CORESTA REPORT ON

Determination of allelism between different sources of Black Shank race 0 (*Phytophthora nicotianae)* resistance and linkage studies between Black Shank race 0 and Angular Leaf Spot race 1 *(Pseudomonas syringae pv. tabaci* Tox-) resistance in tobacco (*Nicotiana tabacum)*

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1. Determination of allelism between different sources of black shank race 0 (*Phytophthora nicotianae)* resistance in tobacco

SUMMARY

Black shank is an important disease affecting all types of cultivated tobacco. Resistance to black shank disease has been introgressed into *N. tabacum* from some wild species among them *N. longiflora* and *N. rustica*. For purposes of parental selection in black shank breeding programmes, it is not known whether the black shank resistance gene from *N. longiflora* in the line L8 can be taken as an allele of the black shank resistance gene from *N. rustica* in the line WZ. The objective of this study was therefore to determine whether the black shank resistance gene from *N. longiflora* is allelic to that from *N. rustica*. The line L8 was hybridised with the line WZ in the summer of the 2002/3 season. Some F_1 (L8 x WZ) plants were selfed and others were crossed with KE1, a black shank susceptible line to create F_2 and testcross populations. The F_2 and testcross populations together with their F_1 and parental populations were exposed to *P. nicotianae*, the black shank pathogen, in the field and in the greenhouse. In all the tests made, segregation for black shank disease was observed in the F_2 and testcross populations. We concluded that the black shank resistance gene from *N. longiflora* in L8 is not allelic to that from *N. rustica* in WZ. Breeders can therefore not consider the two as alleles of each other in black shank breeding programmes.

INTRODUCTION

Black shank is a destructive root and stem rot disease of all types of cultivated tobacco. It occurs worldwide and losses can reach 100 per cent (Shew and Lucas, 1990). Black shank tends to occur sporadically and in some instances pieces of land are known to have been abandoned for tobacco production because of black shank incidence. It is caused by a fungal pathogen namely *Phytophthora nicotianae*. Diseased plants are noticeable by stunting with wilting of leaves that normally turn yellow and hang down the stalk. Roots and the lower part of the stalk (the shank) become black. Later, tissues in the centre part of the lower stalk also become blackened. The pith normally dries and becomes segmented into discs.

Breeding for black shank resistant varieties is a very important way of combating the diseases given the prohibitive costs of chemicals in modern day farming. Valleau, Stokes and Johnson (1960) transferred black shank race 0 resistance from *N. longiflora* into a burley cultivar L8 whilst Chaplin (1962) transferred black shank race 0 resistance from *N. plumbaginifolia* to a stable flue-cured breeding line, PD 468. At the Tobacco Research Board (T.R.B.), Zimbabwe, black shank race 0 resistance was transferred from *N. rustica* into a stable flue-cured breeding line named WZ (unpublished data). Even though great success has been achieved in transferring black shank resistance from wild species into *Nicotiana tabacum,* plant breeders are still yet to fully explore and understand black shank inheritance.

Several reports have indicated that the *N. longiflora* derived black shank race 0 resistance is controlled by a single dominant gene (Valleau, Stokes and Johnson, 1960). Many years of experience with the breeding line WZ at the T.R.B. have also indicated that the *N. rustica* derived black shank race 0 resistance is controlled by a single dominant gene (unpublished data). Whilst the mode of transmission for black shank race 0 resistance derived from *N. plumbaginifolia, N. longiflora and N. rustica is* generally understood and believed to be qualitative, studies pertaining to the genetic relationships existing between these different resistance sources have not yet been exhaustive. For example, Stavely (1979) reported that the genetic locus for black shank resistance derived from *N. longiflora is* the same as that derived from *N. plumbaginifolia*. In this report, Stavely suggested allelism for the black shank race 0

resistance genes from *N. longiflora* and *N. plumbaginifolia*. Since then no work is known to have been done, documented and published regarding the possibility of allelism between the *N. rustica* derived black shank resistance gene and the *N. longiflora* derived black shank resistance gene. Such studies of allelism are particularly important in disease resistance breeding programmes as they guide breeders on parental selection. They are also important in predicting the possibility and extent of getting susceptible recombinants in segregating populations depending on whether the resistance genes of parents used in a programme are allellic or not. The objective of this study was therefore to determine the possibility of allelism for the black shank race 0 resistance genes derived from *N. longiflora* and *N. rustica.*

MATERIALS AND METHODS:

Allelism tests were carried out in the field at Kutsaga Research Station and three times in the greenhouse at Banket Research Station. Both stations belong to the Tobacco Research Board.

Plant materials

The breeding line L8 with dominant monogenic resistance, designated *Phl,* to black shank (black shank referred to the race 0 in this study) from *N. longiflora,* the breeding line WZ with dominant monogenic resistance from *N. rustica,* designated *Phr,* and a recessive susceptible tester line KE1 (genotype *phph*) were used.

Creation of test populations

The breeding line L8 was hybridised with the breeding line WZ in the summer of the 2002/2003 season. In winter 2003, the F_1 (L8 x WZ) hybrid was allowed to self-pollinate by bagging its flowers to generate some F_2 generation seed. Some F_1 (L8 x WZ) hybrid plants were pollinated with KE1, a tester line susceptible to black shank, to generate some testcross seed. The F_2 and testcross generations were then repeatedly used to test for allelism both in the field and in the greenhouse.

Field study

At the Banket Research Station of the Tobacco Research Board, seedlings of WZ, L8, KE1, F_1 (L8 x WZ), F_1 (L8 x WZ) x KE1 and F_2 (L8 x WZ) were raised using the conventional seedling production method. The above six entries were then planted in a black shank infested land at Kutsaga Research Station, Harare, in a randomised complete block design. The trial was bordered with Ky 14, a line highly susceptible to Black Shank disease, which was used as a susceptible check. Each plant was topdressed with 5 grams of ammonium nitrate fertiliser (34.5% N) before inoculation.

Greenhouse study

In the greenhouse, parental, F_1, F_2 and testcross seed was sown in float trays. At about four weeks after sowing, the seedlings were then transplanted into marked individual pots as intact root seedlings and basal fertilised at the rate of 2 g/pot. They were topdressed with ammonium nitrate fertiliser (34.5% N) at the rate of 1 g/pot as soon as they started showing proper establishment. Plants were sprayed twice with an insecticide. A total of three separate allelism tests were carried out in the greenhouse using the F_2 (L8 x WZ) and F_1 (L8 x WZ) x KE1 as the allelism test populations.

Phytophthora nicotianae race 0 inoculum preparation

The inoculum was prepared in accordance with the guidelines developed at the

Tobacco Research Board by Masuka and Sigobodhla (1996). Isolates were grown on Oat Meal Agar for 7 days in darkness, at 25 °C in petri dishes. The mycelial tufts were aseptically collected and placed onto sterile pond water in glass. The suspension was incubated at 25 °C under constant, fluorescent light in the lightbank (light source 50 cm above the inoculum) for 48 hours. The inoculum was then given a "cold shock " in a deepfreezer at – 180 °C for 25 minutes and left to warm up on the bench top at room temperature in the constant temperature room for 60 minutes to release the zoospores from sporangia. Three 1 ml samples were aseptically placed in test tubes and were shaken using a "vibrofix" for 15 seconds at 2000/minute to encyst the zoospores so as to enable the counting procedure. A haemacytometer under the microscope was used to count the zoospores. The inoculum was then diluted to a concentration of 1×10^4 cfu/ml and used for inoculation.

Inoculation and disease assessment

In the field study, each field plant was inoculated, at three weeks after planting, with 10 ml of 1×10^4 concentration of *P. nicotianae* as a way of adding up to the natural inoculum and also evening up the distribution of inoculum in the trial area. Inoculation was done by slightly dibbling the soil around the base of the plant and pouring the *P. nicotianae* inoculum around the plant base. The inoculum was soil covered to avoid dehydration. This method has been extensively used at the Tobacco Research Board in black shank trials. The trial was monitored weekly beginning two weeks after inoculation until 13 weeks after inoculation. On assessing plants for resistance / susceptibility to black shank disease, the mid morning was preferred as some susceptible plants tended to show signs of recovery from wilting especially in the mornings following rain. Plants were recorded as susceptible when they showed the characteristic yellowing and wilting of leaves that hang down the stalk and the blackening of the lower stem. Plants were not scored as their reaction to disease was more qualitative than quantitative. Plants that did not show the above symptoms were recorded as resistant.

In the greenhouse experiments, each plant was inoculated at 10 days alter transplanting with 10 ml of 1×10^4 cfu / ml of *Phytophthora nicotianae*. Plants were also inoculated by first dibbling around the base of the plant, pouring the inoculum around the base of the plant and then covering up to avoid dehydration. Plants were, from thereon, watered just to keep the soil moist and avoid leaching of the inoculum. Disease assessment started at two weeks after inoculation. Plants that showed the characteristic yellowing and wilting of leaves that hang down the stalk were recorded as susceptible to black shank disease and those that did not show these symptoms were recorded as resistant. Plants that did not show obvious symptoms were up-rooted to see the extent of root damage. Plants that had blackened damaged roots were recorded as susceptible.

Collected data was tabulated and each generation had its plants classified as either resistant or susceptible to black shank disease. The presence of segregation in the testcross and F_2 generations was then used to determine the allelic relationship for black shank resistance genes from *N. rustica* (as in the breeding line WZ) and from *N. longiflora* (as in the breeding line L8). If the black shank race 0 resistance genes from *N. longiflora* and *N. rustica* were allelic, then no black shank susceptible plants would be expected in the F_2 and testcross generations. If non-allelic, then some plants in the F_2 and testcross generations would be expected to be susceptible to black shank disease.

RESULTS

In the field tests, black shank symptoms were observed on KE1, the susceptible control, and on some plants in the F_2 generation and the cross of F_1 (L8 x WZ) to the susceptible cultivar KE 1 whilst all F_1 generation plants were resistant to black shank. More susceptible plants were observed in the testcross generation than in the F_2 generation (table 1). All Ky 14 plants that

were planted in the field to border the trial as susceptible checks started showing black shank symptoms within two weeks of inoculation and they eventually died of black shank. Ky 14 showed its highly susceptible nature which it has always shown in other black shank disease trials conducted at the Tobacco Research Board in previous years. Both parents, L8 and WZ, showed resistance to black shank disease.

In the greenhouse, plants showed symptoms earlier than in the field probably as a result of better control of the environmental conditions. The reaction of the F_2 and testcross plants emulated field observations in that segregation was observed in the F_2 and testcross generations as some plants were resistant whilst others were susceptible. The appearance of black shank susceptible plants in the F_2 and testcross populations was a clear indication that the *N. rustica* and *N. longiflora* black shank resistance genes are not allelic. In the second greenhouse tests, all the KE1 plants were susceptible to black shank disease and the parents, L8 and WZ, maintained their resistance to black shank as was observed in the field tests. Segregation was again observed in the F_2 and testcross generations (table 3). The third greenhouse test involved one segregating generation, the testcross. In this test, segregation was also observed in the testcross generation (table 4).

Table 1. Reaction of parental, F_1, F_2 and testcross populations to *P. nicotianae* in field allelism studies (Resistant - R, Susceptible - S)

	Reaction to *P. nicotianae (* black shank race 0)	
Generation	R	S
L8 (*N. longiflora* source)	30	0
WZ (*N. rustica* source)	30	0
KE1	0	30
F_1 (L8 x WZ)	30	0
F_2 (L8 x WZ)	389	11
F_1 (L8 x WZ) x KE1	357	41

Table 2. Reaction of parental, F_1, F_2 and testcross populations to *P. nicotianae* in greenhouse allelism studies (Resistant - R , Susceptible - S) - first greenhouse test

	Reaction to *P. nicotianae (* Black Shank race 01	
Generation	R	S
L8 (*N. longiflora* source*)*	12	0
WZ (*N. rustica* source)	12	0
KE1	0	12
F_1(L8 x WZ)	12	0
F_2 (L8 x WZ)	156	4
F_1 (L8 x WZ) X KE1	109	11

Table 3. Reaction of parental, F_1, F_2 and testcross populations to *P. nicotianae* in greenhouse allelism studies (Resistant - R , Susceptible - S) - second greenhouse test

	Reaction to *P. nicotianae* (Black shank race 0)	
Generation	R	S
L8 *(N. longiflora source)*	12	0
WZ (N. rustica source)	12	0
KE1	0	12
F_1 (L8 x WZ)	12	0
F_2 (L8 x WZ)	154	6
F_l (L8 x WZ) X KE1	103	17

Table 4. Reaction of parental, F_1 and testcross generations to *P. nicotianae* in greenhouse allelism studies (Resistant - R , Susceptible - S) - third greenhouse test

	Reaction to *P. nicotianae (* Black Shank race 0)	
Generation	R	S
L8 *(N. longiflora* source)	30	0
WZ (N. rustica resistance)	30	0
KE1	0	30
F_1 (L8 x WZ)	30	0
F_1 (L8 x WZ) x KE1	187	43

DISCUSSION

Allelism studies have been carried out before for several genetic reasons, not on tobacco alone but also on wheat, soyabeans and a whole lot of other crops. Johnson, Wolff and Wernsman (2002) recently used the concept of allelism to determine the origin of dominant black shank resistance gene, *Ph*, found in the cultivar Coker 371-Gold. They also managed to prove that the black shank resistance gene *Php* from *N. plumbaginifolia* is not allelic to the black shank resistance gene, *Phl*, from the closely related species *N. longiflora* though the two genes can be said to be homologous in function thus putting to rest the long standing debate of homology between these two genes.

We present in this paper four sets of field and greenhouse data suggesting that the black shank resistance gene from *N. longiflora* found in the tobacco line L8 is not allelic to the black shank resistance gene from *N. rustica* found in the tobacco line WZ. Segregation in the F_2 (L8 x WZ) and [F_1(L8 x WZ) x KE1] populations in which plants susceptible to black shank were observed is an indication of the occurrence of recombination thus suggesting that the black shank resistance genes in L8 and WZ are on different loci. Had the two genes been allelic, then the F_2 (L8 x WZ) population would have consisted only of plants with either of three genotypes relative to black shank resistance *i.e.* either a (1) gene pair consisting of both alleles of the *N. longiflora* source, a (2) genotype made up of both alleles from the *N. rustica* source or a (3) genotype made up of a combination of the *N. longiflora* allele and the *N. rustica* allele. These genotypes can be symbolically represented as *Phlphl, PhrPhr* and *PhlPhr* respectively. All these genotypes would be made of dominant black shank resistance genes rendering all the F_2 plants resistant if an allelic situation was prevailing. Similarly, the [F_1(L8 x WZ) x KE1] population

would also be expected to consist only of black shank resistant plants since all gametes produced from the F_1 (L8 x WZ) generation plants would be expected to carry dominant black shank resistance genes (either *Phl* or *Phr)* such that when fertilised with KE1 gametes carrying the recessive gene, *ph,* all the [F1 (L8 x WZ) x KE1] plants would be expected to be resistant by virtue of the dominant resistance genes masking the recessive genes. The same concept was used by Zeller, Lutz and Stephan (1993) when they studied the location of genes for resistance to powdery mildew in common wheat (*Triticum aestivum L.).* They crossed the wheat cultivar Ralle possessing powdery mildew resistance gene designated *Mlk* (Heun and Fischbeck, 1987) but of unknown genetic location with near isogenic wheat lines Asosan/8*Cc, Chul/8*Cc and Sonora/8*Cc, whose powdery mildew resistances were known to be governed by alleles at the *Pm3* locus. All plants in the resulting F_2 populations were found to be resistant to powdery mildew. Basing on the response of the F_2 populations, the authors concluded that the gene *Mlk* in Ralle is allelic to the *Pm3* locus. In our study, however, the appearance of black shank susceptible plants (a result of recombination events between two loci) in the F_2 and testcross populations is a clear indication that the black shank resistance genes in L8 and WZ are on different loci. We therefore conclude that the black shank resistance genes in L8 (from *N. longiflora)* and WZ (from *N. rustica)* are not allelic.

The non-allelism of black shank resistance genes from *N. longiflora* and *N. rustica* is probably so as *N. rustica* is not presumed to be closely related to *N. longiflora* or any other members of section Alatae (Ramsey Lewis, personal communication). A classic study by Moav (1958) indicated that when genes are transferred from one species to another, residual chromosome homology can be involved in determining the exact position in which the alien gene will be inserted in the recipient genome. Moav (1958) also showed that even though residual chromosome homology tends to make interspecific introgression non-random, independent interspecific transfers of the same alien gene into the same recipient genome do not necessarily result in gene insertion on the same chromosome or same locus in the recipient genome. Residual chromosome homology tends to be stronger in more closely related species and tends to be weaker as species become more distantly related. Johnson, Wolff and Wernsman (2002) discovered that the black shank resistance introgressions from *N. longiflora* and *N. plumbaginifolia* into *N. tabacum* were done on the same *N. tabacum* chromosome. This could have been possible as a result of residual chromosome homology since *N. longiflora* and *N. plumbaginifolia* are very closely related species. It is, in fact, still debatable whether *N. plumbaginifolia is* actually a distinct species. In the case of the introgressions involving the distantly related *N. longiflora* and *N. rustica* species, one would argue that other than just residual chromosome homology, the possibility of random translocation having been involved in the introgressions is quite high thus increasing the chances of gene insertion on different chromosomes or loci during these separate introgressions. The non-allelism of black shank resistance genes in L8 and WZ should therefore be unsurprising. Given the high frequencies of black shank susceptible plants in the F_2 and testcross populations tested, it is likely that the two genes are on different chromosomes. If the two genes happen to be on the same chromosome, then the loci are far apart since a lot of recombination was affordable. Higher chances are, however, that they are on different chromosomes as according to Valleau, Stokes and Johnson (1960) having worked with the *N. longiflora* factor for black shank resistance for nine years, they reported that the breeding line L8 appeared to have a pair of *longiflora* chromosomes substituted for a pair of *tabacum* chromosomes.

In the case of *the N. rustica* black shank resistance in the breeding line WZ, the introgression could have only taken place mainly through three possibilities; either a *N. rustica* chromosomal pair carrying the black shank resistance locus was added to the *N. tabacum* genome, a pair of *N. tabacum* chromosomes was substituted for a pair of *N. rustica* chromosomes carrying black shank resistance or a chromosomal segment of *N. rustica* genome

carrying the black shank resistance locus was exchanged with a *N. tabacum* chromosomal segment. The fact that in L8, the black shank resistance locus is on a *N. longiflora* chromosome gives a strong suggestion that the two loci are on different chromosomes since in WZ the black shank locus can only be either on a *N. rustica* chromosome (if a pair of *N. rustica* chromosomes was added to the *N. tabacum* genome or if a pair of *N. tabacum* chromosomes was substituted for a pair of *N. rustica* chromosomes) or on a *N. tabacum* chromosome (if a *N. rustica* segment was substituted for a *N. tabacum* segment in the *N. tabacum* genome). It is apparent and recommendable, therefore, that breeders may not take the black shank resistance gene in the breeding line WZ as an allele of the black shank resistance in the breeding line L8. In the Zimbabwe burley tobacco industry where no black shank resistant cultivars are known to have been developed, it seems as though selection for black shank resistance and the need to plant segregating populations in black shank nurseries (or inoculating populations with *Phytophthora nicotianae)* remains paramount to the breeder should the breeding lines L8 and WZ (or lines with L8 and WZ in their pedigrees) be used in black shank breeding programme as intraspecific parents. Selection for black shank resistance remains important when a breeder uses these two lines as parents since black shank susceptible plants will segregate out in F_2 populations. To lighten the burden of selection using the conventional methods of planting large populations in disease nurseries or using inoculations, which sometimes do not work effectively, breeders world-over are moving towards selection at the molecular level using molecular markers. The discussion of markers is beyond the scope of this study and we shall not take it any further. In an allelic situation, however, the breeder tends to concentrate more on selecting for agronomic and other important traits than concentrating on selecting for black shank resistance because all the F_2 plants will be carrying black shank resistance genes anyway.

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2. Linkage studies between black shank race 0 and angular leaf spot race 1 *(Pseudomonas syringae* pv. *tabaci* Tox-) resistance in tobacco *(Nicotiana tabacum)*

SUMMARY

The tobacco breeding line, WZ, with angular leaf spot (ALS) race 1 resistance was developed at the Tobacco Research Board (TRB), Zimbabwe, following a *N. rustica* introgression programme whose primary objective was to develop a wildfire resistant cultivar for Zimbabwe's tobacco growing Arcturus area. Years of working with the WZ fine as an ALS race 1 resistant fine resulted in the observation that the WZ plants were also showing resistance to black shank race 0 disease in a manner that depicted gene linkage with ALS race 1 resistance although no scientific proof and documentation were available. The objective of this study was therefore to investigate the possibility of gene linkage between black shank race 0 and ALS race 1 resistance in the tobacco breeding line WZ. In the summer of the 2002/3 season, the line WZ was hybridised with KE1, a line susceptible to both black shank and ALS. Some F_1 (KE1 x WZ) plants were selfed to create an F_2 (KE1 x WZ) population and other F_1 (KE1 x WZ) plants were pollinated with KE1 to create a testcross population. The F_2 and testcross populations together with their F_1 and parental populations were exposed to both black shank and ALS diseases in the field and in the greenhouse. The reaction of each plant to each of the two diseases was noted. Chi-square tests for goodness of fit to expected independent assortment ratios were performed on the various populations. The F_1 plants were generally uniformly resistant to both diseases. The F_2 populations conformed to the expected 3:1 ratio when chi-square tests were separately performed on them for each of the two diseases. The testcross populations also conformed to the expected 1:1 ratio when chi-square tests were separately performed on them for each of the two diseases. However, a combined (for the two diseases) co-segregation and linkage analysis showed significant deviation ($P<0.05$) from the expected 9:3:3:1 and 1:1:1:1 independent assortment ratios for F_2 and testcross populations, respectively. We concluded therefore that the failure of the black shank and ALS resistance genes in WZ to segregate independently of each other is because of the linkage that exists between them. Breeders can take advantage of this gene linkage in breeding for combined resistance to black shank race 0 and ALS race 1 (and wildfire).

INTRODUCTION

Angular leaf spot (ALS) is an important tobacco bacterial disease which can cause serious losses in yield and quality (Woodend and Mudzengerere, 1992). In Zimbabwe, ALS is one of the most important tobacco field diseases of economic importance. It is caused by a strain of the pathogen that causes wildfire disease. This pseudomonas is named *Pseudomonas syringae* pv *tabaci* Tox-. Symptoms of ALS are the same both in the nursery and in the field. ALS spots are first water-soaked, later become necrotic, turn brown or black upon drying and have angular margins as reported by Shew and Lucas (1990). At an advanced stage, centres of ALS spots normally fall off giving the leaf a ragged or a shot hole appearance resulting in direct losses to tobacco yield and quality.

It is often observed in breeding work that certain characteristics tend to be inherited together within the same individual. Such characteristics would normally be controlled by separate genes that occur on the same chromosome in which case they are said to be linked. In 1975, a breeding programme was started at the Tobacco Research Board (TRB), Zimbabwe, to develop a cultivar resistant to both races 0 and 1 of wildfire *(Pseudomonas syringae* pv *tabaci* Tox +) and ALS race 1 following the occurrence of wildfire race 1 in the Arcturus area,

Zimbabwe. This breeding programme produced the breeding line WZ through a *N. rustica* introgression that gave it resistance to wildfire (race 0 and 1) and ALS race 1 diseases as was reported by Lyle, Ternouth, Woodend and Matibiri (1988). Later work at the TRB resulted in the discovery that the *N. rustica* introgression in WZ made the WZ plants resistant to both ALS race 1 and black shank race 0 diseases. Plants that do not have this introgression have generally been found to be susceptible to both black shank race 0 and ALS race 1 diseases (unpublished data). It has long been debated therefore that the tobacco breeding line WZ carries both ALS race 1 and black shank race 0 resistances in a linked manner, though no scientific proof and documentation are available. The objective of this study was therefore to determine whether the genes for ALS race 1 and black shank race 0 resistance in the tobacco breeding line WZ are linked or not. If proven to be linked, ALS race 1 and black shank race 0 resistances in tobacco can be bred for simultaneously.

MATERIALS AND METHODS

Plant materials

Linkage tests were carried out once in the field at Kutsaga Research Station and three times in the greenhouse. The flue-cured tobacco breeding line ,WZ , with dominant monogenic black shank resistance (black shank meant the race 0) and dominant monogenic ALS resistance (ALS meant the race 1) and Kutsaga E1 (KE1), a flue-cured breeding line susceptible to both black shank race 0 and ALS race 1 were used.

Creation of test populations

The susceptible breeding line KE1 was hybridised with the resistant breeding line WZ in the summer of the 2002/2003 season. In winter 2003, the F_1 (KE1 x WZ)) hybrid was allowed to self-pollinate by bagging its flowers to give some F_2 generation seed. Some F_1 (L8 x WZ) hybrid plants were testcrossed with KE1 to give some testcross seed. The F_2 and testcross generations together with their parental and F_1 generations were then repeatedly used to test for gene linkage between black shank and ALS resistance both in the field and in the greenhouse.

Field study

Tobacco seedlings of KE1, WZ, F_1 (KE1 x WZ), F_2 (KE1 x WZ) and F_1 (KE1 x WZ) x KE1 were raised at the Banket Research Station of the TRB through the conventional seedling production method. The above five entries were then planted in a black shank infested land at Kutsaga Research Station, Harare, in a randomised complete block design. Two number 22 cups, one each side of the plant, of compound B (4:17:15) were immediately applied as basal fertilisation. Each plant was topdressed with 5 grams of ammonium nitrate fertiliser (34.5% N) before inoculation.

Greenhouse study

The greenhouse linkage experiments were repeated three times and the agronomic practices were basically the same. The parental, F_1, F_2 and testcross seed was sown in float trays. At about four weeks after sowing, the seedlings were transplanted into marked individual pots and basal fertilised with compound B (4:17:15) at the rate of 2 g/pot. They were topdressed with ammonium nitrate fertiliser (34.5% N) at the rate of 1 g/pot as soon as they started showing proper establishment. Seedling establishment was fast as they were transplanted from float trays as intact root seedlings. Plants were sprayed twice with an insecticide.

Phytophthora nicotianae inoculum preparation

The *Phytophthora nicotianae* inoculum for the linkage study was prepared using the same method described under allelism tests.

Pseudomonas syringae pv. *tabaci* inoculum preparation

The *Pseudomonas syringae* pv. *tabaci* inoculum was prepared using guidelines of the method developed at the TRB by Mapuranga (2000). Streaks of isolate PAL 32 were made on King's medium B (KMB) plates and checked for fluorescing after 48 hours. Fluorescing colonies were purified by restreaking on another set of KMB plates. Sterile nutrient broth was then inoculated using 250 ml concentrate for every litre of broth, in flasks. The flasks were covered to exclude light and the bacteria aerated on a shaker for 24 hours at 25 °C. Centrifuge tubes were sterilised with alcohol twice and rinsed with sterile water three times before pouring broth with bacteria into them and balancing them in a centrifuge. The bacteria were washed by centrifuging at 1000 for 15 minutes. The resulting supernatant fluid was decanted and the bacteria resuspended in a quarter strength Ringers' solution. Two washings were done to get rid of toxin. The inoculum was then diluted to a concentration of 1×10^7 colony forming units (cfu)/ml and tested on KE1 and WZ plants for pathogenicity and expected reaction before use on field and greenhouse experiments.

Inoculation and disease assessment

Inoculation and disease assessment were the same for field and greenhouse trials except that greenhouse plants were inoculated at 10 days alter planting and field plants were inoculated at three weeks after planting. Each plant was inoculated with 10 ml of 1×10^4 cfu/ml of *Phytophthora nicotianae* to add up to the natural inoculum and to even up inoculum distribution in the trial area. *Phytophthora nicotianae* inoculation was done by dibbling the soil around the base of the plant and pouring the *Phytophthora nicotianae* zoospores, including mycelial fragments. The inoculum was then covered with soil to avoid dehydration. Since the linkage study sought to investigate the reaction of each particular plant to both black shank and ALS, all plants were also immediately inoculated with *Pseudomonas syringae* pv *tabaci* so that the reaction of each plant to ALS could be assessed before the death of blank shank susceptible plants (for simpler disease assessment, some plants were inoculated with the wildfire form of *Pseudomonas syringae* pv *tabaci,* which produces a conspicuous yellow halo on the leaf of a susceptible plant). *Pseudomonas syringae* pv *tabaci* field inoculation was done using an inoculum concentration of 1×10^7 cfu/ml. A sterilised 20 ml bottle fitted with a sterilised punctured cap was used. The punctured cap on the 20 ml bottle was held against the leaf between the veins. It was then lightly pressed against the leaf to cause some slight damage points which would act as bacterial entry points. The 20 ml bottle would then be tilted whilst still held against the leaf to release the inoculum on the punctured leaf points. Each plant was also inoculated with sterile water as a control. This method has been widely used at the TRB for *Pseudomonas syringae* pv *tabaci* inoculations and has worked satisfactorily (James Lyle, TRB, personal communication).

Plants were monitored for ALS (or wildfire) daily beginning two days alter inoculation until day 10 as under normal circumstances ALS should start showing within 48 hours after inoculation (Desirée Cole, TRB, personal communication). On the final assessment, each plant was recorded as either resistant or susceptible to ALS. Susceptible plants were identified basing on the appearance of brown or black necrotic spots with angular margins around the inoculation points as described by Shew and Lucas (1990). In some instances, these ALS spots would coalesce to form bigger spots. Since ALS resistance is known to be qualitative (Woodend and Mudzengerere, 1992), no scoring was done and plants were assessed basing on the presence (susceptible) or absence of symptoms (resistant) rather than on quantitative differences in

severity of infection (Wannamaker and Rufty, 1989). In experiments where the wildfire form of *Pseudomonas syringae pv tabaci* was used for ease of assessment, the appearance of a conspicuous yellow halo on the inoculation point depicted susceptibility to wildfire (and therefore ALS). As for black shank, plants were monitored weekly beginning two weeks after inoculation until 13 weeks after inoculation. On assessing plants for resistance or susceptibility to black shank disease, the mid morning was preferred as some susceptible plants tended to show signs of recovery from wilting especially in the mornings following rain. Plants were recorded as susceptible when they showed the characteristic yellowing and wilting of leaves that hang down the stalk and the blackening of the lower stem. Plants were also not scored as their reaction to disease was more qualitative than quantitative. Plants that did not show the above symptoms were recorded as resistant.

RESULTS

In the linkage study, plants were first analysed for their reaction to the individual diseases to see whether the individual gene pairs were segregating normally. After individual gene pair analysis, collected data was then subjected to a co-segregation analysis for the two gene pairs to detect the presence or absence of gene linkage between them.

Field studies

Disease expression, particularly the ALS (or its wildfire form), was good in the field as inoculation was timed for February when conditions were ideal. The separate analyses of plant reactions to ALS and black shank diseases showed some good fit to expected ratios (1:1 for testcross and 3:1 for F_2 generation) as shown in tables 1 and 3. The combined analysis for plant reaction to the two diseases, however, showed significant deviation ($P<0.05$) from expected independent assortment ratios (9:3:3:1 for F_2 and 1:1:1:1 for testcross) as shown by the co-segregation and linkage analysis in table 5. Recombination was evident though (table 5).

Greenhouse studies

Disease expression in the greenhouse was also good particularly for the wildfire form of *Pseudomonas syringae* pv *tabaci*. Where the wildfire form was used, disease assessment was simpler (because of the conspicuous yellow halo) than where the ALS form was used.

In the first greenhouse tests, there was a good fit to the expected monogenic inheritance ratios (1:1 for testcross and 3:1 for F2 generation) when a separate analyses for plant reaction to ALS were done (table 2). As for black shank disease, only the testcross had a good fit to a 1:1 ratio, supporting dominant monogenic inheritance, whilst the F_2 generation showed conflicting results as significant deviation ($P<0.05$) from an expected 3:1 ratio was observed (table 4). When a co-segregation and linkage analysis for plant reactions to the two diseases was done, both testcross and F_2 generations showed significant deviations ($P<0.05$) from independent assortment ratios though recombination was still evident in both generations (table 6).

In the second greenhouse tests, both the F_2 and testcross generations showed a good fit to 3:1 and 1:1 ratios, respectively, when separate analyses for plant reactions to each of *Pseudomonas syringae pv tabaci* and *Phytophthora nicotianae* were done (tables 2 and 4). The co-segregation and linkage analyses for resistance to the two diseases again showed significant deviation from expected independent assortment ratios of 9:3:3:1 and 1:1:1:1 respectively at the 5% level of significance but recombinant phenotypes were observed (table 6).

In the third greenhouse tests, the testcross generation showed a good fit to the expected 1:1 independent assortment ratio when plant reactions to each of the two diseases were analysed separately (tables 2 and 4). The co-segregation and linkage analysis for resistance to the two diseases, like in the field and preceding greenhouse tests, showed significant deviation from the

expected independent assortment ratio of 1:1:1:1 at the .5% level of significance (table 6). Recombinant phenotypes were observed again.

Table 1. Segregation ratios for reaction to *P. syringae* pv *tabaci* Tox - alone and chi-square goodness of fit tests (expected 1:1 in testcross and 3:1 in F_2) in field study. Resistant - R, Susceptible -S.

	Reaction to *P. syringae pv tabaci* Tox - (ALS race 1)		
Generation	R	S	Chi-square
KE1	0	40	
WZ	39	0	
F_1 (KE1xWZ)	37	0	
F_1 (KE1xWZ)x KE1	302	312	0.16
F_2 (KE1 x WZ)	480	151	0.39

* indicates significance at 5% level
Critical chi-square value is 3.48 (1 df)

Table 2. Segregation ratios for reaction to *P. syringae pv tabaci* Tox - alone and chi-square goodness of fit tests in greenhouse study (1:1 in testcross and 3:1 in F_2). Resistant – R, Susceptible - S.

	Reaction to *P. syringae pv tabaci* Tox - alone (ALS race 1)		
Generation	R	S	Chi-square
KE1	0	15	
WZ	15	0	
F_1 (KE1xWZ)	15	0	
F_1 (KE1 x WZ) x KE1 - first test	75	67	0.45
F_2 (KE 1 x WZ) - first test	136	44	0.03
F_1 (KE 1 x WZ) x KE 1 - second test	68	72	0.11
F_2 (KE1 x WZ) - second test	129	46	0.15
F_1 (KE1 x WZ) x KE1 - third test	116	98	1.51

* indicates significance at 5% level
Critical chi-square value is 3.84 (1 df)

Table 3. Segregation ratios for reaction to *Phytophthora nicotianae* alone and Chi-square goodness of fit tests in field study (1:1 in testcross and 3:1 in F_2). Resistant - R, Susceptible – S.

	Reaction to *P. nicotianae* alone (Black Shank race 0)		
Generation	R	S	Chi-square
KE1	0	40	
WZ	39	0	
F_i (KE1 x WZ)	37	0	
F_i (KE1 x WZ) x KE1	316	298	0.53
F_2 (KE 1 x WZ)	485	146	1.17

* indicates significance at 5% level
Critical chi-square value is 3.84 (1 df)

Table 4. Segregation ratios for reaction to *Phytophthora nicotianae* alone and chi-square goodness of fit tests in greenhouse study (1:1 in testcross and 3:1 in F_2). Resistant - R, Susceptible - S.

	Reaction to *P. nicotianae* alone (Black shank race 0)		
Generation	R	S	Chi-square
KE1	0	15	
WZ	15	0	
F_1 (KE1xWZ)	15	0	
F_1 (KE1 x WZ) x KE1 - first test	78	64	1.38
F_2 (KE1 x WZ) - first test	147	33	4.27*
F_1 (KE1 x WZ) x KE1 - second test	75	65	0.70
F_2 (KE1 x WZ) - second test	139	36	1.82
F_1 (KE1 x WZ) x KE1 - third test	120	94	3.15

* indicates significance at 5% level
Critical chi-square value is 3.84 (1 df)

Table 5. Co-segregation and linkage analysis for resistance to black shank race 0 and ALS race 1 in test-cross and F_2 generations in field study (1:1:1:1 for testcross and 9:3:3:1 for F_2)

	Generation	
Phenotypic expression	F_1 (KE1 x WZ) x KE1	F. (KE1 X WZ1
B (R) . A (R)	271	451
B (R) . A (S)	45	34
B (S) . A (R)	31	29
B (S) . A (S)	267	117
Chi-square	348.31*	306.03*

*indicates significance at 5 % level
Critical chi-square value is 7.82 (3 df)

B (R) . A (R) - resistant to black shank race 0 and ALS race 1 - *Parental phenotype*
B (R) . A (S) - resistant to black shank race 0 but susceptible to ALS race 1 - *Recombinant*
B (S) . A (S) - susceptible to black shank race 0 but resistant to ALS race 1- *Recombinant*
B (S) . A (S) - susceptible to black shank race 0 and ALS race 1- *Parental phenotype*

Table 6. Co-segregation and linkage analysis for resistance to black shank race 0 and ALS race 1 in test-cross and F_2 generations in greenhouse studies (1:1:1:1 for testcross and 9:3:3:1 for F_2)

	Generation	
Phenotypic expression	F_1 (KE1 x WZ) x KE1	F_2 (KE1 X WZ)
First test		
B (R) . A (R)	70	118
B (R) . A (S)	8	29
B (S) . A (R)	5	18
B (S) . A (S)	59	15
Chi-square	96.52*	12.04*
Second test		
B (R) . A (R)	66	124
B (R) . A (S)	9	15
B (S) . A (R)	2	5
B (S) . A (S)	63	31
Chi-square	100.28*	76.68*
Third test		
B (R) . A (R)	103	
B (R) . A (S)	17	
B (S) . A (R)	13	
B (S) . A (S)	81	
Chi square	115.49*	

* indicates significance at 5 % level
Critical chi-square value is 7.82 (3 df)

B (R) . A (R) - resistant to black shank race 0 and ALS race 1 - *Parental phenotype*
B (R) . A (S) - resistant to black shank race 0 but susceptible to ALS race *1 - Recombinant*
B (S) . A (S) - susceptible to black shank race 0 but resistant to ALS race 1- *Recombinant*
B (S) . A (S) - susceptible to black shank race 0 and ALS race 1- *Parental phenotype*

DISCUSSION

In the tests for linkage, it is apparent that ALS resistance is controlled by a single dominant gene since most of the testcross and F_2 populations (except the F_2 population in the second greenhouse tests) that were tested conformed to the expected Mendelian monogenic ratios of 1:1 and 3:1 respectively (tables 1 and 2). This confirmed work previously done by Woodend and Mudzengerere (1992). Inconsistent ratios such as in the F_2 population of the second greenhouse tests, normally feature in studies such as these. A number of reasons including misclassification, loss of pathogen virulence, improper inoculation of the pathogen or lack of ideal conditions for the proper development of the pathogen among other reasons have been cited in several studies as contributory to such inconsistencies. It is possible that in our study one or a combination of these factors may have caused this departure from the expected 3:1 ratio in this F_2 population. Uniform resistance to black shank disease observed in the F_1 (KE1 x WZ) hybrids and conformation to monogenic ratios of 1:1 and 3:1 in the testcross and F_2 populations (tables 3 and 4) also serves as confirmation of unpublished sources that indicate that black shank resistance introgressed from *N. rustica* into *N. tabacum,* through the breeding line WZ, is controlled by a single dominant gene. Wernsman and Rufty (1987) also noted that disease resistance genes of interspecific origin are usually monogenic and express complete dominance in tobacco.

The conformation of most testcross and F_2 populations to expected monogenic ratios relative to black shank and ALS resistance when plant reaction to each of the two diseases was analysed separately is a clear indication that each gene pair was segregating normally. In explaining the procedure for the detection of linkage, Strickberger (1976) noted that when each of two gene pairs segregates as expected, the failure for the combined gene pairs to segregate independently of each other is a result of linkage between them. If Strickberger's observation is anything to go by, then in our study the failure of the combined black shank and ALS resistance gene pairs to segregate independently of each other (tables 5 and 6) is a result of linkage between them since the individual gene pairs were segregating normally (tables 1 to 4). Other than linkage, one may argue that the independent assortment ratios of 9:3:3:1 and 1:1:1:1 were not observed in the F_2 and testcross generations as a result of pleiotropism, where one gene locus may control both black shank and ALS resistance. In this study, however, pleiotropism is ruled out because of the evidence of recombinant phenotypes in the F_2 and testcross populations. A single locus cannot give recombinant phenotypes but two loci can. In our study, the two gene pairs did not segregate independently of each other. We conclude therefore that black shank and ALS resistance genes in the tobacco breeding line WZ are linked. Recombinant phenotypes in the F_2 and testcross populations are a result of genetic cross-over. We recommend that in Zimbabwe, where ALS and black shank are important tobacco diseases, the breeding line WZ can be used as an important parental line in breeding for combined resistance to black shank and ALS especially in burley tobacco where varieties with such combined resistance are still lacking.

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