^6 cfu/mL) par pulvérisation de certaines zones jusqu'à saturation hydrique et incubation des plantes à 28 °C et 70% RH pendant 10 jours. La réaction à la race 0, mesurée par le diamètre des lésions, est différente de celle précédemment obtenue avec la race 0 (Zim). Les races 0

et 1 (Zim) sont avirulentes sur WZ alors que la race 0 (KY) est bien virulente. D'autres isolats de race 0 ont été reçus du Maryland (MD) et du Tennessee (TN). Les isolats TN ont surmonté la résistance dérivée de *N. longiflora* et *N. rustica*, sauf si les deux séries de gènes étaient présents dans le même cultivar ou variété. Les réactions avec l'isolat de la race 0 (MD) ont été variables, suggérant qu'il s'agit d'une culture mixte. On en conclut qu'il existe au moins quatre races de *Ps. syringae* pv. *tabaci* Tox+ dans le monde entier et que la race 0 (KY) devrait être désignée comme race 3. Sur toutes les variétés, c'est la race 2 qui, de façon régulière, a provoqué les lésions les plus étendues. [Beitr. Tabakforsch. Int. 19 (2001) 353–59]

INTRODUCTION

Wildfire and angular leaf spot, caused by two forms of the same pathogen, *Pseudomonas syringae* pv. *tabaci* (Wolf & Foster) Young, Dye and Wilkie, are important diseases of flue- and air-cured tobacco in Zimbabwe and cause losses of economic significance in yield and quality every season. The management of the diseases is complicated by the occurrence of several races of the pathogen. Control by copper-based bactericides is limited to seedbeds because of residue levels on cured leaf if tobacco is sprayed in the field. Recently, the plant activator, acibenzolar-S-methyl (Bion 50% dg) has been recommended, because it induces systemic acquired resistance to bacterial pathogens, with considerable success against races 0 and 1 of *Ps. syringae* pv. *tabaci* Tox+ and race 1 Tox- (1). It is slightly less effective against race 2 (Tox+ and Tox-) (2). Residues of the compound and its metabolites are within acceptable limits (1).

Breeding for resistance can sometimes be a more effective way of overcoming the effects of the pathogens if resistance is durable. Cultivars with resistance to race 0 of *Ps. syringae* pv. *tabaci* Tox+ (wildfire) have been bred in Zimbabwe using Burley 21. Burley 21 incorporates the major gene for resistance interspecifically derived from *N. longiflora* (3,4,5). *Ps. syringae* pv. *tabaci* Tox+ race 1 was introduced into Zimbabwe on seed imported from Wisconsin in 1971. Breeding programs in both USA and Zimbabwe used the single dominant resistance gene from *N. rustica* cv. Brasilea (6,7) as the source of resistance and this was introduced interspecifically into flue-cured genotypes in Zimbabwe (5). Race 2, which is now the most prevalent form of the pathogen (both the Tox+ and Tox- forms), overcomes all known sources of resistance (8). There are indications that polygenic resistance may offer some reduction in the level of the diseases.

Very few cultivars with no resistance to *Ps. syringae* pv. *tabaci* are grown commercially, and as a result, race 0 (the Tox- form of which has not been reported) has not been isolated from field tobacco in Zimbabwe since 1996 (Figure 1). Because we no longer have a pathogenic culture of *Ps. syringae* pv. *tabaci* Tox+ race 0 for

Figure 2. Comparison of reaction of Zimbabwe and Kentucky isolates of *Pseudomonas syringae* pv. *tabaci* Tox+ race 0; (a) reactions expected, (b) reaction to race 0 (KY). Cultivar K E1 = Kutsaga E1, K M10 = Kutsaga M10, WZ = breeding line, K 35 = Kutsaga 35

breeding tests, we obtained an isolate of race 0 (race 0 KY) from Kentucky, USA, in January 2000 (courtesy of Brenda Kennedy, University of Kentucky, through B. Smeeton). The reaction on indicator cultivars was different from that previously obtained with race 0 (Zim) by CHIBI (9) who used cv. K 51E (no resistance to *Ps. syringae* pv. *tabaci*), K M10 (resistance to race 0 derived from *N. longiflora*) and WZ (resistance to races 0 and 1 derived from *N. rustica*) (Figures 2a and 2b). Race 0 (Zim) did not produce lesions on either cv. K M10 or on WZ plants. Further isolates of race 0 were requested and sent via B. Smeeton from Maryland (race 0 MD [courtesy A. Grybauskas, University of Maryland]) and Tennessee (race 0 TN [courtesy R. Hensley, University of Tennessee]). The research reported below details the reaction of isolates of *Ps. syringae* pv. *tabaci* Tox+ on indicator tobacco cultivars to determine their identity.

MATERIALS AND METHODS

Seedling production

The indicator tobacco cultivars used were as follows: Kutsaga E1 (K E1) no known resistance to *Ps. syringae* pv. *tabaci* (rrll); Kutsaga Mammoth 10 (K M10) and Burley 21 (Bu 21) with homozygous resistance to race 0 derived from *N. longiflora* (rrLL); WZ, a breeding line with homozygous resistance (RRll), and Kutsaga RK6 (K RK6) with heterozygous resistance (Rrll), to races 0 and 1 derived from *N. rustica* and Kutsaga 35 (K 35), a hybrid with heterozygous resistance (RrLl) to races 0 and 0 and 1 derived from *N. longiflora* and *N. rustica* respectively.

Seedlings of the cultivars were raised in a glasshouse (max. 35 °C, min. 15 °C) for 6 weeks in trays containing sandy soil into which a compound fertilizer (N:7, P₂O₅:21, K₂O:7) had been incorporated at 143 g · m⁻², before being planted out individually into 125 mm-diameter pots containing sandy soil and grown for a further 4–6 weeks. At this stage they were inoculated and placed in a controlled environment unit (28 °C, high relative humidity [>70%] and a 13/11 hour L/D photoperiod) for the duration of the experiment.

At least one of each example of the primary four indicator types (susceptible, resistance derived from *N. longiflora* or *N. rustica* and resistance derived from both *N. longiflora* and *N. rustica*) were included in all six experiments. Treatments were arranged in a factorial design with races and cultivars as the two factors, in four randomized complete blocks.

Inoculum preparation

Isolates of the various races of *Ps. syringae* pv. *tabaci* Tox+ were streaked onto King's Medium B or *Pseudomonas* fluorescent medium (PFA) in 90 mm-diameter Petri dishes and incubated at 28 °C for 48 h. Selected blue-fluorescing colonies, indicative of a fluorescing *Pseudomonad* (11), were inoculated into one litre of Oxoid Nutrient Broth in five litre flasks and aerated by continuous agitation (124 rpm⁻¹) on a shaker for 18–24 h at 25 °C.

The resulting bacterial cells were checked for purity by the Gram stain method, centrifuged at 1000 g for 15 min, the bacterial pellet washed twice and finally resuspended in 250 mL quarter-strength Ringer's solution. The inoculum was adjusted to 1 × 10⁶ cfu/mL by dilution plating.

Inoculation technique

A modification of the method by DEALL and COLE (10) was used. Four sites, two on either side of the midrib of the abaxial surface of the two uppermost expanded leaves of potted plants (usually leaves 5 and 6) were sprayed with the appropriate bacterial suspension using an artist's airbrush (Hansa Aero-pro 251, 0.2 mm nozzle). The airbrush was held 20 mm from the surface of the leaf, operating at a pressure of 250 kPa until the tissue below the point of inoculation was just watersoaked (0.25 mL). Leaves were rinsed with sterile distilled water immediately after inoculation and the plants were placed in a controlled environment unit as above. Because there is no damage to leaves by the watersoaking inoculation technique (8,9 and numerous other experiments), water controls were not included.

Assessments

The diameter of necrosis in developing lesions (two measurements at right angles to each other) was mea-

Table 1.
Reaction (lesion size) of races of *Pseudomonas syringae* pv. *tabaci* Tox+ on four indicator tobacco cultivars 10 d after inoculation

Cultivar	Source of resistance	Lesion size ($\sqrt{\text{area}}$ (mm))		
		Race 0 (KY)	Race 1 (PW155)	Race 2 (PW252)
Kutsaga E1	rrll	3.87	5.48	8.49
Kutsaga M10	rrLL	0	5.82	9.91
Kutsaga 35	RrLI ^a	0	0	11.57
WZ	RRll	3.66	0	10.97
SED ^b (race $\times$ cv.)		0.703 ^c		
t (0.05,33 df) = 2.036				

^aRr = *N. rustica*, Ll = *N. longiflora*.

^bSED = standard error of the difference between the means.

^c $P < 0.05$.

Table 2.
Reaction (lesion size) of indicator cultivars of tobacco to races of *Pseudomonas syringae* pv. *tabaci* Tox+, 10 d after inoculation

Cultivar	Source of resistance	Lesion size ($\sqrt{\text{area}}$ (mm))	
		Race 0 (KY)	Race 1 (PW155)
Kutsaga E1	rrll	4.34	7.47
Kutsaga M10	rrLL	0.71	7.48
Burley 21	rrLL	0.51	5.67
Kutsaga 35	RrLI	0.35	0.22
WZ	RRll	4.90	0
WZ-ms	RRll	4.82	0
SED (race $\times$ cv.)		0.784 ^a	
t (0.05,51 df) = 2.009			

^a $P < 0.05$.

sured 7 and 10 d after inoculation. The area of the lesions (transformed to the square root to normalize the data and bring it to the same scale as the diameters) and the mean diameters of the lesions were analysed by the GLM procedure of SAS statistical package, version 6.12 (Statistical Analysis Systems Institute Inc, Cary NC, USA). The limit of resistance was defined as mean diameters of lesions < 2 mm (9) or the square root of the area < 1.8 mm², 10 d after inoculation. CHIBI (9) defined a < 2 mm lesion a hypersensitive reaction because the lesions did not increase in size with time and had no haloes. The centres tended to be parchment-like compared to the dark brown centres of lesions where the pathogen was active.

RESULTS

Experiments 1, 2 and 3

Race 0 (KY) which was in all morphological and biochemical aspects, *Pseudomonas syringae* pv. *tabaci*

Table 3.
Reaction (lesion size) on a range of indicator tobacco cultivars of races of *Pseudomonas syringae* pv. *tabaci* Tox+, 10 d after inoculation

Cultivar	Source of resistance	Lesion size ($\sqrt{\text{area}}$ (mm))		
		Race 0 (KY)	Race 1 (PW155)	Race 2 (PW252)
Kutsaga E1	rrll	4.63	4.63	5.21
Kutsaga M10	rrLL	0	5.05	4.54
Burley 21	rrLL	0	5.35	5.29
Kutsaga 35	RrLI	0	0	5.67
WZ	RRll	3.97	0	4.52
WZ-ms	RRll	4.35	0	5.18
Kutsaga RK6	Rrll	4.36	0	5.29
SED (race $\times$ cv.)		0.41 ^a		
t (0.05,60 df) = 2.000				

^a $P < 0.05$.

Tox+, caused typical wildfire symptoms on K E1, WZ and male sterile WZ, and on K RK6, but not on Bu 21, K M10 or K 35. Race 1 (PW155) caused typical symptoms on K E1, Bu 21 and K M10 but not on either WZ line or K 35. Race 2 (PW252) caused wildfire symptoms on all cultivars. Representative data is presented in Tables 1 and 2.

Experiments 4 and 5

The reaction of race 0 (KY), race 0 (TN), race 0 (MD), race 1 (PW155) and race 2 (PW252) were compared on four indicator cultivars, K E1, K M10, WZ and K 35 in experiment 4 and on six (including Bu 21 and K RK 6) in experiment 5.

Race 1 (PW155) caused wildfire symptoms on K E1, K M10 and Bu 21 but there were no symptoms on WZ, K RK6 or K 35. Race 2 (PW252) caused severe wildfire on all cultivars. Race 0 (KY) caused wildfire symptoms on K E1, WZ and K RK6 (Table 3).

Race 0 (TN) caused severe symptoms on K E1, and symptoms on K M10, WZ and K RK6.

The reaction to race 0 (MD) was variable. There was a slight reaction on K E1 but not on the other indicator cultivars in experiment 4 (data not presented), but in experiment 5, (Table 4) there were wildfire lesions on all cultivars.

Experiment 6

All four isolates of race 0 (KY), i.e. the original culture in sterile distilled water, stored at 4 °C, a reisolation from beads (Protect Beads®, TSC Ltd) stored at -70 °C, a reisolation from lesions on plants, and a suspension of the original in quarter strength Ringers solution, inoculated onto the four indicator cultivars K E1, K M10, WZ and K 35 reacted similarly, overcoming resistance

Table 4.
Reaction (lesion size) on range of indicator tobacco cultivars of three USA "race 0" isolates of *Pseudomonas syringae* pv. *tabaci* Tox+

Cultivar	Source of resistance	Lesion size ($\sqrt{\text{area}}$ (mm))				
		Race 0 (KY)	Race 0 (MD)	Race 0 (TN)	Race 1 (PW155)	Race 2 (PW252)
Kutsaga E1	rrll	2.90	9.40	10.70	5.30	9.90
Kutsaga M10	rrLL	0.90	4.40	4.20	9.00	11.00
Burley 21	rrLL	0.20	2.30	1.70	5.80	8.30
Kutsaga 35	RrLI	1.00	4.30	2.10	0.00	8.90
WZ	RRll	4.00	6.10	6.90	0.00	11.00
Kutsaga RK6	Rrll	3.50	5.60	5.40	0.00	12.00
SED (race $\times$ cv.)			1.09 ^a			
t (0.05,87 df) = 2.00						

^a $P < 0.05$.

Table 5.
Comparison of reaction on indicator tobacco cultivars of original and reisolutions of race 0 (KY) of *Pseudomonas syringae* pv. *tabaci* Tox+

Cultivar	Source of resistance	Lesion size ($\sqrt{\text{area}}$ (mm))				
		Isolate ^a				
		1	2	3	4	Mean
Kutsaga E1	rrll	13.08	12.36	12.64	13.56	12.91
Kutsaga M10	rrLL	0.31	0.00	0.55	0.68	0.39
Kutsaga 35	RrLI	1.76	0.27	0.39	1.96	1.10
WZ	RRll	9.87	8.99	10.87	11.92	10.41
Mean		6.26	5.40	6.11	7.03	6.20
SED	Isolate			0.588		
	Cultivar			0.588 ^b		
	Interaction			1.175		
t (0.05,45 df) = 2.016						

^a1 = Original culture in sterile distilled water (SDW), stored at 4 °C; 2 = reisolation from beads stored at -20 °C;

3 = reisolation from plants; 4 = suspension of original stored in Ringers solution at 4 °C.

^b $P < 0.05$.

derived from *N. rustica*, but not that derived from *N. longiflora* (<0.05) (Table 5).

A summary of the results of the reaction to race 0 (KY) on indicator cultivars in six experiments is given in Figure 3.

DISCUSSION

The resistance genes from *N. longiflora* to race 0 and *N. rustica* to races 0 and 1 are single dominant genes (7,11). The determination of race 0 of *Ps. syringae* pv. *tabaci* has been based on the above information that race 0 cannot infect plants containing the gene for wildfire resistance derived from *N. longiflora* or from *N. rustica*. In 1973, race 1, which was able to overcome the resis-

tance from *N. longiflora* (12) was identified. The only previously known pathogenic isolate in Zimbabwe was therefore classified as race 0. From then until the last isolate of race 0 made from field tobacco in Zimbabwe in 1996, there was an abundance of pathogenic *Ps. syringae* pv. *tabaci* Tox+ isolates that fitted this category (9). More recently, race 2 which is able to overcome resistance to *Ps. syringae* pv. *tabaci* conferred by genes from both *N. longiflora* and *N. rustica* (8), has become the dominant race.

The race 0 we received from the Kentucky collection, 0 (KY), did not conform with the above definition of race 0. It was able to overcome the resistance to *Ps. syringae* pv. *tabaci* conferred by the gene from *N. rustica* (Figure 3). Cultivars containing resistance genes from both *N. longiflora* and *N. rustica*, were resistant to

Figure 3. Cultivar reaction (wildfire symptoms) over 6 experiments to *Pseudomonas syringae* pv. *tabaci* Tox+ race 0 (KY). Key to resistance source Rr = *N. rustica*; LI = *N. longiflora*

Table 6. Disease reaction of indicator cultivars of tobacco to races of *Pseudomonas syringae* pv. *tabaci* Tox+

Indicator cultivar	Resistance gene source			
	rrll (K E1)	rrLL (K M10, Bu 21)	Rrll (WZ)	RrLI (K 35)
Race 0	Suscept.	Resistant	Resistant	Resistant
Race 1	Suscept.	Suscept.	Resistant	Resistant
Race 2	Suscept.	Suscept.	Suscept.	Suscept.
Race (KY)	Suscept.	Resistant	Suscept.	Resistant
Race (TN)	Suscept.	Suscept./Res.	Suscept.	Resistant

to race 0 (KY) because of the presence of the *N. longiflora* gene. However, race 0 (TN) was generally much more virulent on all cultivars and overcame, to some extent, the resistance conferred by *N. longiflora* and *N. rustica*. The relative reaction of the various indicator cultivars to *Ps. syringae* pv. *tabaci* is summarised in Table 6. Race 0 (MD) reactions were variable suggesting that the isolate is not pure, but a mixture of races. The reaction of race 0 (TN) needs to be reinforced by further tests.

We suggest that the isolate of *Ps. syringae* pv. *tabaci* from Kentucky, race 0 (KY), should be classified as race 3, because its reaction on indicator cultivars is different from races 0, 1 and 2. Race 3 is able to overcome the resistance conferred by genes from *N. rustica* but not the resistance conferred by genes from *N. longiflora*. A data search into race classification experiments in Zim-

babwe over the past 9 years has highlighted two isolates of the bacterium which could have been classified as race 3, one in 1992 and the other in 1994. Unfortunately, both isolates lost their viability and were discarded. No other local isolates have given a race 3-type reaction. The isolate, race 0 (KY), from which numerous subcultures have been made and subjected to the tenets of Koch's postulates on many occasions over the past 15 months, is stable, always giving the same reaction. The newly defined race has been very useful in our resistance testing because it establishes whether a particular genotype is carrying *N. longiflora* as well as *N. rustica* resistance genes. With the races available, the *N. longiflora* genes were masked by the *N. rustica* genes if the genotype carried both sets of genes.

Acknowledgements: We thank Mr Brian Smeeton (RJR North Carolina, USA) for sourcing the "race 0" isolates for us. We acknowledge the excellent technical assistance of the plant pathology staff especially Gift Mushonga and Terttu Sigobodhla.

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