

ABSTRACT

VONTIMITTA, VIJAY. Genetic Mapping of Genes Controlling Two Leaf Surface Chemistry Traits and Identification of Quantitative Trait Loci (QTL) Associated with Resistance to *Phytophthora nicotianae* in Tobacco (*Nicotiana tabacum* L.). (Under the direction of Dr. Ramsey Lewis.)

The ultimate objective of a tobacco breeder is to use genetics to improve traits contributing to improved crop production efficiency and that affect product quality. Two leaf surface chemistries, *cis*-abienol and β -methylvaleric acid-containing sucrose esters (BMVSEs), are known to be associated with organoleptic properties of tobacco products and may contribute to resistance against insect pests and fungal pathogens. The black shank disease, caused by *Phytophthora nicotianae*, causes severe crop losses annually in many tobacco growing regions of the world. Multiple races of this pathogen make it difficult to develop tobacco cultivars with high levels of resistance to all races. In the current study, an investigation was carried out to gain insight into the genetics controlling two leaf surface chemistry traits and black shank resistance using the assistance of microsatellite markers. A cigar tobacco line, 'Beinhart 1000,' was chosen as a source of the ability to accumulate the two leaf surface chemistries and as a source of high levels of resistance against multiple races of the black shank pathogen. A doubled haploid population of 118 lines was developed from a cross between Beinhart 1000 and the flue cured cultivar, Hicks, which lacks the ability to produce the two leaf surface chemistries and is highly susceptible to the black shank disease. Field evaluations in disease nurseries were conducted using replicated trials in three environments. A genetic linkage map with 24 linkage groups was constructed by genotyping the mapping population with 190

microsatellite markers. Both genes controlling leaf surface chemistry traits were positioned on linkage group 4 and were separated by a genetic distance of 8.5 centimorgans (cM). Multiple interval mapping (MIM) analysis identified a total of six quantitative trait loci (QTL) (on linkage groups 2, 4, 8, 9, 11, and 14) associated with black shank disease resistance in Beinhart 1000. Two QTLs on linkage groups 4 and 8 were identified as having major effects and explained 20.5% and 23.5% of the phenotypic variation for end percent survival, respectively. The QTL on linkage group 4 was found to be strongly linked with the gene, *Abl*, controlling *cis*-abienol accumulation. Growth chamber evaluations of the mapping population with specific races (Race 0 and Race 1) revealed that the two major QTLs on linkage groups 4 and 8 were significantly associated with resistance to both races. The QTL on linkage group 8 was observed to have consistent large effects in both field and growth chamber evaluations. An attempt was also made to draw genetic comparisons between Beinhart 1000 and the currently most widely exploited source of black shank disease resistance, 'Florida 301'. A mapping population of 125 recombinant inbred lines (RILs) developed from the cross between Florida 301 and Hicks was used for this study. Microsatellite markers representing the six genomic regions identified to be associated with resistance in Beinhart 1000 were tested for their association with resistance in Florida 301. Markers on linkage group 8 and 2 were identified to be associated with resistance in Florida 301. Markers on linkage group 4 and other regions of interest were not significantly associated with resistance in Florida 301. Results may be useful for transferring the two leaf surface chemistry traits and black shank resistance into new cultivars using marker assisted breeding.

Genetic Mapping of Genes Controlling Two Leaf Surface Chemistry Traits and
Identification of Quantitative Trait Loci (QTL) Associated with Resistance to
Phytophthora nicotianae in Tobacco (*Nicotiana tabacum* L.)

by
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DEDICATION

To my beloved parents

BIOGRAPHY

Vijay Vontimitta was born in Andhrapradesh, a state in the southern part of India. Being interested in biology from school days, he opted to study biology at the high-school level, and later decided to pursue an undergraduate degree in agricultural sciences. During his undergraduate studies, he gained knowledge in various aspects of field crops cultivation, and also became interested in genetics and plant breeding. After obtaining a bachelor's degree in agriculture, he received a junior research fellowship awarded by the Indian Council of Agricultural Research to pursue a master's degree in genetics and plant breeding. For his master's research project, under the guidance of Dr. M. V. C. Gowda, he studied aspects of resistance breeding for southern blight disease in peanuts. After the completion of his master's degree, he worked in a sunflower breeding program for one year. In the fall of 2005, he came to North Carolina State University to pursue a doctoral degree in genetics and plant breeding under the guidance of Dr. Ramsey Lewis. As a doctoral student, he received excellent education in statistics and quantitative genetics under the guidance of outstanding teachers in the Crop Science and Statistics departments of North Carolina State University. His future goal is to pursue a career in plant breeding with the objective of carrying out research focused on genetic improvement of field crops.

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CHAPTER 1

LITERATURE REVIEW

Tobacco Classification

The genus *Nicotiana* is the sixth largest genus in the *Solanaceae* family and is represented by 76 recognized species (Knapp et al., 2004). Commercial tobaccos belong to the species *Nicotiana tabacum* L., an allotetraploid species that resulted from natural hybridization between two wild species, *N. sylvestris* and possibly *N. tomentosiformis* (Wernsman & Matzinger, 1980) followed by chromosome doubling. Some researchers have suggested that *N. otophora* may have contributed to the evolution of tobacco (Kenton et al., 1993; Hashimoto et al., 1998; Riechers and Timko, 1999; Chase et al., 2003). Economically important classes of tobacco include flue-cured, light air-cured (includes burley and Maryland), cigar wrapper, cigar filler and oriental or Turkish tobacco. Varieties of flue-cured, burley and oriental tobaccos formed distinct clusters in genetic analyses involving use of amplified fragment length polymorphism (AFLP) and microsatellite (also known as SSR) markers (Julio et al., 2006b; Bindler et al., 2007), indicating that these groups differ genetically. Genetic variation among different species of *Nicotiana* was identified to be higher than that within *Nicotiana tabacum* using randomly amplified polymorphic DNA (RAPD), microsatellite, and AFLP marker analyses (Bogani et al., 1997; Ren and Timko, 2001; Moon et al., 2009).

Artificial Hybridization

Tobacco is a highly self-pollinating crop species with typically less than 5 % cross pollination that may depend on the cultivar, environment, and activity of pollen vectors. The sweet fly (*Didea fasciata*), hawk moth (*Theretra tersa*), bumblebees

(*Bombus spp.*), honey bees (*Apis mellifera*) and hummingbirds (*Archilochus colubris*) are responsible for cross pollination (Wernsman and Rufty, 1987). Morphology of the tobacco flower facilitates easy removal of anthers and pollination for directed hybridizations. A detailed description of artificial hybridization as well as self pollination was provided by Wernsman and Matzinger (1980). Each capsule is expected to contain about 3000 seeds and shelled seed viability can be maintained for several years under proper storage conditions (Wernsman and Matzinger, 1980).

Tobacco Breeding

The goal of any tobacco breeding program is to develop cultivars with traits desired by growers and manufacturers of tobacco products. An interesting observation that accessions from different geographical locations with similar manufacturing qualities have similar AFLP patterns (Ren and Timko, 2001; Arslan and Okumus, 2006) indicates that definitive factors may be influencing these characters and hence may be targeted for genetic improvement.

Tobacco does not exhibit significant levels of inbreeding depression and exhibits limited heterosis in hybrids. Hence, most breeders have focused on development of pureline cultivars. However, for varietal protection and deployment of certain disease resistance genes in heterozygous condition, F_1 hybrids are now the primary focus of modern tobacco breeders. Initial tobacco breeding efforts were confined to mass selection within heterogeneous cultivars. Later, pedigree selection was widely used for tobacco

cultivar development. However, other methods like backcross breeding and recurrent selection are also used in tobacco breeding (Wernsman and Rufty, 1987).

A major task for tobacco breeders is to incorporate disease resistance traits into cultivars without adversely affecting desirable agronomic characteristics. The flue cured tobacco cultivar Coker 371-Gold (with the *Ph* gene conferring resistance to race 0 of the black shank pathogen) was not widely accepted by farmers due to its poor agronomic traits such as low yielding ability, lodging susceptibility, premature flowering, and poor curing properties. In 1997, NC 71 (with the same *Ph* gene) was released and was readily accepted by farmers due to its increased yields and higher levels of disease resistance. Undesirable negative correlations are often observed between characters of interest such as yield and percent total alkaloids (Lewis, 2006). Research efforts should be directed towards developing cultivars with a wide range of desirable characteristics.

Doubled Haploids (DHs) in Tobacco

Being highly responsive to haploid production and chromosome doubling, tobacco has been widely used as a model system for doubled haploid research. Tobacco haploids can be produced using methods such as anther culture (Bourgin and Nitsch, 1967), pollination with irradiated pollen (Kumashiro and Oinuma, 1985), and pollination with *N. africana* (Burk et al., 1979). Among these different methods, pollination with *N. africana* is the most widely used. When this method is used, 0.25 to 1.42 percent of the progeny survive and can readily be identified as either interspecific hybrids or maternal haploids (Burk et al., 1979). Haploids with desirable characteristics can be selected for

traits of interest prior to chromosome doubling, so that the doubled haploids developed from selected plants will be homozygous for desirable traits. Selected haploids can be chromosome-doubled using the leaf midvein tissue culture method developed by Kasperbauer and Collins (1972). This method saves a lot of time and can make breeding more efficient and cost effective. Selection of haploids for resistance to the black shank disease was effective and resistance of haploids was maintained in doubled haploids derived from them (Campbell and Wernsman, 1994). Selection for characters like plant type and maturity has not been successful due to the morphological differences between haploids and doubled haploids (Wilhite, 1990). Significant correlations between the ranks of haploids and doubled haploids have been observed for yield and quality traits (Witherspoon and Wernsman, 1989). Selection for black shank resistance in haploid population was reported to be more effective than selection in F_2 populations (Campbell and Wernsman, 1994). However, getting doubled haploids from field-grown haploids is difficult because of fungal and bacterial contamination of midveins (Witherspoon, 1987). The number of plants needed for achieving desired levels of genetic variation is much less for doubled haploid populations as compared to F_2 populations that are widely used in conventional cultivar development programs (Griffing, 1975). In doubled haploid populations, all genetic variability is partitioned into differences among doubled haploid lines and hence can be readily subjected to selection.

Doubled haploid populations of tobacco have been successfully used in research related to molecular markers (Johnson et al., 2002b; Milla et al., 2005) and QTL mapping

studies (Nishi et al., 2003). A doubled haploid population was helpful to conclude that the genetic nature of the resistance gene (*Ph*) in Coker 371-Gold variety is qualitative (Carlson et al., 1997). RAPD markers with bulked segregant analysis (BSA) were used to identify markers linked to root knot nematode resistance in a doubled haploid population (Yi et al., 1998b).

Leaf Surface Chemistry of Tobacco

Surfaces of many plants are described to be pubescent or bearing of small epidermal protrusions referred to as ‘trichomes.’ The botanical literature is said to contain more than 300 descriptions of trichomes to characterize various morphological types (Behnke, 1984). Trichomes can be divided into two general types, the non-glandular type and the glandular secreting type. Glandular trichomes of *Solanaceous* plants are of particular interest because of their ability to produce chemical substances that are ecologically and sometimes economically important. Since the report that leaf surface chemicals were associated with tobacco aroma (Michie and Reid, 1968), researchers have focused on tobacco leaf surface chemistry and later realized its economic importance after finding the association of these chemical traits with insect resistance (Johnson and Severson, 1982; Severson et al., 1983; Johnson and Severson, 1984), possible antifungal properties (Cruickshank et al., 1977), and growth regulatory activities (Cutler et al., 1977). Apart from producing the exudates, trichomes also have a role in affecting leaf wettability and interaction of the plant with light (Duffey, 1986; Fahn, 1988; Brewer et al., 1991; Wagner, 1991). A comprehensive review written by Severson et al. (1985)

serves as a good source of information that gives insight into many aspects of tobacco leaf surface chemistry.

The five main classes of tobacco leaf surface chemicals are diterpenes, sugar esters, surface waxes, volatiles, and other minor components (Wagner, 1999). Among these the diterpenes (subdivided into duvanes and labdanoids) and sucrose esters (SE) make up major portion of the leaf surface chemicals (Severson et al., 1985). The combination of labdanoids and SE-derived chemicals present in oriental tobacco comprises the major aroma forming components in that tobacco type (Leffingwell and Leffingwell, 1988).

Among the labdanoids, *cis*-abienol is a major component that plays a vital role in influencing flavor of the smoke. Oxidative breakdown of *cis*-abienol results in attractive flavor properties and imparts a cedar-like aroma to the smoke (Enzell, 1976). It has also been demonstrated that *cis*-abienol can exhibit plant growth regulating properties (Cutler et al., 1977). It was established that the ability to produce *cis*-abienol is governed by a single dominant gene designated as *Abl* (Tomita et al., 1980). Severson et al. (1985) and Tomita et al. (1980) suggested that *cis*-abienol must have originated from the genome of progenitor species, *N. tomentosiformis*. The enzyme responsible for the synthesis of *cis*-abienol was found to be similar to terpene cyclases in a study investigating the biosynthesis of *cis*-abienol using extracts from trichomes (Guo et al., 1994). The exact nature of the enzyme and its mode of action have yet to be elucidated in detail.

The other vital component in tobacco trichome exudates is a group of six SE isomers that differ one from the other by one carbon unit (Severson et al., 1985). Oriental tobaccos contain β -methyl valeric acid-containing SEs that impart them with a characteristic aroma and smoke flavor (Severson et al., 1985). The ability to produce β -methyl valeric acid-containing SEs in tobacco was reported to be governed by a single dominant gene designated as *BMVSE* (Gwynn et al., 1985). It was reported that SEs are also associated with aphid resistance in tobacco (Severson et al., 1985) and possibly resistance against the fungus that causes the blue mold disease (caused by *Peronospora tabacina*) of tobacco (Kennedy et al., 1992). Sucrose esters have also been reported in other species such as wild tomato, wild potato, and petunias (King et al., 1988; King et al., 1993; Kays et al., 1994), and were reported as being associated with aphid resistance in wild potato (Neal et al., 1990). In addition to the above mentioned characteristics, SEs have been documented to have an inhibiting effect on cancer development in mouse skin (Okabe et al., 1999). Different researchers, after finding the insecticidal activity of SEs, have explored the possibility of using synthetic SEs as insecticides and found them to be effective against whiteflies (Chortyk et al., 1996; Natwick, 1999; McKenzie et al., 2005; Song et al., 2006), aphids (McKenzie et al., 2004), kitchen pests (Wadleigh et al., 2005) and mites (Parker et al., 2007). Usage of these synthetic SEs was confirmed to be safe for some beneficial insects (McKenzie and Puterka, 2004; McKenzie et al., 2004; Michaud and McKenzie, 2004).

Apart from being influenced by genetics, the levels of leaf surface chemical components are also dependent on different factors like growing conditions of the plant (Gamou and Kawashima, 1979), stage of plant development (Gamou and Kawashima, 1979; Severson et al., 1982; Chang and Grunwald, 1976), and geographical location (Heeman et al., 1981). Hence, with available methodology, it is difficult to precisely phenotype breeding materials for their ability to produce these surface chemicals. Due to the importance of trichome constituents as part of tobacco product aroma, and also because of their contribution to chemical defense systems, alteration of trichome metabolism could be a good strategy to improve tobacco quality and enhance the species resistance to insects and disease.

Black Shank in Tobacco

The black shank pathogen (*Phytophthora nicotianae*) of tobacco was first reported by Breda de Haan (1896) in Indonesia and, since then, the disease has been reported in almost all tobacco growing countries (Erwin and Ribeiro, 1996). In 1915, black shank was first reported in the United States in southern Georgia, and in 1931 in North Carolina.

Breda de Haan (1896) named the pathogen as *Phytophthora nicotianae* but could not provide adequate description of the species. Dastur (1913), from India, also reported the species and named it *P. parasitica*. Tucker (1931) chose the name *P. parasitica* var. *nicotianae* because of its host preference for tobacco. Waterhouse (1963) replaced the name *P. parasitica* with *P. nicotianae*. This change has not been universally accepted and

hence the name *P. parasitica* is still in use (Erwin and Ribeiro, 1996). Although, the species *P. nicotianae* has a very wide host range, isolates from tobacco are generally pathogenic only to tobacco (Lucas, 1975).

The pathogen is an oomycete that causes a disease that affects the root system and basal part of the tobacco stem, hence named 'black shank'. A detailed description of the pathogen and its disease symptoms were presented by Lucas (1975). Young tobacco seedlings, when infected, damp off and the stem near the soil level becomes dark brown or black. When plants of a foot tall are attacked, sudden wilting and drooping of the leaves occur, followed by yellowing of the leaves. Finally, the root system and base of the stalk decay. In older plants, the stem above the ground turns black, the leaves turn from yellow to brown, and in a few days the plant typically dies. When the stem of a disease-infected plant is split longitudinally, the pith appears dry and is separated into plate-like disks. This is a diagnostic symptom of the black shank disease (Lucas, 1975). Apart from the stage of the plant, tissues have been reported to have differential responses to the disease. Cortex tissue is more vulnerable to infection as compared to pith tissue (Wills and Moore, 1971). The atypical symptoms (also known as stem black shank) of this disease include symptoms on the stem just above the ground level without infection on the root system (Dukes et al., 1965; Wills and Moore, 1971).

Disease initiation is caused by chlamydospores in the soil that are released from decomposed tissues of black shank-infected plants. Chlamydospores produce germ tubes that can either infect the plant or produce a sporangium. The sporangia, in turn, produce

zoospores that can travel greater distances and serve as primary infective propagules (Gallup et al., 2006). New chlamydospores formed in the infected tissue can either germinate to initiate new infections or serve as a survival structures until the next cropping season (Gallup et al., 2006). Chlamydospores can survive in soil for up to five years in the absence of host plants (Lucas, 1975).

Temperature, precipitation, and duration of drought conditions have been reported to have significant influence on disease progress (Jacobi et al., 1983). Cloudy weather, prolonged dampness, and temperatures above 20° C are favorable for outbreak of the black shank disease (Lucas, 1975). When a severe drought is followed by high rainfall, there is a high chance of disease occurrence even in highly resistant cultivars. The stage of the plant 6-8 weeks after transplanting was reported to be most susceptible, and considered the most appropriate stage for studies focusing on loss assessment due to the disease (Jacobi et al., 1983). As compared to other species of *Phytophthora*, the black shank pathogen requires a much lower inoculum density for disease development (Kannwischer and Mitchell, 1981). Even in soil diluted 100 times with sand, inoculum can be sufficient to infect and kill tobacco plants (Nusbaum, 1952).

Crews et al. (1964) reported that disease occurrence in a particular genotype varies depending on the genotypes it is grown together with (*i.e.*, higher disease occurs in the presence of susceptible varieties). The principle behind this mechanism was predicted to be root exudates, inoculum build-up, or other unknown factors. Resistance to the pathogen does not prevent infection, but stops growth and reproduction to help decrease

the population of the pathogen associated with the plant (Shew and Shew, 1994). It has been observed that development of black shank symptoms depends on initial inoculum density and the level of partial resistance expressed by the host plant (Ferrin and Mitchell, 1986; Shew, 1987; Jones and Shew, 1995). In a morphometric root analysis, it was established that first order roots were found to be more susceptible than second order roots (English and Mitchell, 1989). A significant association between the size of tobacco root systems and black shank resistance was reported after observing that susceptible cultivars have two to three times larger root systems as compared to resistant cultivars (Jones and Shew, 1995). Having smaller root systems might make resistant cultivars less likely to come into contact with the pathogen, and hence may be a mechanism to avoid infection (Shew and Shew, 1994). Tissue culture techniques have been used to study the differential response of tobacco tissues against the black shank pathogen. The colonization level of the pathogen on callus from resistant genotypes was less as compared to that of susceptible genotypes, which indicates that the tissue has some genetic expression of resistance (Helgeson et al., 1972). The variation in resistance could be observed among different cultivars, among plants within a cultivar, and among different parts of the same plant. There is therefore a need for devising additional analytical methods for studying the molecular basis of resistance to black shank pathogen (Wills and Moore, 1971).

Host-pathogen Interactions

Based on observations of many isolates of the black shank pathogen, Apple (1957) hypothesized that the black shank pathogen was the result of a rare mutation in *P. parasitica*. The species, *Phytophthora parasitica*, is emerging as a model system for studying molecular level host-pathogen interactions that operate in diseases caused by oomycete pathogens (Attard et al., 2008). A characteristic feature of species belonging to the genus *Phytophthora* is production of elicitors (Ricci, 1997). Elicitors have the ability to induce a hypersensitive responses (HR) and systemic acquired resistance (SAR) (Kamoun et al., 1998; Bonnet et al., 1996; Keller et al., 1996). Pathogen cultures isolated from black shank affected tobacco plants have generally been found to lack the ability to produce elicitors (Kamoun et al., 1994; Bonnet et al., 1994; Colas et al., 1998). It has thus been hypothesized that it is the inability to produce elicitor that may make the black shank pathogen specific to its host, tobacco. However, some isolates that produce elicitor were found to be weakly or moderately virulent on tobacco and are frequently pathogenic on tomato (Colas et al., 1998). The non-elicitor producing isolates from tobacco exhibited distinct molecular patterns in RFLP studies (Colas et al., 1998). It has been established that a gene, *parI*, that belongs to a gene family responsible for the production of elicitor is down regulated by the pathogen in order to overcome the hypersensitive response by a host plant (Colas et al., 2001). Information regarding the interaction between the black shank pathogen and tobacco plants is not well developed and needs to be elucidated using recent molecular tools.

Races of the Black Shank Pathogen

In 1957, Apple, based on observation of 200 isolates of the black shank pathogen from different tobacco fields, reported that different isolates show varying levels of pathogenicity. Heggestad and Lautz (1957) also showed that there is a variation in virulence of different isolates, but could not prove whether or not there was a physiological specialization among those isolates. The isolates collected from fields of resistant cultivars were found to be more aggressive as compared to isolates from susceptible cultivars (Dukes and Apple, 1961). It was later confirmed that variations in aggressiveness resulted from population shifts towards a prevalence of more aggressive isolates (Jaarsveld et al., 2002).

For many years, the black-shank pathogen was considered to be a relatively stable organism. In 1958, a strain of this pathogen was found to be highly pathogenic to *N. longiflora* and *N. plumbaginifolia*, and less pathogenic to tobacco cultivars with the Florida 301 type of resistance (Smith and Clayton, 1958). Later, it was also demonstrated that the new strain differed physiologically from the earlier known strains and hence the strain pathogenic to *N. plumbaginifolia* was designated as 'Race 1' and the one nonpathogenic to *N. plumbaginifolia* was designated as 'Race 0.' Even though Race 1 is observed to be a naturally occurring form of the pathogen, the most common strain that is found in the United States is race 0 (Sullivan et al., 2005a). Race 1 is relatively less fit as compared to Race 0 and becomes more prevalent only under intense selection pressure that results from continuous cultivation of Race 0 resistant cultivars (Apple, 1962a;

Apple, 1967; Csinos and Bertrand, 1994; Sullivan et al., 2005b). Aggressiveness differs among different isolates of the same race, however (Apple, 1954; Apple, 1957; Dukes and Apple, 1961; Jaarsveld et al., 2002; Csinos, 2005). Race 0 is generally more aggressive as compared to Race 1 (Carlson et al., 1997; Sullivan et al., 2005b). Csinos (2005) reported that race 1 has a wide range of aggressiveness, however, some isolates are more aggressive than race 0. Even though race 1 is generally less aggressive than race 0, it causes severe stunting, reduced leaf quality, and weakened root system (Sullivan et al., 2005b).

Although race 1 was identified as early as 1958, it was only in the 1990's that it became widespread due to the extensive use of cultivars with resistance against Race 0 conferred by the *Ph* gene (Carlson et al., 1997). Races 2 and 3 have been reported in South Africa, and Connecticut respectively (Prinsloo and Pauer, 1974; McIntyre and Taylor, 1978). McIntyre and Hankin (1977) reported that Race 0 and Race 1 can be distinguished from race 3 by observing ketose production in race 0 and 1 but not in race 3. Recently, in the counties of North Carolina, race 3 was discovered that is capable of overcoming the resistant gene (*Phl*) from *N. longiflora* but not the gene (*Php*) from *N. plumbaginifolia* (Gallup et al., 2008).

Molecular Variation in the Black Shank Pathogen

In contrast to its nature of exhibiting multiple races and different levels of aggressiveness within a race, the black shank pathogen was found to exhibit much less variation at the molecular level as revealed by diversity studies based on isozymes

(Oudemans and Coffey, 1991), RAPDs (Zhang et al., 2003), mitochondrial DNA markers (Lacourt et al., 1994; Forster et al., 1990), and nuclear RFLPs (Colas et al., 1998). With advanced molecular marker techniques, it may be possible in the future to detect molecular variation in this pathogen.

Breeding for Black Shank Resistance

Tisdale (1931), in Florida, crossed selections of ‘Big Cuba’ and ‘Little Cuba’ and derived through inbreeding a resistant line, Florida 301. Initial investigations into the genetics of this resistance resulted in different interpretations. Smith and Clayton (1948) reported the resistance of this line to be polygenic. Even though the resistance was considered as polygenic, a major gene thought to contribute to resistance was suggested to be inherited in a simple recessive fashion (Clayton, 1958). Moore and Powell (1959), using material derived from Florida 301, hypothesized that resistance is partially dominant and is influenced by modifiers present in susceptible parents. Crews et al. (1964) and Chaplin (1966) observed that hybrids of resistant \times susceptible varieties show intermediate levels of resistance and suggested that the resistance in hybrids is influenced by modifying genes present in susceptible parents. Because of its partial and multigenic nature, the resistance in Florida 301 is a typical example of the quantitative type of disease resistance in plants. The Florida 301 type of resistance has less of an effect in stems as compared to root systems and, as a result, the vascular system can easily be invaded leading to wilting of the plant (Hendrix and Apple, 1967). Irrespective of different interpretations and conclusions about the genetics of this resistance, it has

proved to be partially effective against both race 0 and race 1 (Wernsman et al., 1974). Florida 301, for a long time, has been used as the primary source of resistance in the development of most black shank resistant cultivars. Severe yield losses in tobacco cultivars with this type of resistance have been reported under high drought stress (Valleau et al., 1960), however. This prompted the search for alternative sources of resistance.

Two wild *Nicotiana* species, *N. longiflora* and *N. plumbaginifolia*, were found to be highly resistant to the pathogen and hence were used as resistance sources for the incorporation of disease resistance into cultivated tobacco. Black shank disease resistance was transferred to tobacco from *N. plumbaginifolia* by Apple (1962b) and Chaplin (1962) and from *N. longiflora* by Valleau et al., (1960). Smith and Earley (1964) transferred *N. longiflora* resistance into the cultivar, Hicks, and developed varieties McNair 20 and 30 that were released in 1963.

Chaplin's (1962) data indicated that the *N. plumbaginifolia* resistance is governed by a partially dominant single gene. Apple (1962b) reported that *N. plumbaginifolia* resistance is controlled by a single gene, possibly modified by suppressor or complementary genes. Goins and Apple (1970) could obtain lower proportions of homozygous genotypes for the *N. plumbaginifolia* resistance gene (*Php*) than expected. Valleau et al. (1960) experienced the same problem with the *N. longiflora* gene (*Phl*). Later, it was confirmed that the resistance in both the sources is governed by a single gene and was incorporated on the same tobacco chromosome. It was thus hypothesized

that both types of resistance are governed by the same gene in these two sources (Collins et al., 1971).

A flue-cured tobacco cultivar, Coker 371-Gold, released in 1986, exhibits a high level of resistance to the black shank disease. Ambiguity in its origin made it difficult to determine the resistance source in initial studies. It was reported to be resistant to race 0 and partially resistant to race 1 (Nielsen, 1995). Carlson et al. (1997) reported that the resistance in Coker 371-Gold is controlled by polygenes derived from Florida 301 and a major dominant gene (*Ph*) that was predicted to be derived from either *N. plumbaginifolia* or *N. longiflora*. Later, it was concluded that the *Ph* gene present in Coker 371-Gold was derived from *N. plumbaginifolia* (Johnson et al., 2002a).

A selection from the cigar tobacco variety ‘Quin Diaz’ grown in the Dominican Republic, Beinhart 1000, was reported to be highly resistant against multiple races of the black shank pathogen tested in many experiments (Heggstad and Lautz, 1957; Chaplin, 1966; Wills, 1971; Tedford and Nielsen, 1990; Nielsen, 1992). F₁ hybrids resulting from crossing Beinhart 1000 with susceptible varieties were compared to the performance of hybrids derived from other resistance sources. Hybrids derived from Beinhart 1000 tended to be highly resistant as compared to hybrids of lines derived from other resistance sources (Silber and Heggstad, 1963). The resistance was found to be partially dominant and may be modified by parental genetic background (Silber and Heggstad, 1963). Unlike in Florida 301, the resistance in Beinhart 1000 is expressed both in the leaves and stem (Wills, 1971). Beinhart 1000 has not successfully been utilized in tobacco breeding

programs for black shank resistance because of associations between the resistance and the undesirable cigar type characteristics (Nielsen, 1992). In a study comparing different resistance sources including Florida 301, Coker 187 (has Florida 301 resistance), PD 468 (has resistance from *N. plumbaginifolia*), and Beinhart 1000, it was established that expression of the resistance varies based on the genetic background of a particular genotype (Chaplin, 1966). In a recurrent selection experiment, a base population was created by intercrossing susceptible, moderately resistant, and highly resistant cultivars. With the progress of the selection cycles, the percent survival of the plants increased from cycle C₀ to cycle C₃, reflecting a gradual increase in frequency of favorable alleles (Tedford and Nielsen, 1990).

Since there have been only limited sources of resistance reported, there needs to be more research focus on finding different sources of resistance in cultivated tobacco and in different species of *Nicotiana*. It was reported that four species from section *Alatae* of *Nicotiana* contain high resistance to black shank (Valleau et al., 1960). As the existence of resistance from other species of *Nicotiana* like *N. debneyi*, *N. repanda*, *N. megalosiphon* and *N. suaveolens* have been reported (Li et al., 2006), there should be more focus on investigation for finding more resistance sources in tobacco and other closely related species.

Black Shank Disease Management

If not managed properly, black shank can lead to complete yield losses under severe disease conditions. Apart from planting resistant cultivars, an effective disease

management strategy for black shank also includes cultural and chemical practices. The goal of cultural practices would be to reduce pathogen populations in the soil that initiate black shank disease. Cultural practices like planting on raised beds, stalk and root destruction after harvest, and crop rotation with a non host crops can efficiently reduce initial inoculum (Gallup et al., 2006). Chemical control is effective only when used in conjunction with the use of resistant cultivars and above mentioned cultural practices. Metalaxyl and Mefenoxam are the only two chemicals that are recommended for the control of the black shank pathogen. These two chemicals are expensive, however, and require perfect application timing (Moore et al., 2003). The variation among cultivars for sensitivity to metalaxyl can make it difficult to determine proper concentrations for each cultivar and, as a result, application at higher concentrations can make the cost of cultivation prohibitive (Csinos and Bertrand, 1994). The importance of combining the *Ph* gene and partial resistance for controlling both races has been stated, and it has been suggested that cultivars with the *Ph* gene should be rotated with partially resistant cultivars to reduce possible yield losses (Sullivan, 2005a).

Black shank is prevalent when the crop is infested with other diseases like Granville wilt, black root rot, and Fusarium wilt (Lucas, 1975). A significant association between the presence of nematodes and black shank infestation has been reported in several experiments (Sasser et al., 1955; Powell and Nusbaum, 1960). The control of nematodes by fumigation or avoiding them by crop rotation has been reported to decrease black shank severity (Nusbaum and Chaplin, 1952). Soil fumigants such as chloropicrin

and fumigant nematicides like 1,3- dichloropropene can be use to reduce nematode populations (Gallup et al., 2006). The transport of infected seedlings to different regions of cultivation may lead to appearance of new strains and races in a particular region (Jaarsveld et al., 2002). Hence, care should be taken to transport only disease-free seedlings.

Disease Screening

Screening germplasm, breeding lines, and cultivars for their levels of resistance is a common practice in breeding for disease resistance. Field evaluation requires considerable resources and hence can be limiting step in black shank resistance breeding (Campbell and Wernsman, 1994). Field experiments can furthermore be complicated by non-uniform inoculum and presence of more than one race at a time (Csinos et al., 1984). Different methods that can be used as alternatives to field evaluation have been reported by different researchers. In different screening experiments using callus tissue, leaves, and seedlings, it has been commonly reported that results are not correlated with field observations (Hendrix and Apple, 1967; Wills, 1971; Hegelson et al., 1972; Tedford et al., 1990).

Wills and Crews (1964), for the first time, reported that leaf tissue could be used for disease screening, and the results were consistent with methods involving whole plants. Being non-destructive, methods using leaf tissue would facilitate collection of seed from selected resistant plants. Even though leaf tissue was reported to be a reliable target for inoculation in disease screening experiments (Wills and Crews, 1964), it was

later established that leaf resistance was not correlated with whole plant response (Wills, 1971). It was suggested, however, that investigations related to leaf resistance could aid in understanding the biochemical mechanisms involved in host plant interactions (Wills, 1971). It has commonly been observed that the level of resistance varies between the stem and root of a plant (Hendrix and Apple, 1967; Csinos, 1999; Jaarsveld et al., 2003). It was later established that stem resistance was better correlated with whole plant resistance, and thus stem tissue, particularly stem epidermis, may be considered for the study of the nature of resistance (Wills and Moore, 1971).

The fact that resistant plants can express resistance even from the seedling stage (Lucas, 1975) makes it possible to screen genotypes at very early stages of growth. Many workers have therefore tried to find an efficient way to screen tobacco plants at the seedling stage. Stokes and Litton (1966) used plastic tubes with vermiculite and dipped the tubes with seedlings in inoculum solution. A slight modification of this procedure was suggested where tubes would be incubated in water tanks containing inoculum for three days (Litton et al., 1970). Carlson et al. (1997) reported that greenhouse inoculations (inoculum is poured in holes in soil near stem) were efficient for screening. In another study, using greenhouse methods, cultivars and breeding lines were evaluated and some breeding lines showing substantial resistance were identified (Csinos et al., 1984). A nondestructive method of inoculating a detached-leaf was developed by Tedford et al. (1990) where results were highly correlated with field results. A rapid seedling technique was developed by Jaarsveld et al. (2003) that correlated with adult plant inoculation

studies with respect to both identification of resistant lines and differentiating races. Even though this method is useful for screening a large number of genotypes, it is difficult to distinguish moderately resistant plants.

The use of the physiological stage of the plant rather than calendar time serves as the proper criteria in studies related to disease progress (Jacobi et al., 1983). Studying population dynamics of races is as important as disease screening for resistant genotypes, because recommendations made to farmers would depend on the race that is most prevalent in their areas (Csinos, 2005; Gutierrez and Mila, 2007). As race 3 was recently discovered in North Carolina recently (Gallup et al., 2008), it is necessary to update the differential hosts as well as the techniques. An efficient screening technique, which requires less space and time as compared to the earlier reported greenhouse techniques, has been developed and was reported to be reliable for differentiating races of the black shank pathogen (Gutierrez and Mila, 2007). In this technique, colonized *Catharantus roseus* (*Vinca rosea*) leaf discs were used to inoculate 25 days old seedlings in the greenhouse.

Molecular Markers in Tobacco

Molecular markers closely linked to numerous traits of economic importance have been developed in many crops and might facilitate indirect selection for desirable traits in early segregating generations. Indirect selection can generally be very effective due to its ability to permit pyramiding of genes for different traits of interest. Earlier studies based on RAPD analysis reported low levels of polymorphism in cultivated tobacco (Bai et al.,

1995). The apparent low level of genetic diversity may be due to a bottleneck genetic effect on variation contributed by few genotypes that served as progenitors in the evolution of modern day tobacco. With the advent of better and more reliable markers like AFLP and SSRs, much larger amounts of molecular polymorphism can be observed within cultivated tobacco (Ren and Timko, 2001; Julio et al., 2006a; Julio et al., 2006b; Bindler et al., 2007; Moon et al., 2009).

Molecular markers associated with resistance to different diseases of tobacco like black root rot (Bai et al., 1995; Julio et al., 2006b), wildfire (Yi et al., 1998a), black shank (Johnson et al., 2002b), potato virus Y (Lewis, 2005; Julio et al., 2006b), tobacco mosaic virus (Lewis et al., 2005) and blue mold (Milla et al., 2005; Julio et al., 2006b) have been reported. In tobacco, RAPD markers associated with *Rk* gene that controls resistance to root knot nematode (*Meloidogyne incognita*) have also been identified (Yi and Rufty, 1998; Yi et al., 1998b). Using RAPD analysis combined with SCAR marker, the probable source of disease resistance for blue mold (caused by *Pernospora tabacina*) was traced back to the wild species *N. debneyi* (Milla et al., 2005). In a similar study, the origin of the *Ph* gene in Coker 371-Gold was found to be *N. plumbaginifolia* (Johnson et al., 2002a). Markers associated with resistance genes from wild species can facilitate precise transfer of the traits to elite lines and may assist in reducing linkage drag effects. RAPD and SSR markers were used in phylogenetic studies of the *Nicotiana* genus and yielded results similar to those produced using morphological and cytological data (Bogani et al., 1997; Moon et al., 2008). Molecular markers, hence, can also be useful in

phylogenetic studies in the cultivated species as well as for the *Nicotiana* genus as a whole.

As many of the most agronomically important traits exhibit polygenic inheritance, identification and location of quantitative trait loci on genetic maps has become an important area of research in plant breeding. The experiments conducted by East (1916) with regard to corolla length of *Nicotiana langsdorffii* helped lay the foundation for quantitative genetics in plants. A large number of QTL studies have been conducted for a number of crop species (Bernardo, 2008). Relatively little progress has been made in the area of QTL mapping in tobacco due to the lack of a detailed genetic map. The earliest genetic linkage map published for the *Nicotiana* genus was constructed using the cross of *N. plumbaginifolia*/*N. longiflora* and was based on RFLP and RAPD markers (Lin et al., 2001). In another study, a total of 184 AFLP, inter simple sequence repeat (ISSR), sequence specific amplified polymorphism (SSAP), and sequence characterized amplified region (SCAR) were used to construct a partial genetic map in tobacco (Julio et al., 2006a). A microsatellite linkage map was recently published by Bindler et al. (2007) which can serve as a framework for QTL studies in tobacco.

A few studies have identified some QTLs in tobacco using molecular markers. A QTL associated with bacterial wilt resistance was identified using AFLP markers (Nishi et al., 2003), and another QTL associated with blue mold resistance was mapped using RAPD markers (Milla et al., 2005). In another study, using a partial genetic map of 18

linkage groups, QTL analysis was conducted for a total of 59 traits related to diverse agronomic, leaf quality, chemical composition, and smoke properties (Julio et al., 2006a).

With advanced statistical procedures for mapping QTLs such as interval mapping (Lander and Botstein, 1989), composite interval mapping (Zeng, 1994) and multiple interval mapping (Kao and Zeng, 1997; Kao et al., 1999) it is possible to precisely identify and locate QTL on linkage maps in order to utilize them in marker assisted selection.

Tobacco Genomics

Complete nucleotide sequence information of a genome serves as an ultimate resource for understanding the genome structure and organization of genes for a particular organism. The tobacco genome, with 4500 million base pairs, is 1.5 times size of the human genome (Arumuganathan and Earle, 1991), and most of it is presumed to be comprised of highly repetitive sequences (Gregor et al., 2004). Complete nucleotide sequences of the chloroplast and mitochondrial genomes of *N. tabacum* have been published (Shinozaki et al., 1986; Sugiyama et al., 2005). Yukawa et al. (2006) published the nucleotide sequences of the chloroplast genomes for the two tobacco probable progenitor species *N. tomentosiformis* and *N. sylvestris*. Complete sequencing of the tobacco genome would be expensive and tedious, and so a major focus of researchers has been sequencing most of the expressed regions of the tobacco genome. The first of these efforts was that of Matsuoka et al. (2004) who sequenced 9,190 expressed sequence tags (ESTs) that are available to the public at their website

(<http://mrg.psc.riken.go.jp/strc/index.htm>). Later, a total of 46,546 EST sequences were made available in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) by the 'European Sequencing in Tobacco' project (<http://www.estobacco.info>). The Dana Farber Cancer Institute (DFCI) maintains the '*Nicotiana tabacum* Gene Index' (NtGI) data base (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=tobacco>) that gives a non-redundant view of tobacco ESTs and genes by integrating information from different EST sequencing and gene research projects across the world. A major project, the Tobacco Genome Initiative (TGI), for sequencing the tobacco genome was carried out at North Carolina State University, USA (Gadani et al., 2003; Opperman et al., 2007). By using only methyl-filtered genomic clones, TGI project sequenced approximately 95% of the expressed genomic regions (Opperman et al., 2007). The TGI project also sequenced a total of 85,000 ESTs (Opperman et al., 2007) and made their sequence information available for the public in the GenBank data base and TGI website: <http://www.pngg.org/tgi/>. This information for expressed regions may serve as a valuable resource for identification of genes responsible for different traits in tobacco.

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CHAPTER 2
GENETIC MAPPING OF TWO LEAF SURFACE CHEMISTRY TRAITS IN
TOBACCO

ABSTRACT

Leaf surface chemistry traits in tobacco are economically important because of their role in influencing smoke flavor and aroma as well as resistance to insect pests and possibly fungal diseases. Among leaf trichome exudates, *cis*-abienol and β -methyl valeric acid-containing sucrose esters (BMVSEs) are two chemical components that have been implicated in affecting smoke flavor and pest resistance. Studying the inheritance pattern of these traits and identifying molecular markers associated with them would be of value to plant breeders wishing to incorporate them into new cultivars. A doubled haploid (DH) mapping population produced from the cross between Beinhart 1000 (produces both BMVSEs and *cis*-abienol) and Hicks (lacks the production of both components) was used in the present study. The inheritance pattern for synthesis of the two leaf surface chemistries was investigated and confirmed to be governed by single genes. Segregation distortion for the gene affecting BMVSEs accumulation (*BMVSE*) was highly significant ($P = 0.0073$). Joint segregation analysis revealed highly significant ($P < 0.0001$) linkage between the genes controlling these two traits with a logarithm of odds (LOD) score of 17.46. Two microsatellite markers (PT30209 and PT20315) with BMVSEs and one marker (PT30124) with the *cis*-abienol trait were identified as being strongly linked with zero percent recombination. In order to map the genes to a particular linkage group, a genetic map of 190 microsatellite markers was generated by genotyping the doubled haploid population. The genetic map consisted of 24 linkage groups. Genes encoding for these traits were mapped to linkage group 4 and separated by a genetic distance of 8.5 centi morgans (cM). High segregation distortion rates were found to be associated with

the majority of the markers on linkage group 4. The chromosomal region containing the genes affecting the two traits was densely mapped with a total of 17 markers. Using this information, plant breeders can incorporate one or both of these traits into new cultivars in a cost effective manner.

INTRODUCTION

Trichomes were among the first anatomical features of plants that were observed by early microscopists (Behnke, 1984). Glandular trichomes of *Solanaceous* plants are of particular interest because of their ability to produce chemical substances that have economic importance due to their antifungal and pest resistance properties. In tobacco, the two chemical components that make up a major proportion of trichome exudates are the diterpenes (subdivided into duvanes and labdanoids) and sucrose esters (SEs) (Severson et al., 1985). The combination of labdanoids and SE derived chemicals present in oriental tobacco comprises the major aroma forming component of this tobacco class (Leffingwell and Leffingwell, 1988).

Among the labdanoids, *cis*-abienol is a major component that plays a vital role in influencing smoke flavor. Oxidative breakdown of *cis*-abienol results in attractive flavor properties and imparts a cedar-like aroma to smoke (Enzell, 1976). It has also been established that *cis*-abienol can exhibit plant growth regulating properties (Cutler et al., 1977). Tomita et al. (1980) and Severson et al. (1985) suggested that the ability to synthesize *cis*-abienol must have originated from the genome of progenitor species, *N. tomentosiformis*. A study investigating the inheritance of *cis*-abienol revealed that it is

governed by a single dominant gene designated as *Abl* (Tomita et al., 1980). The process of biosynthesis of *cis*-abienol was investigated using trichome extracts and it was concluded that the enzyme responsible for synthesis is similar to terpene cyclases (Guo et al., 1994). The exact nature of the enzyme and its mode of action still have to be elucidated in detail.

The second important class of compounds in tobacco trichome exudates is a group of six SE isomers that differ from each other by one carbon unit (Severson et al., 1985). Oriental tobaccos contain β -methyl valeric acid-containing SEs that impart them with a characteristic aroma and smoke flavor (Severson et al., 1985). Gwynn et al. (1985) reported that a single dominant gene designated as *BMVSE* controls the biosynthesis of β -methyl valeric acid-containing sucrose esters.

SEs have been reported to be associated with aphid resistance in tobacco (Severson et al., 1985). Sucrose esters were also reported in other species like wild tomato, wild potato, and petunias (King et al., 1988; King et al., 1993; Kays et al., 1994) and have been related to aphid resistance (Neal et al., 1990). Antifungal properties of SEs were confirmed after observing an inhibiting effect on the blue mold fungus (*Peronospora tabacina*) (Kennedy et al., 1992). Different researchers, after noting the insecticidal activity of SEs, have explored the possibility of using synthetic SEs as insecticides and found that they are effective against whiteflies (Chortyk et al., 1996; Natwick, 1999; McKenzie et al., 2005; Song et al., 2006), aphids (McKenzie et al., 2004), kitchen pests (Wadleigh et al., 2005), and mites (Parker et al., 2007). Use of these

synthetic SEs was confirmed to be safe for some beneficial insects (McKenzie and Puterka, 2004; McKenzie et al., 2004; Michaud and McKenzie, 2004). In addition to the above mentioned characteristics, SEs have also been documented to have an inhibiting effect on cancer development (Okabe et al., 1999).

Using monosomic analysis, earlier researchers located both genes, *Abl* and *BMVSE*, on chromosome A of the *N. tabacum* genome (Kubo et al., 1982; Danehower et al., 1989). It would be of a great value for tobacco breeders to have molecular markers associated with these leaf chemistry traits in order to develop cultivars with the ability to produce one or both of these components. Of equal value would be to have a dense genetic map with molecular markers in order to map these traits along with other traits of interest like pest resistance, disease resistance, and other agronomic characters. Molecular markers associated with these traits might also facilitate the isolation of genes responsible for producing these two chemicals. No efforts, thus far, have been reported to identify molecular markers associated with leaf surface chemistry traits in tobacco. Except for the genetic map reported by Bindler et al., (2007), there has been no dense genetic map available for tobacco breeders. With the advent of tobacco microsatellite markers or simple sequence repeats (SSRs) markers, there are enhanced opportunities to identify marker trait associations and to map genes underlying important traits on a genetic map.

The first objective of the present investigation was to confirm the inheritance pattern for production of the two chemical components, *cis*-abienol and BMVSEs. The

second objective was to identify microsatellite markers associated with the two leaf chemistry traits. The third objective was to generate a genetic linkage map and to locate genes controlling these two traits on the linkage map.

MATERIALS AND METHODS

Production of Doubled Haploid Population

The cigar tobacco cultivar, 'Beinhart 1000,' was chosen as a source of the ability to produce the two chemistries, *cis*-abienol and BMVSEs. This line was hybridized with flue cured cultivar 'Hicks' that lacks the ability to produce those two chemistries. F₁ progeny from this cross were hybridized as females with *Nicotiana africana* to generate maternally derived haploids (Burk et al., 1979). A doubled haploid population of 117 lines was produced from these haploid plants using the midvein tissue culture procedure of Kasperbauer and Collins (1972).

Field Planting and Sample Collection

A total of 117 doubled haploid lines, along with the parental lines, were evaluated in 3 m plots containing 5 plants per plot in a randomized complete block design with two replications during 2008 at Central Crops Research Station, Clayton, NC. Inter-row and within-row spacing were 1.14 m and 0.56 m, respectively. From each plot, 10 leaf discs (two per plant) each measuring 1.6 cm in diameter were punched at points approximately one-third and two-third of the distance from the tip of leaf about 2-3 cm from the midrib as described by Daneshmandi et al. (1987).

Gas Chromatography

Sample preparation was a modification of the procedure described by Severson et al. (1984). In the laboratory, disks were washed twice with 7.5 mL of HPLC grade CH_2Cl_2 (Fisher Scientific, Pittsburg, PA) by vortexing each 7.5 mL aliquot for 30 seconds. The combined washes contain over 99% of the total leaf surface components (Danehower et al., 1987). Approximately 0.5 g of anhydrous Na_2SO_4 was added to each scintillation vial and vials were allowed to stand for at least two hours. Extracts were then filtered through 10-20 μm porosity fritted glass funnels into 25 mL screw cap test tubes and the vials were rinsed twice with 1 mL of CH_2Cl_2 . 1.00 mL of an internal standard solution of heptadecane (C-17 alkane) and heptadecanol (C-17 alcohol) in toluene was added. The solvent was then evaporated by heating at 40° C under an N_2 gas stream using a Pierce Reacti-therm heating module and Reacti-vap III manifold (Pierce Chemical Co., Rockford, IL). 100 μL of a 1:1 mixture of BSTFA: DMF (Pierce Chemical Co.) was added, the test tube was capped tightly under a head of N_2 , and the sample was heated at 75 °C for 30 minutes. After cooling, 100 μL of a 1:1 BSA: Pyridine solution was added to dissolve hydrocarbons. Samples were stored at -20° C until analysis. Prior to analysis, samples were brought to room temperature and vortexed.

Gas chromatographic analysis was conducted using an Agilent HP 6890 GC-FID (Santa Clara, CA) and a 30 meter DB-5 column (J & W Scientific, 0.32 mm I.D. and 0.25 micron film thickness). The carrier gas was Helium at a linear gas velocity of approximately 28 cm per second. The injector was set at 240° C and the detector at 375°

C. The analysis consisted of a temperature program from 160 to 310° C at 4 degrees per minute followed by a hold at 310° C for 10 minutes.

The identity of the compounds of interest (β -methyl valeric acid-containing sucrose esters, and *cis*-abienol) was verified by comparison of retention times to that of authentic standards and/or confirmed by GC-MS analysis using an HP 5890 GC equipped with an HP5972 Series MSD Detector. Quantitation of compounds utilized an internal standard method and an authentic standard of *cis*-abienol prepared in our labs via preparative low pressure liquid chromatography (Lawson et al, 1988). Because the sucrose esters are a complex mixture for which no purified standard was available, quantitation of sucrose esters was based upon an internal standard method calibration prepared using synthetic sucrose octa-acetate (Sigma-Aldrich, St. Louis, MO). Data was collected and analyzed using Agilent ChemStation software (Agilent Technologies, Palo Alto, CA). Quantitation of the sucrose esters was carried out using multiple peak summing to simplify reporting of the SE data.

Genotyping

Genomic DNA isolation was carried out according to the protocol developed by Johnson et al. (1995) with the exception that a BIO 101 Fast Prep machine (BIO 101, Holbrook, N.Y.) was used to grind the leaf samples. A total of 433 SSR markers were used to screen the parental lines to identify those that amplified polymorphic bands. The identified polymorphic markers were used to screen the entire population. An economic

method developed by Schuelke (2000) was used by tailing the forward primers with M-13 sequence to facilitate the detection of amplified product. PCR was performed in 15 μ l volumes containing 25 ng of template DNA, 1X PCR buffer (10 mM KCl, 20 mM Tris-HCl pH 8.8, 2 mM MgSO_4), 0.015 μ M of forward primer, 0.06 μ M of reverse primer, 0.06 μ M of M-13 primer, 0.2 mM dNTPs and 1U of *Taq* polymerase. Thermocycling started with a denaturation step for 5 min at 94°C followed by 29 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec and 7 cycles for M-13 activity were run as 94°C for 30 sec, 52°C for 45 sec, 72 for 45 sec with a final extension process for 5 min at 72°C. The microsatellite marker alleles were separated using 8 % polyacrylamide gels and a LI-COR 4300 DNA Analysis System (LI-COR Biosciences, Lincoln, Nebraska) under the following conditions: 1500V, 40mA, 40W, and 45°C for 2.5 hr. IRDye 700- or 800-labeled molecular weight standards (50 – 700 bp) were loaded on each gel for identifying the allele size. The alleles were scored using AFLP Quantar 1.0 software (KeyGene Products B.V., Wageningen, The Netherlands).

Construction of Linkage Map

A genetic linkage map was generated by Mapmaker/EXP 3.0 (Lander et al., 1987) on the basis of a minimum logarithm of odds (LOD) score of 3.0 and a linkage threshold of 40 cM by executing the ‘Group’ command. Map distances in centimorgan (cM) units were estimated from recombination fractions using the Haldane mapping function (Haldane, 1919). Linkage order was established by first selecting a subset of eight markers and then identifying the best order using the ‘Compare’ command. Remaining

markers were added using the 'Try' command. The final marker order was confirmed by executing the 'ripple' command. A graphical representation of the linkage map was generated using MAPCHART software (Voorrips, 2002).

RESULTS

Variation in the Content of cis-abienol and BMVSEs

As evident from the chromatograms (Figure 1), Beinhart 1000 produced significant levels of *cis*-abienol and BMVSEs, while Hicks did not produce detectable levels of these chemical components. The mean production levels for Beinhart 1000 were observed to be 34.0 and 27.7 $\mu\text{g}/\text{cm}^2$ for *cis*-abienol and BMVSEs, respectively. Among the doubled haploid lines that produced these two components, *cis*-abienol content levels ranged from 9.0 – 51.8 $\mu\text{g}/\text{cm}^2$, while BMVSEs content levels ranged from 8.0 – 58.6 $\mu\text{g}/\text{cm}^2$ (Appendix I). However, the concentration of these chemicals is highly affected by the growth stage of the plant, growing conditions of the plant, and trichome density on the leaf. Even though great variation was observed among the doubled haploid lines for these traits, considering the above mentioned complications in quantitatively characterizing a genotype, these characters were considered to be qualitative traits for the current study. Segregation and mapping analyses were carried out by categorizing the DH lines as either producing or not producing these chemical components. Presence and absence of these chemicals in the genotypes were consistent between the two replications for all the entries.

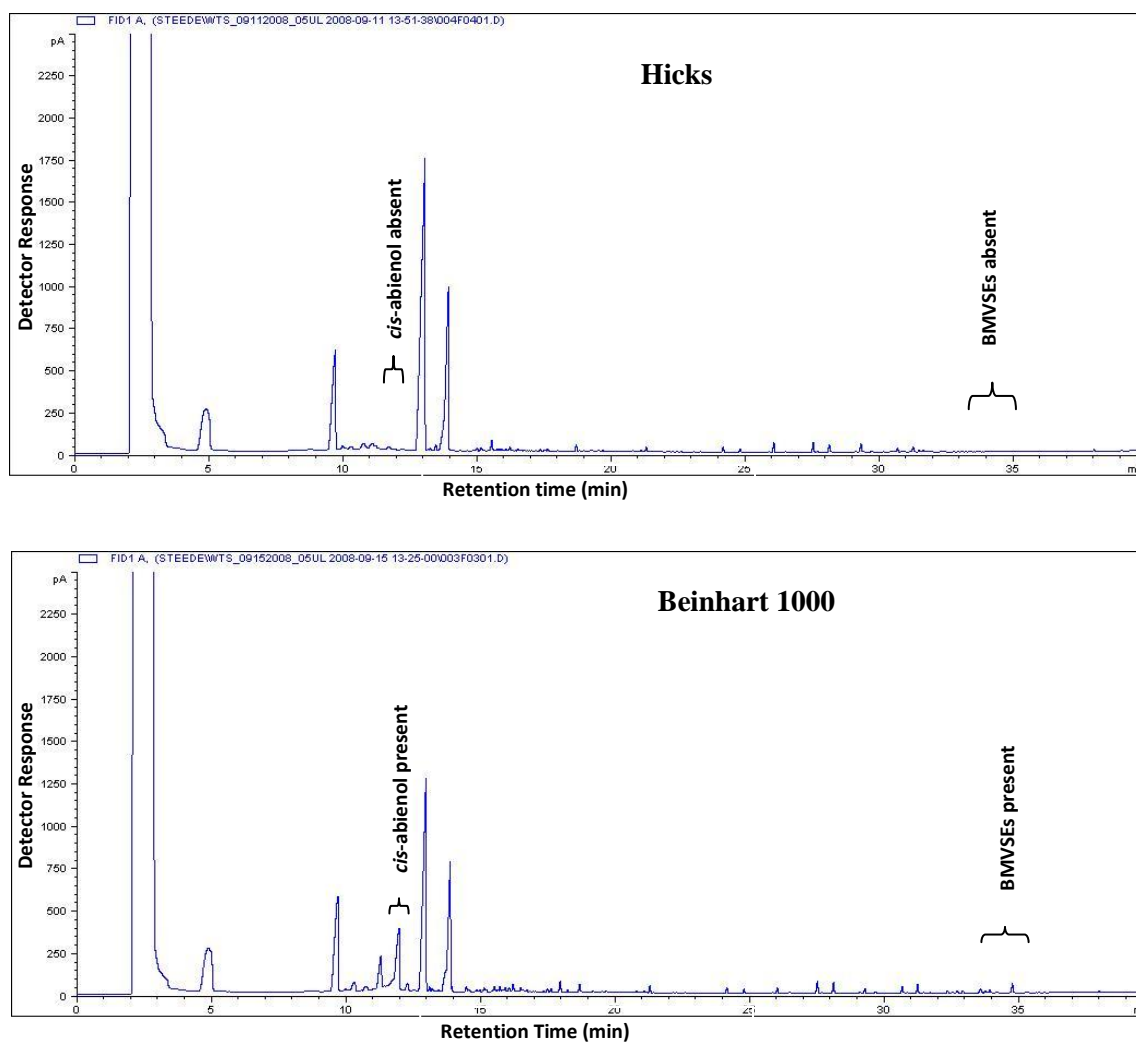


Figure 1. Gas chromatograms showing difference in the ability to accumulate two leaf chemistries by the parental lines, Hicks and Beinhart 1000.

Test for Segregation Distortion

The results of chi-square analyses to test for segregation distortion are presented in Table 1. Significant segregation distortion was observed for the BMVSEs trait, while the distortion for *cis*-abienol trait ($P = 0.079$) was not significant at the $\alpha = 0.05$ significance level. In both cases, the bias was towards the Beinhart 1000 allele (*i.e.*, there were more genotypes that have the ability to produce the chemical components).

Table 1. Chi-square analysis to test segregation distortion for two leaf surface chemistry traits.

Trait	Number of DH lines				χ^2 value	P-value
	Expected		Observed			
	+	-	+	-		
BMVSEs	58.5	58.5	73	44	7.19	0.0073
<i>cis</i> -abienol	58.5	58.5	68	49	3.09	0.0790

Joint Segregation Analysis for BMVSEs and cis-abienol Traits

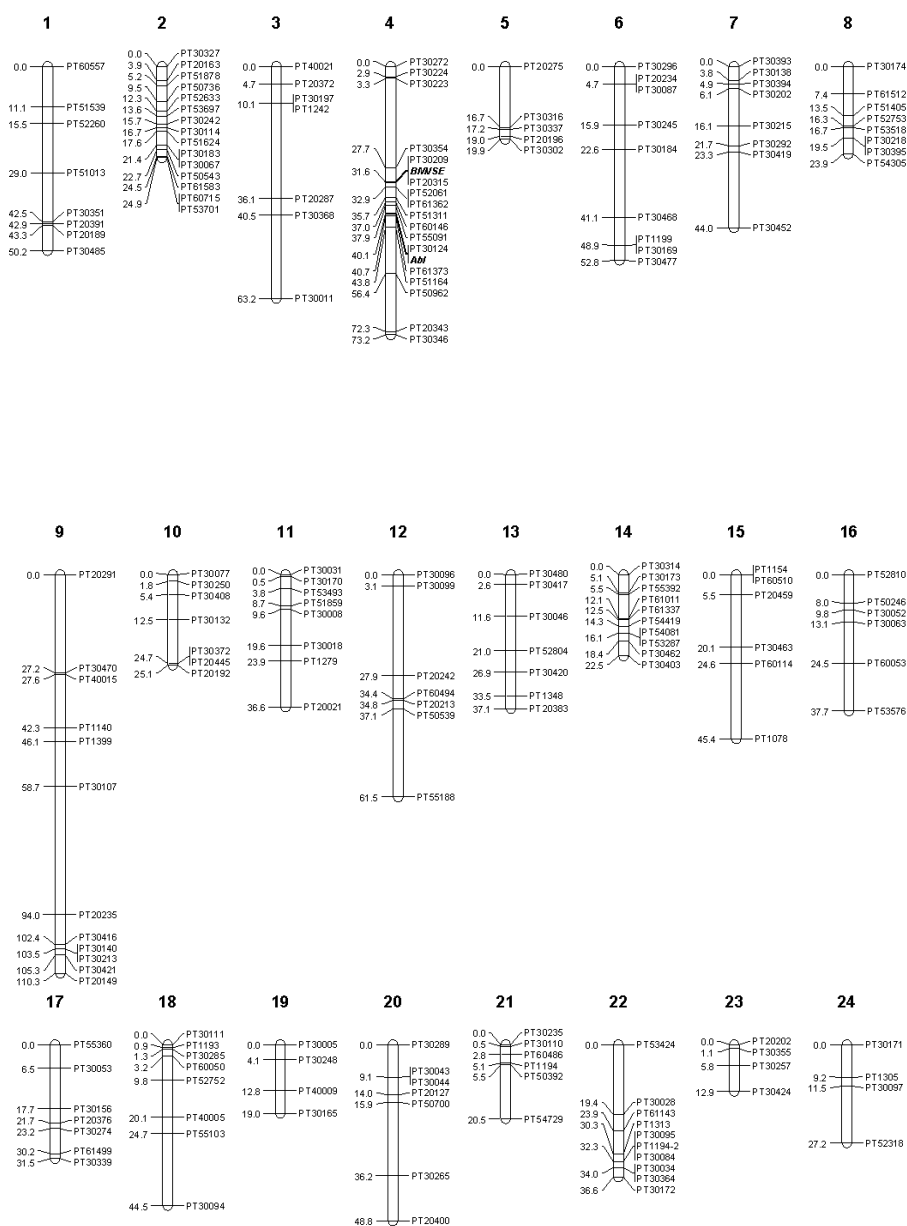
Presented in Table 2 is a two way classification of the doubled haploid lines that were used for joint segregation analysis of the two traits. A significantly ($P < 0.0001$) high chi-square value of 69.6, in this test of independence, suggested unambiguous genetic linkage between the two traits. A high LOD score of 17.46 was estimated from the two point linkage test.

Table 2. Two way classification of doubled haploid lines for BMVSEs and *cis*-abienol traits.

Genotype	BMVSEs		Total
	(+/+)	(-/-)	
<i>cis</i> -abienol (+/+)	64	4	68
<i>cis</i> -abienol (-/-)	9	40	49
Total	73	44	117

Genetic Map

Out of the 433 primer pairs used to screen the parental lines, 197 were observed to be polymorphic and all of them produced a single band except in the case of two markers, PT1194 and PT20291, which amplified two polymorphic bands. Only 190 markers, out of the total 199 markers, were grouped into 24 linkage groups. Nine markers remained unlinked (Figure 2). The total genetic distance for the map was 969.3 cM. Each linkage group consisted of 4 to 17 markers per group with an average distance of 5.1 cM between two markers. In the individual tests for segregation distortion, 32 markers, which make up 16.1% of the total markers, were observed to exhibit significant distortion at the $\alpha = 0.05$ significance level (Appendix II). Out of the 32 distorted markers, 23 of them had Beinhart 1000 alleles as the predominant allele, whereas, nine of them had Hicks allele as the predominant ones. Markers showing significant segregation distortion belonged to genomic regions representing seven different linkage groups.



Unlinked

PT20165, PT20286, PT20289, PT53795, PT60183, PT61153, PT55416, PT61019, and PT20291-2

Figure 2. Microsatellite marker linkage map derived from Beinhart 1000/Hicks doubled haploid population.

Genetic Mapping of the Two Leaf Surface Chemistry Genes, Abl and BMVSE

The genes affecting production of *cis*-abienol and BMVSEs (*Abl* and *BMVSE*) were mapped to linkage group 4 and were separated by a distance of 8.5 cM (Figure 2). Two SSR markers, PT30209 and PT20315, are strongly linked with the *BMVSE* gene with zero recombination and LOD scores values of 34.83 and 33.93 respectively. One marker, PT30124, was identified to be tightly linked with *cis*-abienol with a LOD score value of 34.23. This linkage group extended for a map distance of 73.2 cM with 17 densely placed markers. Among them, 9 markers exhibited significant segregation distortion by having Beinhart 1000 alleles predominantly in the population (Table 3). The markers adjacent to *BMVSE* gene exhibited significant distortion, whereas, the ones adjacent to the *cis*-abienol were less significantly distorted.

Table 3. Segregation distortion test for microsatellite markers present on linkage group 4.

Marker	Position on linkage group (cM)	Frequency of the allele		χ^2 -value ^a	P-value
		Beinhart 1000	Hicks		
PT30272	0.0	0.51	0.49	0.08	0.7778
PT30224	2.9	0.52	0.48	0.21	0.6439
PT30223	3.3	0.51	0.49	0.04	0.8501
PT30354	27.7	0.69	0.31	16.08	0.0001
PT30209	31.6	0.62	0.38	6.23	0.0126
PT20315	31.6	0.62	0.38	6.88	0.0087
PT52061	32.9	0.62	0.38	6.76	0.0093
PT61362	32.9	0.63	0.37	8.04	0.0046
PT51311	35.7	0.63	0.37	7.63	0.0058
PT60146	37.0	0.60	0.40	4.52	0.0335
PT55091	37.9	0.60	0.40	4.68	0.0305
PT30124	40.1	0.57	0.43	2.51	0.1129
PT61373	40.7	0.57	0.43	2.51	0.1129
PT51164	43.8	0.55	0.45	1.07	0.3008
PT50962	56.4	0.67	0.33	12.67	0.0004
PT20343	72.3	0.53	0.47	0.55	0.4576
PT30346	73.2	0.54	0.46	0.69	0.4054

^a χ^2 -value indicates the test statistic for 1:1 segregation of makers in the DH population.

DISCUSSION

Apart from being influenced by genetics, chemical makeup of leaf surfaces is also dependent on different factors like growing conditions of the plant (Gamou and Kawashima, 1979; Court et al., 1984), stage of plant development (Court, 1982; Gamou and Kawashima, 1979; Severson et al., 1982; Chang and Grunwald, 1976) and geographical location (Heeman et al., 1981). Despite the availability of different accurate scientific quantification methods for *cis*-abienol and BMVSEs, because of the above stated reasons, it is always difficult to determine the actual quantitative ability of a particular genotype to produce these leaf chemistries. Hence in the present study, presence or absence of the ability to produce these two components was used as the criterion for characterizing a particular genotype. As long as presence and absence of the compounds are considered, the doubled haploid lines exhibited consistency for both the replications.

Segregation distortion revealed by chi-square tests was highly significant for the BMVSEs trait, while it was less significant for the *cis*-abienol trait. Out of the 32 distorted markers 23 of the markers had Beinhart 1000 allele as the predominant one. The regions might contain loci having a strong influence on meiotic drag, the main cause of segregation distortion. On the 4th linkage group, all the cases of distortions are due to the predominant allele coming from the parental line, Beinhart 1000. Considering the fact that there was very high significant distortion for the markers adjacent to *BMVSE* gene, it can be assumed that the *BMVSE* gene is also more prone to this kind of deviation. Molecular markers with segregation ratios that significantly deviate from expected

Mendelian ratios are often reported in many crop species including barley (Devaux et al., 1995), rice (Xu et al., 1997), wheat (Peng et al., 2000) and maize (Lu et al., 2002). In a previous study, a significant proportion (47%) of molecular markers in tobacco was also reported to exhibit segregation distortion (Julio et al., 2006).

Earlier researchers could locate both genes on chromosome A (Kubo et al., 1982; Danehower et al., 1989), but the actual genetic distance between the two genes was not determined. The present study confirmed the linkage between these two genes and located them on the same linkage group but with a genetic distance of 8.5 cM between them. This information about the genetic distance between the two genes is of value for tobacco breeders who need to decide on appropriate population sizes for incorporating one or both traits into new cultivars. Another significant result from this study is that it is now possible to tag the chromosome A with microsatellite markers on linkage group 4.

Closely linked molecular markers might be of value in future efforts to identify these genes. In the present study, microsatellite markers strongly associated with the two genes were identified, which could effectively be used in future tobacco breeding. However, no information about the biosynthetic pathways involved in synthesis of these two chemicals has been elucidated in the present study. It was earlier suggested that *Abl* may encode for *cis*-abienol synthase (Wagner, 1999). More efforts are needed to get information about the biosynthesis of these chemistries.

Once the relevant genes and enzymes involved in the pathways are characterized, it might be possible to modify *N. tabacum* to produce greater quantities of these

compounds. Up regulation of these genes could increase natural pest resistance or flavor and aroma properties.

CONCLUSIONS

The objective of identifying molecular markers associated with genes influencing aroma and smoke flavor was achieved. With the availability of the genetic map it is now possible to locate genes controlling different economically important traits on the map. Information from the present investigation may facilitate easy incorporation of these traits into new cultivars. Future work should concentrate on purification and characterization of the enzymes involved in the production of *cis*-abienol and BMVSEs. The mode of action in influencing resistance to insect pests and possible fungal pathogens has to be elucidated.

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CHAPTER 3

MAPPING QUANTITATIVE TRAIT LOCI AFFECTING BLACK SHANK RESISTANCE IN THE TOBACCO LINE, 'BEINHART 1000'

ABSTRACT

Black shank, caused by *Phytophthora nicotianae*, is an economically important tobacco disease that causes extensive crop loss annually. Major sources of genetic resistance to this disease have largely been limited to that derived from *Nicotiana longiflora*, *N. plumbaginifolia*, and the *N. tabacum* source 'Florida 301'. Additional sources of resistance may be of value, and DNA markers linked to genes controlling the resistance could facilitate incorporation of resistance into new cultivars. The cigar tobacco line, 'Beinhart 1000,' has one of the highest levels of resistance reported in *N. tabacum* and is believed to be effective against all recognized races of the pathogen. This resistance is controlled by genes located at multiple positions across the genome. The objective of this work was to map quantitative trait loci (QTL) controlling this resistance using microsatellite markers and a doubled haploid (DH) mapping population produced from a cross between Beinhart 1000 and the susceptible cultivar, 'Hicks'. A total of 190 microsatellite markers were mapped in this population and 24 linkage groups were established. Nine markers remained unlinked. Using disease data collected from replicated testing at three environments, thirty one markers were found to be putatively associated with black shank resistance. Multiple interval mapping analysis suggested that at least six QTLs may contribute to resistance in Beinhart 1000. The two largest QTLs explained 23.5% and 20.5% of the total observed phenotypic variation for end percent survival. With a model including all 6 significant QTLs, 62.46% of the phenotypic variation and 71.15% of the genetic variation could be explained. The identified QTLs and associated markers may be of use for tobacco breeding programs aiming to

incorporate black shank resistance into new cultivars. The QTL on linkage group 4 was identified to have strong genetic association with *Abl* gene that controls the production of leaf surface chemical component, *cis*-abienol. Future research efforts have to focus on identifying the probable association between black shank resistance and *cis*-abienol accumulation.

INTRODUCTION

The black shank disease caused by *Phytophthora nicotianae*, is the most important disease affecting tobacco production in the United States. Nearly complete loss can occur in certain areas, and leaves harvested from severely infected plants are of little commercial value (Wernsman and Rufty, 1987). Incorporation of genetic resistance into commercial varieties is the most efficient, cost effective, and environmentally friendly means of reducing economic losses caused by this disease.

In tobacco breeding, monogenic resistance to black shank has been derived from two wild species, *N. longiflora* and *N. plumbaginifolia* and is governed by a gene, designated as *Ph*, which confers complete resistance to race 0 but no resistance against race 1 (Carlson et al., 1997). Due to extensive cultivation of hybrids with this resistance, race 1 has become prevalent in many areas of tobacco cultivation (Sullivan et al., 2005). The tobacco cultivar, 'Florida 301' (Tisdale, 1931) has been the other major source of resistance. This type of resistance is polygenic and effective against multiple races. This type of resistance is only partial in nature and has been associated with reduced yields in flue-cured tobacco, however. Hence, there is an urge to investigate different sources of resistance to black shank disease. A cigar tobacco line, Beinhart 1000, has one of the highest levels of resistance reported in tobacco (Heggestad and Lautz, 1957; Silber and Heggestad, 1963; Chaplin, 1966; Wills, 1971; Tedford and Nielsen, 1990; Nielsen, 1992). Past efforts to incorporate resistance from this line into burley and flue-cured tobacco have not been successful due to unfavorable associations between black shank

resistance and undesirable cigar type characteristics (Nielsen, 1992). The resistance of this line is effective against all recognized races, and probably controlled by genes located at multiple positions across the tobacco genome. Molecular markers linked to genes controlling this resistance might be of value for precisely transferring this resistance to commercial burley and flue-cured cultivars while selecting against undesired cigar type characteristics of Beinhart 1000.

To date, molecular marker studies in tobacco have been mostly limited to identifying associations between markers and resistance genes introgressed from wild relatives like those contributing to resistance to wildfire (Yi et al., 1998), black shank (Johnson et al., 2002), potato virus Y (PVY) (Lewis, 2005) and the blue mold disease (Milla et al., 2005). The report of linkage between randomly amplified polymorphic DNA (RAPD) markers and the *Ph* gene (Johnson et al., 2002) demonstrated the first potential use of marker-assisted selection in breeding for black shank resistance. Limited amounts of genetic polymorphism for RAPD and amplified fragment length polymorphism (AFLP) markers (Bai et al., 1995; Ren and Timko, 2001) had generally prevented genome wide mapping experiments in tobacco. A partial genetic linkage map of tobacco with 18 linkage groups was published using a total of 184 AFLP, inter simple sequence repeat (ISSR), sequence specific amplified polymorphism (SSAP), sequence characterized amplified region (SCAR) markers (Julio et al., 2006). Bindler et al. (2007) published a genetic linkage map based on microsatellites or simple sequence repeat (SSR) markers. These markers are more polymorphic within *N. tabacum*. Only a few

QTL mapping experiments for tobacco have been published to date (Nishi et al., 2003; Julio et al., 2006). With the availability of better and more reliable SSR markers, much larger amounts of molecular polymorphism can be observed among cultivated tobacco lines (Bindler et al., 2007; Moon et al., 2009). As was pointed out by Bindler et al. (2007), however, the genetic map has to be improved by adding more markers in order to achieve complete genomic coverage. Once a dense map is available for tobacco, QTLs responsible for different characters can be located on the map and consequently be used in tobacco breeding programs.

The present investigation was carried out with the first objective of generating a doubled haploid mapping population from the cross between Beinhart 1000 and a black shank susceptible cultivar, Hicks. The second objective was to gain insight into the genetics of resistance contained in Beinhart 1000. The third objective was to construct a genetic linkage map using microsatellite markers, and to identify markers associated QTLs affecting black shank resistance in Beinhart 1000.

MATERIALS AND METHODS

Production of the Doubled Haploid Mapping Population

The cigar tobacco cultivar, Beinhart 1000, (highly resistant to black shank) and flue cured cultivar, Hicks (susceptible to black shank), were hybridized to get F₁ hybrid individuals. F₁ progeny from this cross were hybridized as females with *Nicotiana africana* to generate maternally derived haploids according to the procedure described by Burk et al. (1979). A total of 118 doubled haploid plants were generated from these haploid plants by the midvein tissue culture procedure of Kasperbauer and Collins (1972).

Field Evaluation

The doubled haploid population, along with the parental lines, was evaluated in black shank nurseries at two locations (Cunningham Research Station, Kinston, NC and Upper Coastal Plain Research Station, Rocky Mount, NC) during 2007 and at one location (Cunningham Research Station, Kinston, NC) during 2008. The experimental design was a randomized complete block (RCBD) with 3 replications in each environment. The doubled haploid lines were evaluated in 7 m long plots containing 12 plants with an inter-row spacing of 120 cm and inter-plant spacing of 56 cm. The partially resistant cultivar, 'K 326', was planted in every third plot in each environment to test the uniformity of disease pressure in the disease nurseries. At 15 day intervals after transplanting, the number of plants killed by black shank was recorded throughout the growing season. At the end of season (120 days after transplanting), end percent survival

was calculated from the total number of plants unaffected by black shank disease. A disease index was also calculated by using the formula described by Csinos et al. (1984):

$$\text{Disease Index} = \frac{\sum_{i=1}^n X_i \left[100 - (i-1) \frac{100}{n} \right]}{I},$$

where i = Ordinal evaluation number, n = Number of evaluations (excluding initial stand count), X = Number of dead plants since last count, and I = Initial number of plants in the plot.

Agronomic Trait Evaluation

The DH lines and parental lines were also evaluated for yield, flowering time, plant height, and leaf number in two disease-free environments (at Central Crops Research Station, Clayton, NC) during 2007 and 2008. The experimental design was randomized complete block design (RCBD) with two replications per environment. Each experimental plot was a 3 m long row containing 5 plants. Inter row and within row spacings were 1.14 m and 0.56 m, respectively. Plant height and leaf number were collected for each plant and averaged to get the plot values. Yield data was collected as whole plot yield and averaged to get yield per plant. Number of days to flower was recorded as the average days to flowering for the plants in each plot after transplanting.

Genotyping

DNA was isolated according to the protocol developed by Johnson et al. (1995), except that a BIO 101 FastPrep machine (BIO 101, Holbrook, N.Y.) was used to grind the leaf samples. A total of 433 SSR primer pairs were screened to identify those that amplified polymorphic bands between the parental lines. An economical method developed by Schuelke (2000) was used for labeling the primers to facilitate the detection of amplified products. PCR reactions were performed in 15 µl volumes containing 25 ng of template DNA, 1X PCR buffer (10 mM KCl, 20 mM Tris-HCl pH 8.8, 2 mM MgSO₄), 0.015 µM of forward primer, 0.06 µM of reverse primer, 0.06 µM of M-13 primer, 0.2 mM dNTPs and 1U of *Taq* polymerase. Thermocycling started with a denaturation step for 5 min at 94°C, and 29 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec, followed by 7 cycles of 94°C for 30 sec, 52°C for 45 sec, 72 for 45 sec, and a final extension step for 5 min at 72°C. Amplification products were separated using 8 % polyacrylamide gels and a LI-COR 4300 DNA Analysis System (LI-COR Biosciences, Lincoln, Nebraska) under the following conditions: 1500V, 40mA, 40W, and 45°C for 2.5 hr. IRDye 700- or 800-labeled molecular weight standards (50 – 700 bp) were loaded on each gel for identifying allele sizes. Alleles were scored using AFLP Quantar 1.0 software (KeyGene Products B.V., Wageningen, The Netherlands).

Statistical Analysis

In order to achieve homogeneity of residual variance, data of end percent survival was transformed with arcsine function *i.e.*, $\sin^{-1} \sqrt{\text{end percent survival}}$. Single marker analyses were performed as simple F-tests that identify the significant differences between the two marker groups with respect to the character of concern, in this case, the arcsine transformed end percent survival. Arcsine transformed data was also used for composite interval mapping and multiple interval mapping analyses. Correlation coefficients were calculated using the ‘PROC CORR’ program of SAS 9.1 (SAS Institute, Cary, NC). Heritability on a plot basis and entry mean basis were calculated using univariate mixed model analysis (Holland et al., 2003).

Linkage Map Construction

Linkage analysis and genetic map construction were carried out using Mapmaker/EXP 3.0 (Lander et al., 1987). Linkage was determined on the basis of a minimum logarithm of odds (LOD) score of 3.0 and maximum map distance of 40 cM. Marker order was established by first selecting a subset of eight markers and then identifying the best order using the ‘Compare’ command. Remaining markers were added using the ‘Try’ command. The final marker order was confirmed by executing the ‘Ripple’ command. Map distances (centimorgan, cM) were estimated from recombination fractions using the Haldane mapping function (Haldane, 1919). Graphical representations of the of the linkage map were created using MAPCHART (Voorrips, 2002).

QTL Detection

Single marker analysis was carried out using the ‘PROC GLM’ procedure of SAS 9.1 (SAS Institute, Cary, NC) to identify associations between markers and end percent survival for the doubled haploid lines. Composite interval mapping (CIM) (Zeng, 1994) was performed using Windows QTL Cartographer V2.5 (Wang et al., 2007). Log-likelihood values for statistically significant associations between genotype and arcsine transformed end percent survival were generated by calculating LOD scores at 0.5 cM intervals along linkage groups. The LOD threshold significance level was determined using 1000 permutations of the method of Churchill and Doerge (1994). Composite interval mapping was executed only to provide an initial model for further analysis using the multiple interval mapping (MIM) method (Kao and Zeng, 1997; Kao et al., 1999). LOD score peaks from the CIM analysis with a value more than the threshold value of 2.5 were used in the initial models for MIM. Models were created and tested in an iterative, stepwise fashion, searching for new QTLs, and testing the significance of the QTL after each search cycle. New models were accepted only when they had a decreasing effect on the value of the Bayesian Information Criterion (BIC) (Piepho and Gauch, 2001). After the QTLs were added using the BIC criterion, all possible interactions between the QTLs were tested for their significance and were retained in the model if they decreased the BIC. While developing the model using MIM, there is always a possibility of over fitting the model. To avoid model over fitting, it was made sure that the proportion of total phenotypic variation explained by QTLs did not exceed the trait

heritability on plot basis. The model with the minimum BIC was selected, and the QTL effects were estimated using the “summary” command of QTL Cartographer. Genetic variability explained by QTLs was estimated by dividing the total explained phenotypic variation by entry mean heritability of the trait. Confidence intervals for the identified QTLs were calculated using 1 LOD score support interval as specified by Lander and Botstein (1989).

RESULTS

Phenotypic Variation in the Mapping Population

The two parameters, disease index and end percent survival were found to be highly correlated with a correlation coefficient of 0.91 ($P < 0.0001$) and hence end percent survival alone was considered for further analysis because of its simplicity and convenience for presentation and interpretation. The resistant parent, Beinhart 1000 (Figure 1), proved to be highly superior (93.48% average end survival) compared to the susceptible parent Hicks (0% survival). The doubled haploid population, that included 118 lines, showed wide variation in percent survival ranging from 0% to 91.68% (Appendix I). As is evident from Figure 2, the distribution for end percent survival was not normal but skewed towards susceptibility with 46 lines showing less than 10 % survival and only 10 lines showing more than 75% survival. Arcsine transformed percent survival was subjected to analysis of variance (Table 1) that confirmed not only significant genotypic differences but also significant environmental effects and genotype \times environment interaction (GEI). Despite significant GEI, the correlations between the means from three environments were found to be always greater than $r = 0.81$. Because of these high correlations, means over three environments were considered appropriate for QTL identification analyses. From a separate ANOVA carried out for only K 326 plots, no significant differences were found for disease pressure between the plots within a block across all the three environments. Both the heritability on a plot basis ($h^2 = 0.70$) and heritability on an entry mean basis ($h^2 = 0.88$) for end percent survival were found to

be high. Pair wise comparisons were made on the means of arcsine transformed values using Duncan's multiple comparison procedure. For convenience of presentation, data for the most resistant lines was back transformed and presented in Table 2. Even after the transformation, ranking of genotypes was not changed and hence the two doubled haploid lines, DH05B-1252-300 and DH06B-162-16, were ranked as the two most resistant lines.

Associations between measured agronomic traits and end percent survival were studied by calculating Pearson's correlation coefficients (Table 3). Leaf yield per plant exhibited significant positive association with plant height, leaf number and days to flower with P values of <0.0001 , 0.0003 , and 0.0079 , respectively. A negative association was identified between the mean values for end percent survival and yield per plant. The correlation coefficient ($r = -0.16$) between the two traits was not statistically significant ($P = 0.0931$), however.

Table 1. ANOVA for arcsine transformed end percent survival for doubled haploid population.

Source	DF ^a	SS ^b	MSS ^c	F-value	P-value
Environment	2	0.8911	0.4456	9.50	<0.0001
Block (Environment)	6	3.1247	0.5208	11.10	<0.0001
Genotype	117	162.3566	1.3877	29.59	<0.0001
Genotype × Environment	234	20.5617	0.0879	1.87	<0.0001
Error	702	32.9220	0.0469	-	-

$R^2 = 0.85$

C.V. = 46.32

^aDF- Degrees of freedom; ^bSS- Sum of squares; ^cMSS-Mean sum of squares.



Figure 1: Beinhart 1000 (right) beside a susceptible line (left) in the disease nursery at Rocky Mount, NC.

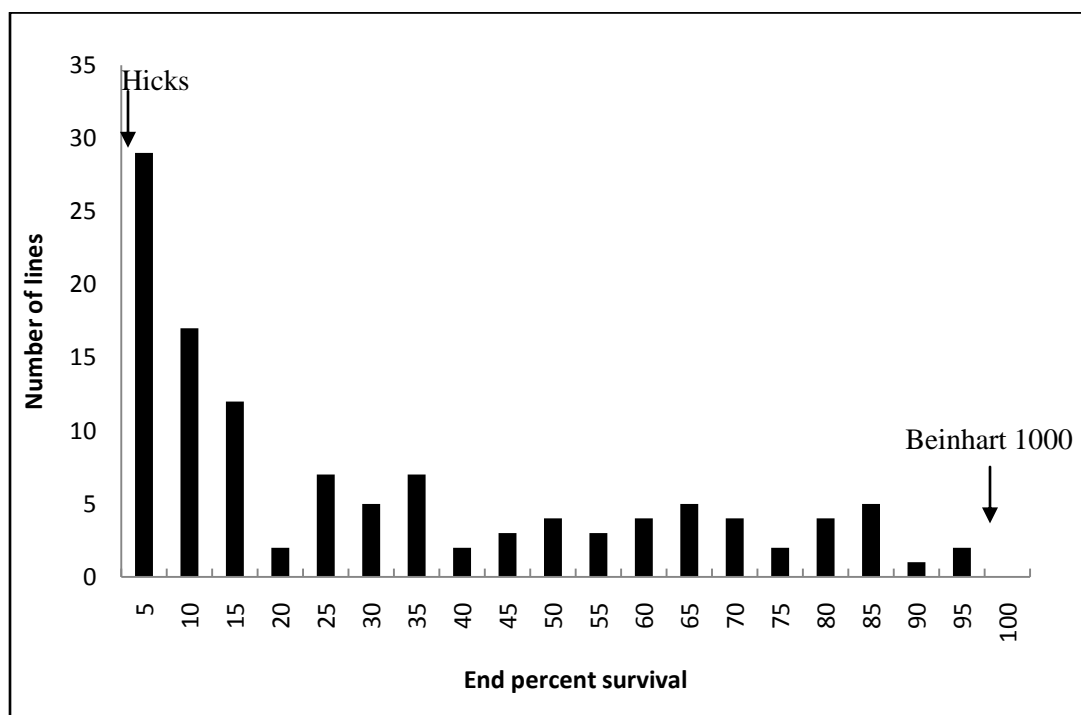


Figure 2: Frequency histogram for end percent survival for doubled haploid population.

Table 2. List of resistant doubled haploid lines exhibiting more than 70 end percent survival rates.

Doubled Haploid	End percent survival ^a	
DH05B 1252-300	96.40	A ^b
DH06B 162-16	95.62	A
DH06B 173-46	91.78	AB
DH05B 1252-100	88.19	ABC
DH05B 1252-240	86.87	ABC
DH06B 173-49	84.78	ABCD
DH05B 1252-116	84.05	ABCDE
DH06B 173-47	83.31	ABCDE
DH06B 162-8	82.56	ABCDEF
DH05B 1252-293	80.23	BCDEFG
DH06B 173-87	79.43	BCDEFG
DH05B 1252-233	79.43	BCDEFG
DH06B 173-20	78.61	BCDEFGH
DH06B 162-27	76.95	BCDEFGHI
DH06B 173-18	72.61	CDEFGHIJ
DH06B 162-19	71.71	CDEFGHIJ

^a End percent survival indicates the back transformed value to approximate the original value of end percent survival

^b End percent survival values with the same letters are not significantly different ($\alpha=0.05$) as revealed by Duncan's multiple range test

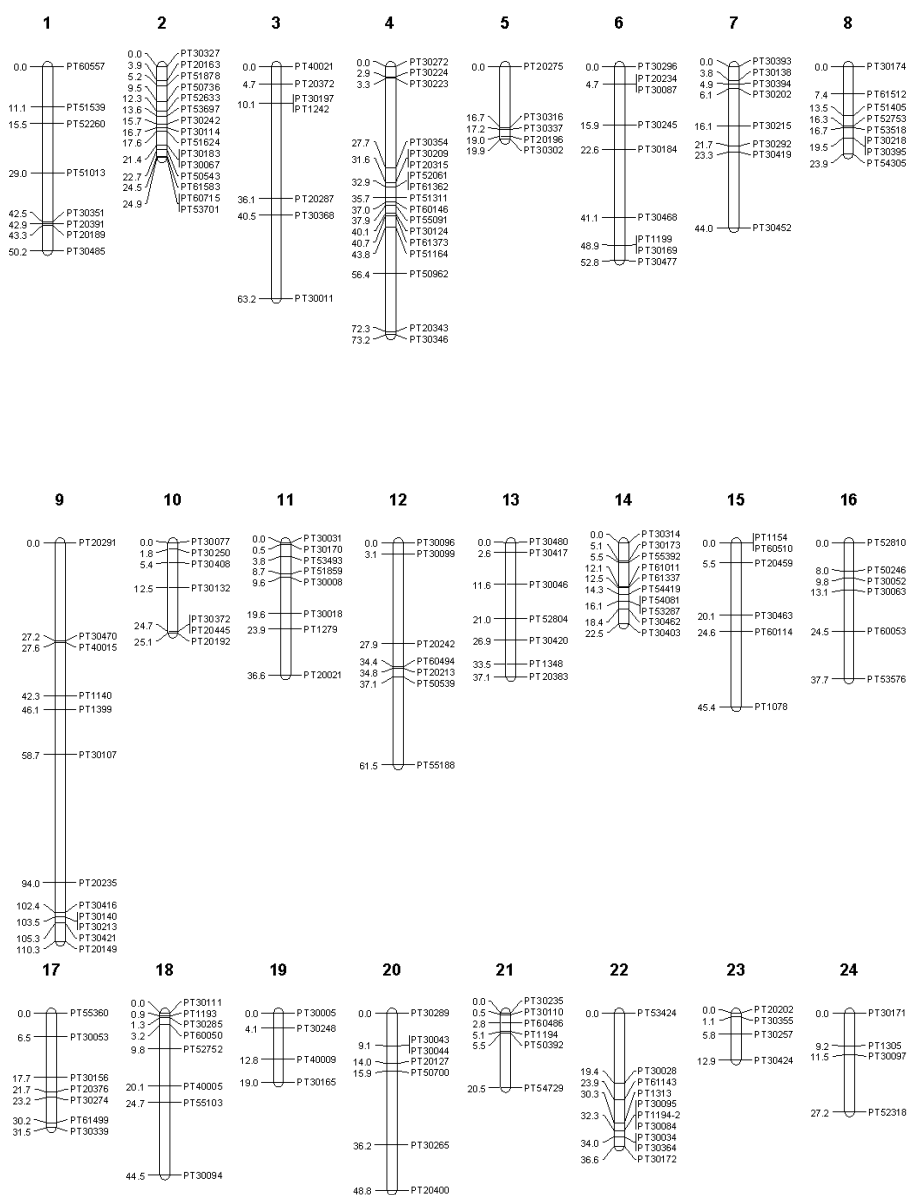
Table 3. Correlation coefficients between different agronomic traits and end percent survival in black shank disease evaluation.

Trait	Leaf Number	Plant Yield	Days to flower	End percent survival
Plant Height	0.61 (< 0.0001) ^a	0.44 (<0.0001)	0.68 (<0.0001)	-0.02 (0.8007)
Leaf Number		0.33 (0.0003)	0.65 (<0.0001)	-0.12 (0.1922)
Plant Yield			0.24 (0.0079)	-0.15 (0.0931)
Days to flower				0.06 (0.5269)

^aValues in parenthesis indicate *P*-values.

Construction of Genetic Map

Out of 433 microsatellite primers screened, a total of 197 primers were identified to be polymorphic between the parental lines while the rest of them were either monomorphic or non-functional with the protocol used. Generally, each primer amplified only one polymorphic band. Two primers (PT1194 and PT20291), however, amplified two polymorphic bands each, making the total number of markers 199. Only 190 markers were grouped into 24 linkage groups on the map, leaving 9 markers unlinked (Figure 3). The total length of the map was 969.3 cM, with each linkage group consisting of 4 to 17 markers per group. Out of the total amplified fragments (23,482 alleles) across the population, Beinhart 1000 contributed 48.89% and Hicks contributed 47.37%, with missing data points accounting for the remainder (3.73%). A total of 32 microsatellite markers exhibited significant levels ($\alpha = 0.05$) of segregation distortion (Appendix II). Out of the 32 distorted markers, 23 of them had Beinhart 1000 alleles predominant while nine had Hicks alleles as most prevalent.



Unlinked markers

PT20165 PT20286 PT20289 PT53795 PT60183 PT61153 PT55416 PT61019 PT20291-2

Figure 3. Microsatellite marker linkage map generated using Beinhart 1000/Hicks doubled haploid population.

Single Marker Analysis

A total of 31 markers were found to be significantly associated with arcsine transformed end percent survival at the $\alpha = 0.05$ level of significance (Table 4.). Out of the 31 markers, two markers were unlinked and hence were not assigned to any linkage group. The *R*-square values for markers significantly associated with end percent survival ranged from 3.45% (PT52633) to 20.44% (PT30174). Two markers, PT30174 and PT61373, explained 20.4% and 19.4% of the observed phenotypic variation, respectively. A gel image of polymorphic bands amplified by marker PT30346 in the DH population is presented in Figure 4. In all cases, except for one marker (PT61153), the alleles corresponding to Beinhart 1000 contributed to greater end percent survival. The significantly associated markers represented 6 genomic regions on six different linkage groups (Linkage groups 2, 4, 8, 9, 11, and 14).

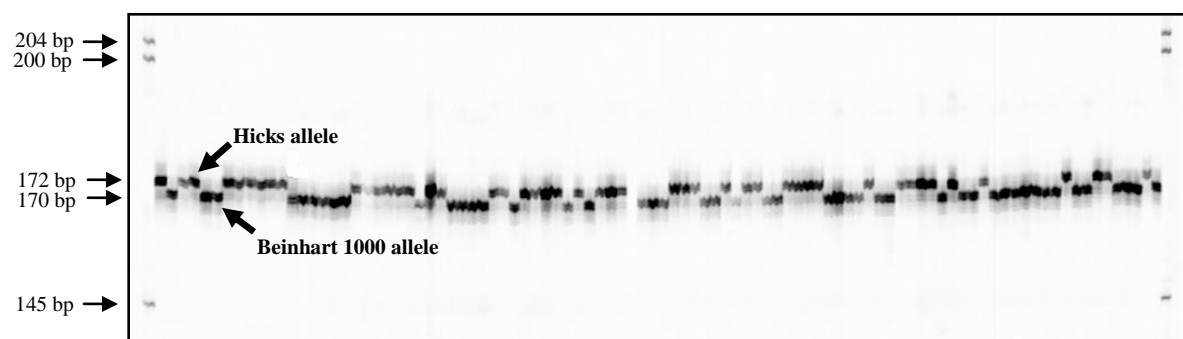


Fig 4: Gel image of polymorphic bands in the doubled haploid population amplified by microsatellite primer pair PT30346.

Table 4. List of significant markers with their R^2 -values, P -values, and effects for arcsine transformed end percent survival.

Marker	Linkage group	R^2 -value	P -value	Effect ^a
PT52633	2	0.0345	0.0439	2.10
PT30067	2	0.0382	0.0389	2.29
PT30354	4	0.0425	0.0270	2.99
PT30209	4	0.1014	0.0005	6.41
PT20315	4	0.0888	0.0013	5.65
PT52061	4	0.1311	<0.0001	8.20
PT61362	4	0.1359	<0.0001	8.87
PT51311	4	0.1647	<0.0001	10.39
PT60146	4	0.1339	<0.0001	8.29
PT55091	4	0.1364	<0.0001	8.51
PT30124	4	0.1604	<0.0001	9.29
PT61373	4	0.1940	<0.0001	11.61
PT51164	4	0.1616	<0.0001	9.51
PT50962	4	0.1087	0.0003	7.22
PT20343	4	0.0768	0.0026	4.67
PT30346	4	0.0929	0.0008	5.62
PT30174	8	0.2044	<0.0001	12.15
PT61512	8	0.1128	0.0002	6.86
PT51405	8	0.0727	0.0036	4.49
PT52753	8	0.1199	0.0001	7.25
PT53518	8	0.0753	0.0030	4.73
PT30218	8	0.0713	0.0038	4.53
PT30395	8	0.0877	0.0012	5.48
PT54305	8	0.0440	0.0232	2.70
PT40015	9	0.0402	0.0303	2.45
PT1140	9	0.0486	0.0165	2.94
PT51859	11	0.0359	0.0399	2.19
PT30173	14	0.0564	0.0102	3.41
PT55392	14	0.0517	0.0141	3.13
PT53795	Unlinked	0.0764	0.0024	4.83
PT61153	Unlinked	0.0425	0.0278	-2.63

^aEffect is presented as the back transformed value to approximate the original value of end percent survival.

QTL by Environment Interaction

In separate analyses carried out for each of the three environments, the QTLs present on Linkage groups 2, and 9 were not significantly associated with resistance in one of the three environments, while the QTL on linkage group 11 was not significantly associated in two environments (Table 5). The QTLs present on linkage groups 4, 8 and 14 were identified to have significant effects in all the three environments. This confirms that these three QTLs are reliable and consistent for a wide range of environments. Two markers that were significant in the overall single marker analysis but not assigned to any linkage group, were significant in all the three environments except in the case of marker PT61153 in summer, 2008, at Kinston.

Table 5. Presence (+) or absence (-) of significant QTLs in separate analyses for three environments.

Environment	Linkage group					
	8	4	14	11	9	2
Kinston, 2007	+	+	+	-	-	+
Rocky Mount, 2007	+	+	+	-	+	+
Kinston, 2008	+	+	+	+	+	-

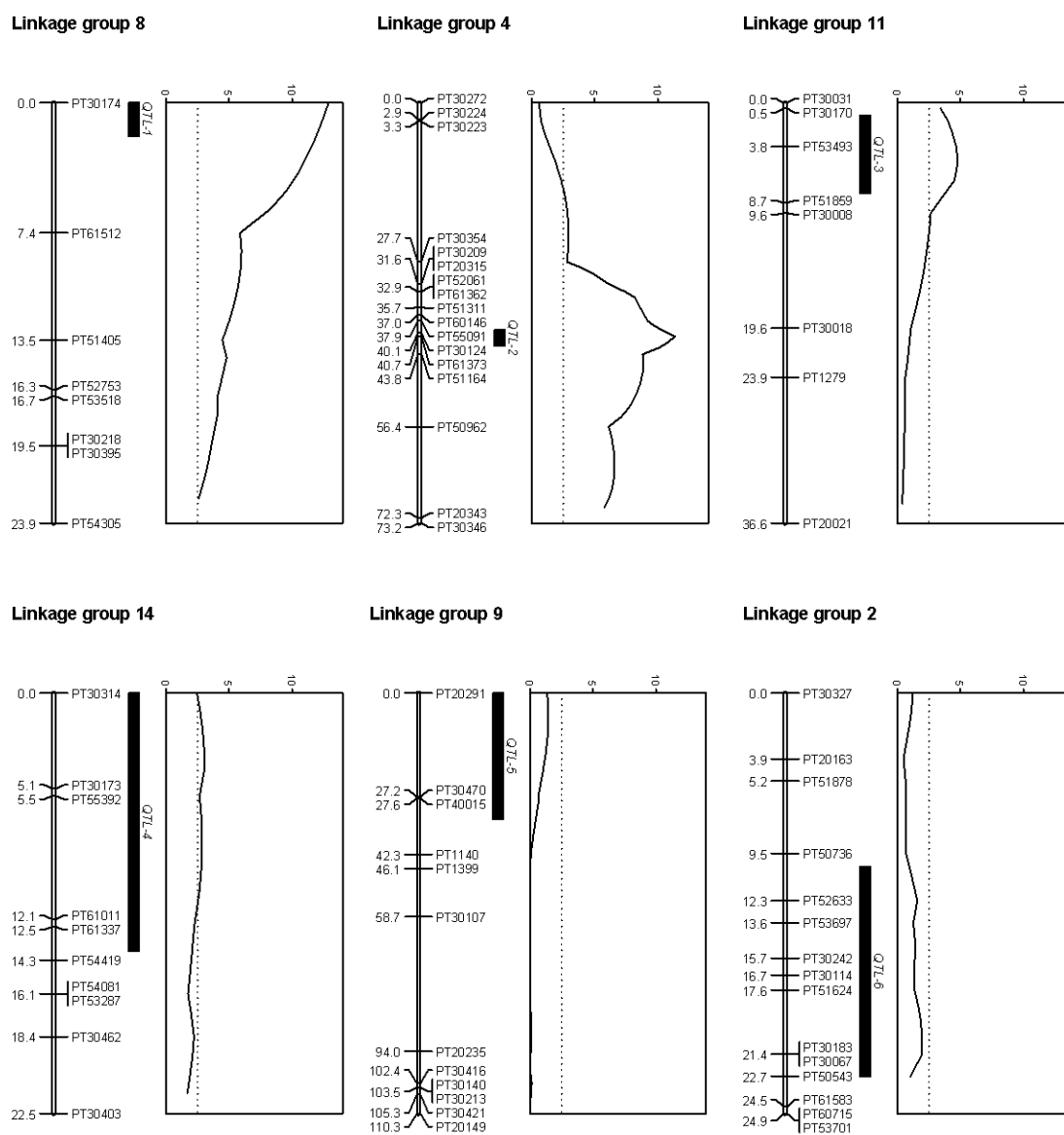
Multiple Interval Mapping

The four QTL regions that were identified in CIM analysis were included in the initial model for MIM analysis. Additional QTL and interaction effects were then added based on the BIC value criterion. After addition of the QTLs present on linkage groups 2 and 9, the BIC value was decreased and hence those two QTLs were also included in the model. All interaction effects between the QTL regions were found to be not significant and hence the final model (Table 6) contained only six QTL regions. As the explained phenotypic variation (62.44%) by this final model was less than the observed heritability (70.49%), it was confirmed that there was no problem of over fitting the model. The effects ranged from 4.8 to 14.4 end percent survival. The QTLs on linkage groups 4 and 8 were associated with higher effects (14.2 and 14.4 percent survival, respectively). The percent of variation explained by each QTL ranged from 3.2% to 23.5%. The top two QTLs together explained 44% of the variation. The total genetic variation explained by the proposed model was 71.1%. The LOD scores for the QTLs on linkage groups 4 and 8 were observed to be 11.32 and 12.85 respectively. The LOD score peaks generated for all six QTLs are presented in Figure 5.

Table 6. Estimates of QTL positions, effects, and explained variation from MIM analysis.

QTL	Linkage group	Left marker	Right marker	Position	LOD	Effect^a	% of phenotypic variation explained
1	8	PT30174	PT61512	0.10	12.85	14.43	23.50
2	4	PT61373	PT51164	40.80	11.32	14.20	20.50
3	11	PT53493	PT51859	5.80	4.71	9.86	6.40
4	14	PT30314	PT30173	4.00	3.09	6.16	5.30
5	9	PT20291	PT30470	6.00	1.45	4.85	3.60
6	2	PT30067	PT50543	21.50	1.92	6.47	3.20

^aEffects are presented as back transformed values to approximate the corresponding end percent survival.



Note: Length of the QTL bars indicate confidence intervals (96.8%) calculated with a support interval of 1 LOD score.

Figure 5. LOD score peaks generated from MIM analysis for arcsine transformed end percent survival.

DISCUSSION

Phenotyping has always been a key component of QTL studies in order to achieve substantial reliability and practical applicability of the identified loci in breeding programs. Particularly in the case of phenotyping of experimental lines for resistance to soilborne diseases, it has to be made sure that they are evaluated in a field with uniform disease pressure. In the present study, in order to verify that disease pressure was uniform in the field environments, partially resistant variety K 326 was planted every two rows. In an ANOVA analysis for only the K 326 plots, the difference between the plots within a block was not significant. This confirmed the suitability of the disease nurseries for evaluating the experimental population.

If end percent survival alone was considered, the two most resistant lines DH05B-1252-300 and DH06B-162-16 exhibited 91.7% and 90.7% survival rates, respectively. This level of resistance is nearly as high as that exhibited by resistant parent, Beinhart 1000 (93.48% survival). It can therefore be concluded that these lines likely captured most, but possibly not all, of the desirable alleles from the resistant parent. The range for end percent survival also suggests that there are probably multiple genomic regions controlling the high level of resistance in Beinhart 1000, and that large segregating populations are probably required for capturing all of these genomic regions. Having half of their genome derived from the flue cured tobacco parent, the identified resistant doubled haploid lines might serve as a good starting point to begin incorporating

resistance into promising tobacco cultivars using a marker assisted back crossing program.

A total of 190 microsatellite markers were grouped into 24 linkage groups to generate a linkage map with a genetic length of 969.3 cM. This likely represents less than 50% of total genomic coverage. This, in addition to the fact that nine markers were not linked to any linkage group, demonstrates the need to add more markers to the map to achieve increased genomic coverage.

A total of 32 (16% of total markers) markers exhibited significant levels of segregation distortion. The majority of these cases had increased frequencies of Beinhart 1000 alleles. Segregation distortion is often encountered and has been reported in many crop species including barley (Devaux et al., 1995), rice (Xu et al., 1997), wheat (Peng et al., 2000), and maize (Lu et al., 2002). A significant proportion (47%) of molecular markers in tobacco was also reported to exhibit segregation distortion (Julio et al., 2006). Mechanisms of segregation distortion have been less studied in plants, and efforts are needed to unravel the mechanism for this phenomenon.

Single marker analysis revealed significant associations between disease resistance and 31 microsatellite markers representing 6 genomic regions. The Beinhart 1000 alleles were always associated with higher resistance except in the case of one marker (PT61153), where Hicks contributed the favorable allele. This suggests that incorporation of Beinhart 1000 chromosomal segments into elite genetic backgrounds may be a practical means of achieving increased levels of black shank resistance. To

more precisely establish the actual interval for QTLs, MIM analysis was conducted to gain insight into the genetic architecture of the black shank resistance. The two QTLs with largest effects explained 23.5% and 20.5% of the total phenotypic variation, respectively. The final model with six QTLs explained 62.46% of the phenotypic and 71.15% of the genetic variation. This demonstrates the likelihood of additional genomic regions that may contribute to the high level of disease resistance exhibited by Beinhart 1000. Additional efforts are needed to increase genome coverage with additional markers. It is likely that additional QTLs will be identified that contribute to black shank resistance in this line.

As was emphasized in a review by Bernardo (2008) there have been thousands of reports about marker-trait associations for many traits in different plant species, but there are far fewer reports of successful exploitation of mapped QTLs. Exploitation of QTLs is sometimes complicated by genetic background effects that are responsible for making an identified QTL ineffective when incorporated into alternative genetic backgrounds. The QTLs identified in the present study should therefore be evaluated in different genetic backgrounds for their role in disease resistance. Other factors that can sometimes limit the utility of QTLs are interaction effects that can exist between the QTL and environments. In the present study, three QTLs have a significant effect in all the three environments, while 2 QTLs are significant in two environments and one QTL was significant only in one location. This might be due to inadequacy of statistical procedures to resolve the QTLs with smaller effects. The two major QTLs have highly

significant effects across all the three environments and constitute about 44% of the total phenotypic variation. Hence these QTLs might successfully be implemented in a breeding program that aims at development of resistant cultivars for a wide range of environments.

When using wild relatives or genetically distant genotypes as the source of desirable genes, the challenge sometimes lies in precise incorporation of only the desirable regions without problems of linkage drag. In the present study, as all of the doubled haploid lines derived half of their genome from the flue cured parent, it is better to use one of the best doubled haploid lines as the source of resistance for incorporation of desirable resistant alleles into flue cured and burley tobacco cultivars.

As can be observed from the previous and present chapters, the two leaf surface chemistry traits, *cis*-abienol and β -methyl valeric acid-containing sucrose esters (BMVSEs) were closely mapped with the very significant QTL present on linkage group 4. Linkage between the two leaf surface chemistry traits and black shank resistance might be the reason for past difficulties in using Beinhart 1000 as a black shank resistance source. These undesirable cigar-type characteristics might have hindered the efforts of tobacco breeders to incorporate Beinhart 1000 resistance into flue-cured and burley cultivars. Considering the possible antifungal properties of the leaf surface chemistries (Cruickshank et al., 1977), the possible relationship between these leaf chemistry traits and black shank resistance should be investigated in the future. The negative association between black shank resistance and plant yield demonstrates the importance of more

research efforts to unravel the genetic architecture of Beinhart 1000 resistance in order to be able to incorporate this resistance into new tobacco cultivars.

CONCLUSIONS

Since a large fraction of the genetic variance for end percent survival was explained by the QTLs, described here, it might now be possible to incorporate most of the Beinhart 1000 resistance into elite varieties using a marker assisted back crossing scheme. More efforts are needed to find additional regions that affect black shank resistance. The identified QTLs have to be validated in different genetic and environmental backgrounds.

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CHAPTER 4

GENETIC COMPARISONS BETWEEN TWO POLYGENIC SOURCES OF BLACK SHANK DISEASE RESISTANCE IN TOBACCO

ABSTRACT

The cigar tobacco cultivar, 'Florida 301,' has to this point in time served as the primary source of resistance in development of many cultivars resistant to the black shank disease caused by *Phytophthora nicotianae*. The resistance of Florida 301 has been reported to be governed by genes at multiple loci and provides partial resistance against multiple races of the pathogen. Another polygenic source that exhibits greater levels of resistance against all races is the cigar tobacco line, 'Beinhart 1000.' Having previously identified molecular markers linked to six quantitative trait loci (QTLs) putatively contributing to resistance in Beinhart 1000, the current investigation was carried out to determine whether or not the same genomic regions contribute to resistance in Florida 301. Phenotyping and genotyping experiments were carried out using a population of 125 F_{5,6} recombinant inbred lines (RILs) developed from a cross between Florida 301 and a susceptible cultivar, 'Hicks.' Out of 21 microsatellite markers representing all six genomic regions of interest in Beinhart 1000, six markers representing two regions (on linkage groups 2 and 8) were found to be associated with resistance contained in Florida 301. The QTL on linkage group 8 was found to be very important in influencing black shank resistance in both Beinhart 1000 and Florida 301. The region on linkage group 2 was a minor QTL affecting resistance in Beinhart 1000 resistance, but was observed to be a major QTL for Florida 301. Interestingly, the genomic region on linkage group 4 that was identified to have a very large effect on resistance in Beinhart 1000 showed no significant association with resistance in Florida 301. More detailed genotyping is needed

to identify additional important regions that contribute to the high level of partial resistance in Florida 301. The QTL information for both of these sources may be valuable information for tobacco breeders attempting to deploy polygenic resistance for the control of the black shank disease.

INTRODUCTION

The black shank disease of tobacco caused by *Phytophthora nicotianae* poses a tough challenge for tobacco breeders to develop cultivars resistant to its multiple races. Out of the four reported races (Race 0, 1, 2, and 3), race 0 and race 1 have been economically important because of their prevalence in tobacco fields in many cultivated regions worldwide. Cigar wrapper tobacco cultivar, Florida 301, was developed by Tisdale (1931) from a cross between Big Cuba and Little Cuba and has served as the primary source of quantitative resistance in the development of many black shank resistant cultivars. The Florida 301 type of resistance can provide partial resistance to race 0 and race 1 in moist seasons, but severe crop losses can be observed during dry seasons (Valleau et al., 1960). Breeding efforts to develop cultivars highly resistant to race 0 have been successful due to the availability of monogenic resistance (conferred by the *Ph* gene) derived from two *Nicotiana* species, *N. plumbaginifolia* and *N. longiflora* (Valleau et al., 1960; Chaplin, 1962). Extensive cultivation of tobacco cultivars possessing the *Ph* gene has resulted in major shifts in pathogen populations towards increased prevalence of race 1 (Sullivan et al., 2005). Race 1, therefore, poses a severe threat to economical tobacco production in many regions. The problem of race shifts in

pathogen populations is a universal phenomenon that occurs when monogenic resistance is implemented in breeding programs. A solution for overcoming this predicament is to incorporate polygenic resistance effective against multiple races (Csinos, 2005).

Another cigar tobacco line, Beinhart 1000, has been reported to exhibit the highest level of polygenic resistance effective against multiple races of the black shank pathogen (Heggestad and Lautz, 1957; Silber and Heggestad, 1963; Chaplin 1966; Wills 1971; Tedford and Nielsen, 1990; Nielsen, 1992). Past efforts to incorporate resistance from this line into burley and flue-cured tobacco have been complicated by associations between resistance and undesirable cigar type characteristics (Nielsen, 1992). Increased understanding of the genetic basis of polygenic resistance in the two available sources, Florida 301 and Beinhart 1000, might be useful for comprehensive breeding programs with the goal of developing new disease-resistant tobacco cultivars. No research efforts, thus far, have investigated the possible genetic similarities between these two polygenic sources of resistance. Having a fairly detailed genetic map of microsatellite makers in hand (Chapter 3), it might now be possible to compare the effects of genomic regions contributing to the resistance in these two sources.

A report of linkage between RAPD markers and the *Ph* gene by Johnson *et al.*, (2002) demonstrated the first potential use of marker-assisted selection in breeding for black shank resistance. With the availability of more precise and highly polymorphic microsatellite markers and a detailed genetic map (Bindler *et al.*, 2007; Chapter 3), marker assisted selection for quantitative types of disease resistance may be a valuable

tool for tobacco breeders. A thorough understanding of these regions might also facilitate initiation of research with a focus on determining the modes of action for black shank resistance.

The present investigation was carried out to gain insight on the extent of similarities of genetic control of black shank resistance between the two tobacco cultivars, Beinhart 1000 and Florida 301. The first objective was to develop a recombinant inbred population from the cross between Florida 301 and Hicks. The second objective was to evaluate the population in multiple environments for black shank resistance, and then to genotype the population with selected markers representing regions of interest in Beinhart 1000 to determine whether or not the same regions influence black shank resistance in the two sources.

MATERIALS AND METHODS

Development of the Mapping Population and Field Evaluation

A population of 125 F_{5:6} recombinant inbred lines (RILs) was developed using single seed descent from a cross between partially resistant cultivar, Florida 301, and the susceptible cultivar, Hicks. The RIL population, along with the parental lines, was evaluated in black shank nurseries at three locations (Cunningham Research Station, Kinston, NC, Upper Coastal Plain Research Station, Rocky Mount, NC, and Oxford Tobacco Research Station, Oxford, NC) in the summer of 2008. The experimental design was a randomized complete block design with three replications in each environment. Plots consisted 7 m long single rows of 12 plants each. Inter-row spacing was 120 cm and inter-plant spacing was 56 cm. At 15 day intervals, the number of plants killed by disease was recorded throughout the growing season. At the end of season (120 days after transplanting), end percent survival was calculated from the total number of plants unaffected by black shank disease. Disease index was calculated by following the formula of Csinos et al. (1984):

$$\text{Disease Index} = \frac{\sum_{i=1}^n X_i \left[100 - (i-1) \frac{100}{n} \right]}{I},$$

where i = ordinal evaluation number, n = number of evaluations (excluding initial stand count), X = number of dead plants since last count, and I = initial number of plants in the plot.

Genotyping

DNA was isolated according to the protocol developed by Johnson et al. (1995) with the exception that a BIO 101 FastPrep machine (BIO 101, Holbrook, N.Y.) was used to grind the leaf samples. A total of 21 SSR markers linked to six QTLs associated with black shank resistance in Beinhart 1000 (Chapter 3) were used to genotype the RIL population. An economic method developed by Schuelke (2000) was used for labeling the primers to facilitate the detection of amplified product. PCR reactions were performed in 15 µl volumes containing 25 ng of template DNA, 1X PCR buffer (10 mM KCl, 20 mM Tris-HCl pH 8.8, 2 mM MgSO₄), 0.015 µM of forward primer, 0.06 µM of reverse primer, 0.06 µM of M-13 primer, 0.2 mM dNTPs, and 1U of *Taq* polymerase (New England Biolabs). Thermocycling started with a denaturation step for 5 min at 94°C followed by 29 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec, and 7 cycles of 94°C for 30 sec, 52°C for 45 sec, 72 for 45 sec, and a final extension step of 5 min at 72°C. Amplification products were separated using 8 % polyacrylamide gels and a LI-COR 4300 DNA Analysis System (LI-COR Biosciences, Lincoln, Nebraska) under the following conditions: 1500V, 40mA, 40W, and 45°C for 2.5 hr. IRDye 700- or 800-labeled molecular weight standards (50 – 700 bp) were loaded on each gel for identifying the allele size. The alleles were scored using AFLP Quantar 1.0 software (KeyGene Products B.V., Wageningen, The Netherlands).

Data Analysis

Analysis of variance, Duncan's multiple range test (Duncan, 1955), and single marker F-tests were carried out using the PROC GLM procedure in SAS 9.1 (SAS Institute, Cary, NC) to identify associations between markers and end percent survival. Heritability on a plot basis and entry mean basis were calculated using univariate mixed model analysis (Holland et al., 2003).

RESULTS

Phenotypic Variation for End Percent Survival

A significant correlation between the mean values for end percent survival and disease index was observed ($r = 0.81$; $P < 0.0001$). End percent survival alone was therefore considered for further analysis because of its convenience for presentation and interpretation. As shown in Table 1, the analysis of variance revealed significant difference between the RILs as well as significant genotype \times environment interaction ($P < 0.0001$). Florida 301 (Figure 1), with an end percent survival of 71.32%, was found to be moderately resistant as compared to Hicks, which exhibited zero end percent survival. The population exhibited a large range of variation for end percent survival that ranged from 60.8% end percent survival to complete susceptibility with zero end percent survival (Figure 2). The frequency distribution of the RILs was not normal, but skewed towards susceptibility (Figure 2). Heritability on a plot basis was found to be low ($h^2 = 0.35$) as compared to heritability on entry mean basis ($h^2 = 0.62$). The genotypes GH07B-959 and GH07B-966 were found to exhibit the highest end percent survival (Appendix III).



Figure 1. Florida 301 (left) beside a susceptible RIL line (right).

Table 1. ANOVA table for end percent survival exhibited by recombinant inbred lines.

Source	DF ^a	SS ^b	MSS ^c	F-value	P-value
Environment	2	34435.19	17217.59	105.95	<0.0001
Replication (Environment)	6	4517.97	753.00	4.63	0.0001
Genotype	124	142343.30	1147.93	7.06	<0.0001
Genotype × Environment	248	58898.87	237.50	1.46	<0.0001
Error	744	120905.58	162.51	-	-

^a DF- Degrees of freedom; ^b SS- Sum of squares; ^c MSS-Mean sum of squares.

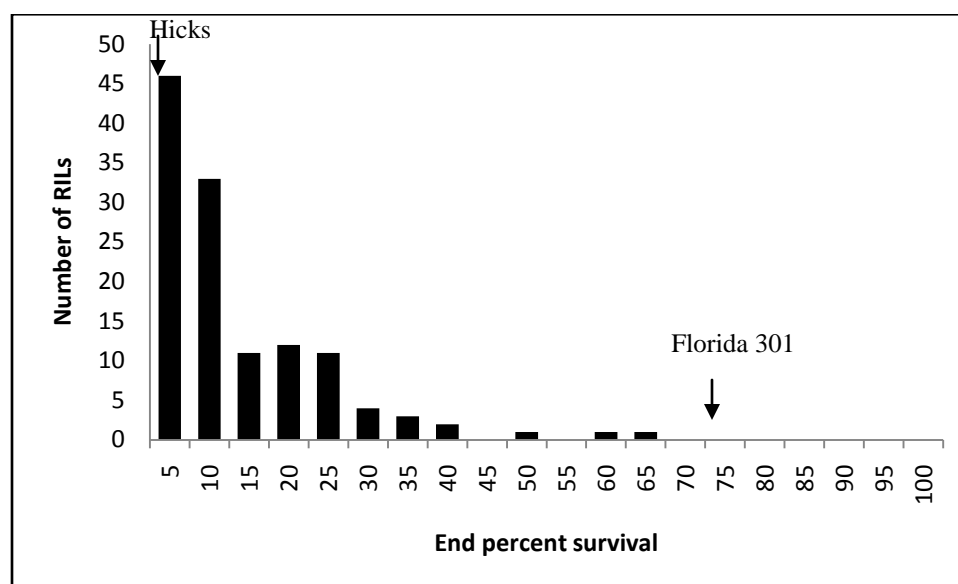


Figure 2. Frequency histogram of RILs developed from the cross Florida 301/Hicks for end percent survival.

Single Marker Analysis

In single marker analysis (Table 2), out of 21 markers representing six regions of interest, six markers representing two of those regions were found to be significantly associated with resistance in Florida 301. In the Beinhart 1000/Hicks population, marker PT52633 belonging to linkage group 2 was found to explain a minor portion of the variation with an R^2 -value of 0.035, where as in Florida 301, it was found to explain a substantial part of the variation with an R^2 -value of 0.136. The other two markers (PT30114, and PT51624) representing the same region also explained a greater degree of variation (7.2%, and 9.5% respectively) in the Florida 301/Hicks population as compared to the Beinhart 1000/Hicks population (2.97%, and 2.95% respectively). The PT30174 marker on linkage group 8 was found to be significantly associated with resistance in both the Florida 301/Hicks and Beinhart 1000/Hicks populations with R -square values of 0.163 and 0.204 respectively. Other markers belonging to linkage group 8 were surprisingly not significantly associated with resistance in the Florida 301/Hicks population, while they were found to be significantly associated with resistance in the Beinhart 1000/Hicks population. The marker, PT61153, that was not assigned to any linkage group explained more variation (7.4%) in Florida301/Hicks population as compared to the explained variation (4.2%) in Beinhart 1000/Hicks population. The markers on linkage groups 4, 9, 11, and 14 were not identified as being significantly associated with resistance in the Florida 301/Hicks population.

For all markers associated with resistance, the allele associated with resistance originated from Florida 301. A total of 12 markers, out of 21, exhibited some degree of heterozygosity in small proportions of the population that ranged from 0.83 to 8.26 %. The average heterozygosity over all loci in the population was found to be 1.9%, which is a little higher than the expected 1.56% for a population in the $F_{5:6}$ generation.

Table 2. Comparison of R^2 -values, P -values, and effects estimated for microsatellite markers in the Florida 301/Hicks and Beinhart 1000/Hicks mapping populations.

Linkage group	Marker	Florida 301/Hicks population			Beinhart 1000/Hicks population		
		R^2 -value	P -value	Effect	R^2 -value	P -value	Effect ^a
2	PT52633	0.1365	<0.0001	8.51	0.0345	0.0439	2.10
2	PT30114	0.0717	0.0193	6.19	0.0297	0.0655	1.83
2	PT51624	0.0948	0.0042	7.19	0.0295	0.0631	1.79
2	PT30183	0.0063	0.3944	-1.85	0.0282	0.0838	1.72
4	PT30346	0.0251	0.2233	-3.32	0.0929	0.0008	5.62
4	PT61373	0.0120	0.4876	-1.33	0.1940	<0.0001	11.61
4	PT30124	0.0059	0.4358	-1.60	0.1604	<0.0001	9.29
4	PT52061	0.0122	0.2341	-2.53	0.1311	<0.0001	8.20
8	PT30174	0.1630	<0.0001	9.07	0.2044	<0.0001	12.15
8	PT61512	0.0119	0.5034	2.39	0.1128	0.0002	6.86
8	PT51405	0.0009	0.9485	0.38	0.0727	0.0036	4.49
8	PT52753	0.0021	0.8947	1.08	0.1199	<0.0001	7.25
8	PT53518	0.0074	0.6518	1.39	0.0753	0.0030	4.73
8	PT30218	0.0247	0.2283	2.76	0.0713	0.0038	4.53
8	PT30395	0.0177	0.2008	3.44	0.0877	0.0012	5.48
9	PT1140	0.0266	0.2041	-0.81	0.0486	0.0165	2.94
11	PT51859	0.0184	0.3508	-3.12	0.0359	0.0399	2.19
14	PT55392	0.0037	0.5186	1.37	0.0517	0.0141	3.13
14	PT30173	0.0005	0.8057	-0.52	0.0564	0.0102	3.41
Unlinked	PT53795	0.0084	0.3162	2.07	0.0764	0.0024	4.83
Unlinked	PT61153	0.0747	0.0022	6.21	0.0425	0.0278	2.63

^aEffect in Beinhart 1000/Hicks population indicate back transformed value to approximate their original value of end percent survival.

DISCUSSION

The best way to reduce crop losses from a pathogen having multiple races is to incorporate resistance effective against all prevalent races into new cultivars. Monogenic resistance generally results in the evolution or prevalence of more virulent and aggressive races. A practical way to utilize all available sources of resistance would therefore be to pyramid monogenic and polygenic resistance into new cultivars. In the case of black shank, monogenic resistance conferred by the *Ph* gene can successfully reduce crop loss from race 0, but can result in population shifts towards race 1. The polygenic resistance in Florida 301 has been the only successfully used source of polygenic resistance in flue-cured and burley tobacco breeding. This genetic system confers only partial resistance against race 0 and race 1. A second potential resistance source is Beinhart 1000 that has been reported to be highly effective against race 0 and race 1. Beinhart 1000 has not been used successfully in flue cured and burley tobacco breeding programs because of negative associations between resistance and cured leaf quality. As reported in Chapter 3, a total of six genomic regions were found to significantly influence resistance in Beinhart 1000. Both polygenic sources, being cigar tobacco types, might have the same or closely related mechanisms working against the black shank pathogen. It was previously shown that the loci conferring black shank resistance in *N. plumbaginifolia* and *N. longiflora* are homologous and possibly identical (Collins et al., 1971). Considering this finding in the present investigation, the effects of the molecular markers representing those six regions

were estimated in an attempt to draw genetic comparisons between the two sources of resistance.

The RIL population exhibited a substantial amount of variation for disease resistance and two lines exhibited survival rates comparable to the resistant parent, Florida 301. The fact that no RIL was as resistant as Florida 301 suggests that the complete resistance of this genotype is governed by multiple genomic regions, and that a large population is required to recover genotypes capturing all genomic regions. Only two genomic regions out of the six regions of interest were found to be associated with resistance in Florida 301. The R^2 -square values for PT30174 in the Beinhart 1000/Hicks and Florida 301/Hicks populations ($R^2 = 0.204$ and $R^2 = 0.163$, respectively) suggests that the genomic region on linkage group 8 plays an important role in influencing resistance in both of these sources. This also established support for the hypothesis that a similar mechanism might be working in both of these lines. Other markers in the same linkage group, having a significant effect in the Beinhart 1000/Hicks population, were surprisingly found to be not significantly associated with resistance in Florida 301. This demonstrates the need to fine map the region close to PT30174 in order to more precisely locate the QTL.

All the markers belonging to linkage group 2 were found to explain a greater amount of variation in the Florida 301/Hicks population as compared to the Beinhart 1000/Hicks population. Interestingly, the markers linked to the QTL on linkage group 4 (2nd largest QTL for Beinhart 1000 resistance) were not significantly associated with

resistance in Florida 301. This may partially explain the partial uniqueness of the Beinhart 1000 resistance and also suggests that the superiority of Beinhart 1000 over that of Florida 301 may be due to a different allele in this line. This region also has significance in that it carries genes affecting the production of two chemicals produced by glandular trichomes, β -methylvaleric acid-containing sucrose esters and *cis*-abienol. Beinhart 1000 has the ability to produce these two chemical components whereas Florida 301 lacks this ability. Linkage between black shank resistance and undesirable cigar-type characteristics like the leaf surface chemistry traits may partially explain past difficulties in using Beinhart 1000 as a source of black shank resistance. The possibility that these chemicals may directly or indirectly contribute to resistance acting in Beinhart 1000 needs to be investigated.

It is likely that additional yet-to-be-identified genomic regions contribute to the high partial resistance of Florida 301. A more detailed genotyping of the population with all available markers and evaluation of the population in a controlled environment might result in an increased understanding of the genetics contributing to the resistance in this line. A more thorough understanding of the genomic regions controlling resistance in both sources would make it possible for tobacco breeders to achieve the maximum level of resistance in new cultivars. As the existence of resistance from other species of *Nicotiana* like *N. debneyi*, *N. repanda*, *N. megalosiphon* and *N. suaveolens* was reported (Li et al., 2006), there should be more focus on identifying resistance from other species and within tobacco.

CONCLUSIONS

The objective of finding similarities and dissimilarities between Beinhart 1000 and Florida 301 was partially achieved by finding that similarity exists with respect to one major QTL. Dissimilarity was observed with respect to another major QTL. More extensive genotyping of the Florida 301 population may help to make concrete conclusions regarding the comparison of these two types of resistance.

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CHAPTER 5

**GROWTH CHAMBER EVALUATIONS OF RACE SPECIFICITY FOR
QUANTITATIVE TRAIT LOCI (QTL) ASSOCIATED WITH BLACK SHANK
RESISTANCE OF THE TOBACCO LINE ‘BEINHART 1000’**

ABSTRACT

The black shank disease of tobacco caused by *Phytophthora nicotianae* is a difficult disease for tobacco breeders to work with because of the existence of multiple races and its soilborne nature. A disease screening method in a controlled environment could facilitate evaluation of resistance to specific races of the pathogen. Results would have to be comparable to field performance to be of practical value, however. The current study was carried out to investigate the race specificity (Race 0 and Race 1) of black shank resistance QTLs identified for Beinhart 1000, and to validate the precision of growth chamber evaluation as compared to field evaluation. A doubled haploid population developed from the cross between Beinhart 1000 and susceptible line, Hicks, was used in the current study. The reliability of the growth chamber evaluation was confirmed by the finding that all the doubled haploid lines that exhibited high field resistance also proved to be highly resistant in the growth chamber experiment. The two major QTLs present on linkage groups 4 and 8 that were identified using field data were also found to be significant in the growth chamber experiment. The QTL on linkage group 8 exhibited a highly significant effect for resistance against both races and was therefore confirmed to be a major genomic region influencing black shank resistance. The QTL on linkage group 4 exhibited a larger effect for resistance against race 1 as compared race 0. A QTL on linkage group 13 that was not identified using field evaluation was found to have a significant effect on resistance against both the races. Two QTLs on linkage groups 15 and 23 had significant effects on resistance against race 1 but not on resistance against race 0.

INTRODUCTION

The pathogen, *Phytophthora nicotianae*, that causes the black shank disease of tobacco exists in the form of multiple races and poses a serious challenge for tobacco breeders to breed cultivars with resistance against all races. A cigar tobacco cultivar, Florida 301 (Tisdale, 1931), has served as the primary source of polygenic resistance in the development of many flue-cured and burley tobacco cultivars and provides partial resistance to both race 0 and race 1 (Wernsman et al., 1974). Development of cultivars highly resistant to race 0 has been successful due to the availability of monogenic resistance (conferred by *Ph* gene) introgressed from two *Nicotiana* species, *N. plumbaginifolia* and *N. longiflora* (Chaplin, 1962; Valleau et al., 1960). An unexploited source of resistance, Beinhart 1000, has been reported to exhibit perhaps the highest levels of resistance against both the races of the pathogen (Heggestad and Lautz, 1957; Silber and Heggestad, 1963; Chaplin 1966; Wills, 1971; Tedford and Nielsen, 1990; Nielsen, 1992). Pyramiding of high levels of polygenic resistance along with the *Ph* gene might contribute to an efficient system of disease management (Carlson et al., 1997). To increase the efficiency of breeding for black shank resistance, it would be of value to precisely identify genomic regions that influence resistance against multiple races. Incorporation of these multiple genomic regions into new varieties might be aided by the use of molecular markers.

The availability of microsatellite markers and a detailed genetic map for tobacco (Bindler et al., 2007; Chapter 3) increase possibilities for identifying genomic regions

associated with the polygenic type of black shank resistance. Six quantitative trait loci (QTLs) have been identified as being associated with resistance in Beinhart 1000 (Chapter 3). It would be valuable to know whether or not these genomic regions influence resistance in a race specific manner. It was also previously determined that the QTL on linkage group 8 contributes very significantly to resistance in both Florida 301 and Beinhart 1000 (Chapter 4).

It is difficult, under field conditions, to study race specific resistance because of the existence of inoculum as a population mixture of races. Moreover, field evaluations for black shank resistance can be complicated by non-uniform inoculum distributions. Interest therefore exists to identify a method that is reliable and that provides unambiguous data. Resistance to black shank has previously been evaluated using several different systems including inoculation of leaves (Wills, 1971; Tedford et al., 1990), stems (Csinos and Bertrand, 1994; Csinos, 1999) and roots (Johnson et al., 2002). Results from these methods are often not well correlated with field observations (Hendrix and Apple, 1967; Wills, 1971; Hegelson et al., 1972; Tedford et al., 1990), however. Methods involving inoculation of young plants may be the most rapid and reliable means to obtain meaningful results (Litton et al., 1970; Jaarsveld et al., 2003). Moreover, screening in growth chambers with controlled temperatures and humidity might make results more consistent and reliable. In the controlled environments of growth chambers, it would also be possible to evaluate genotypes for resistance against specific races.

The primary objective of the present experiment was to investigate the race specificity of the QTLs that were significantly associated with the field resistance in Beinhart 1000. The second objective was to validate the precision of the growth chamber evaluation method by comparing results with those from field evaluations. This work was also carried out to determine the possible existence of additional genomic regions that might be associated with black shank resistance in this line.

MATERIALS AND METHODS

Population Development

The cigar tobacco line, Beinhart 1000, was hybridized with susceptible cultivar, Hicks. F₁ progeny resulting from this cross were hybridized as females with *Nicotiana africana* to generate maternally derived haploids according to Burk et al. (1979). A population of 118 doubled haploid lines was generated from these haploid plants using the midvein tissue culture procedure of Kasperbauer and Collins (1972).

Preparation of Inoculum

For culturing race 0 and race 1 of the black shank pathogen, carrot agar medium was prepared by mixing 50 ml natural carrot juice (Bolthouse Juice Products LLC, Bakersfield, CA) and 20 g agar (Sigma chemical Co. St Louis, MO) in 950 ml of distilled water. Autoclaved medium was poured into 100 × 20 mm extra deep petri plates. An agar plug containing the pathogen of the specified race was placed in the middle of the petri plate and allowed to grow for four days before sterilized oat grains were spread over the

plate in a single layer. Cultures were considered ready to use when hyphae could be seen covering most of the oat grains, generally after 15 days.

Growth Chamber Evaluation

Growth chamber evaluations for race specific resistance were conducted at the North Carolina State University Phytotron facility. The experiment was conducted using a spit plot design with three replications. The pathogen race was applied as the main plot factor and the doubled haploid lines along with parental lines (subplots) were randomized within main plots. A total of six plants per genotype were used for each subplot. Two-week old seedlings were transplanted to individual cells (6 cm × 4 cm × 5 cm) of tray inserts containing a mixture of peat and sand in the proportion of 1:1. Plants were allowed to grow for 15 days prior to inoculation with the pathogen. The experiment was maintained at 25°C with 16 hours of daylight until the time of inoculation. At the time of inoculation, two oat grains per cell were inserted in the soil at the corners of each cell. After inoculation, the temperature of the growth chamber was increased to 30°C with 16 hours daylight and 25°C night temperature. The number of plants that were unaffected by disease was recorded after 30 days and end percent survival was calculated.

QTL detection

The genetic map that was generated as described in Chapter 3 was used for identifying QTL associated with black shank resistance as measured by end percent survival. Composite interval mapping (CIM) was performed using Windows QTL

Cartographer V2.5 (Wang et al., 2007). Log-likelihood values for statistically significant associations between genotype and resistance were generated by calculating LOD scores at 0.5-cM intervals along the linkage groups. The LOD threshold significance level was determined using 1000 permutations of the procedure of Churchill and Doerge (1994). Composite interval mapping was executed only to provide an initial model for further analysis by the multiple interval mapping (MIM) method (Kao and Zeng, 1997; Kao et al., 1999). LOD score peaks from the CIM analysis, with a value of more than 2.5, were used in the initial models for MIM analysis. Models were created and tested in an iterative, stepwise fashion, searching for new QTL, and testing the significance of the QTL after each search cycle. New models were accepted only when they had a decreasing effect on the value of the Bayesian Information Criterion (BIC) (Piepho and Gauch, 2001). After the QTLs were added using the BIC criterion, all possible interactions between the QTLs were tested for their significance and were retained in the model if they decreased the BIC. While developing the model using MIM, there is always a possibility of over fitting the model. To avoid model over fitting, it was made sure that the proportion of total phenotypic variation explained by QTLs did not exceed the trait heritability. The model with minimum BIC was selected and the QTL effects were estimated using the “summary” command of QTL Cartographer. Genetic variability explained by QTLs was estimated by dividing the total explained phenotypic variation by the entry mean heritability of the trait.

Statistical Analysis

Analysis of variance was carried out using the 'PROC GLM' program of SAS 9.1 (SAS Institute, Cary, NC). The error term 'replication \times race' was used to test the significance of the difference between the main plots, whereas, the experimental error was used to test the differences among the subplots as specified by Steel and Torrie (1980). Heritability on a plot basis and entry mean basis were calculated using univariate mixed model analysis (Holland et al., 2003).

RESULTS

Growth Chamber Evaluation

Growth chamber evaluations effectively differentiated the two parental lines as Beinhart 1000 exhibited 100% survival and Hicks exhibited 100% death in all the replications for both races. The evaluation also revealed significant difference ($P < 0.0001$) among the doubled haploid lines with respect to end percent survival (Table 1, Figure 1). The reliability of the evaluation was evident from the fact that lines that exhibited high resistance in field environments were also observed to be showing a high level of resistance in the growth chamber experiment (Table 2). The genotypic means for percent survival in field evaluations were highly correlated with the means for end percent survival exhibited in growth chamber evaluations with $r = 0.63$ ($P < 0.0001$) and $r = 0.77$ ($P < 0.0001$), for race 0 and race 1 respectively. The doubled haploid lines,

DH05B-1252-300 and DH06B-162-16, consistently exhibited high levels of resistance in both field and growth chamber experiments (Table 2).

The ANOVA for end percent survival revealed statistically significant difference ($P = 0.0207$) between the two races. A statistically significant ($P < 0.0001$) interaction between genotype and race was also found in the ANOVA analysis (Table 1). Average percent survivals for the population inoculated with race 0 and race 1 were found to be 71.3% and 53.3% respectively. The difference between the two races was also evident from the frequency distribution of the doubled haploid lines for end percent survival (Figure 2). Distribution of the doubled haploid lines in the evaluation with race 0 was skewed towards a higher survival rate, and the distribution in the case of race 1 resembled that of the field evaluation (*i.e.*, skewed towards lower survival rate). In the field analysis, none of the lines showed a survival rate of more than 95%, whereas, in growth chamber evaluation 26 lines for race 0 and 13 lines for race 1 exhibited complete resistance to the disease with a survival rate of 100%. Because of significant interaction between race and entries, percent survivals for the two races are presented separately (Appendix I). The mean end percent survival rates for race 0 and race 1 were used for QTL identification. The heritability of resistance to race 0 was estimated to be $h^2 = 0.68$ and $h^2 = 0.86$ for a plot basis and entry mean basis, respectively. The heritability on a plot basis and entry mean basis for resistance against race 1 were estimated to be $h^2 = 0.78$ and $h^2 = 0.91$ respectively. The heritability values for resistance in the field ($h^2 = 0.70$ and $h^2 = 0.88$ on

plot and entry mean basis respectively) were estimated to be higher than the values for resistance against race 0 and slightly lower than those for resistance against race 1.

Table 1. ANOVA for end percent survival in growth chamber evaluations of the doubled haploid population.

Source	DF ^a	SS ^b	MSS ^c	F-value	P-value
Replication	2	8296.01	4148.01	11.27	<0.0001
Race	1	57551.58	57551.58	46.74	0.0207
Replication × Race	2	2462.64	1231.32	3.35	0.0361
Entry	117	723824.03	6186.53	16.81	<0.0001
Entry × Race	117	78046.57	667.06	1.81	<0.0001
Error	468	172200.94	367.95	-	-

$R^2 = 0.8348$ C.V. = 30.78

^a DF- Degrees of freedom; ^b SS- Sum of squares; ^c MSS-Mean sum of squares.

Table 2. Comparison of most resistant lines for their performance in field and growth chamber evaluations.

Doubled Haploid Line	End percent survival		
	Field	Growth chamber	
		Race 0	Race 1
DH05B 1252-300	91.67	94.44	94.44
DH06B 162-16	90.74	100.00	100.00
DH06B 173-46	89.08	100.00	88.89
DH05B 1252-240	84.76	100.00	94.44
DH06B 173-49	82.28	100.00	94.44
DH05B 1252-100	81.97	100.00	100.00
DH05B 1252-116	81.53	100.00	94.44
DH06B 173-47	80.42	77.78	83.33
DH06B 162-8	77.71	88.89	94.44
DH06B 173-87	76.87	100.00	100.00
DH05B 1252-233	75.96	100.00	94.44
DH06B 173-20	75.24	100.00	100.00
DH05B 1252-293	73.91	94.44	100.00
DH06B 162-27	70.67	100.00	100.00



Figure 1. Differences in survival rate as shown by resistant and susceptible doubled haploid lines in growth chamber evaluation.

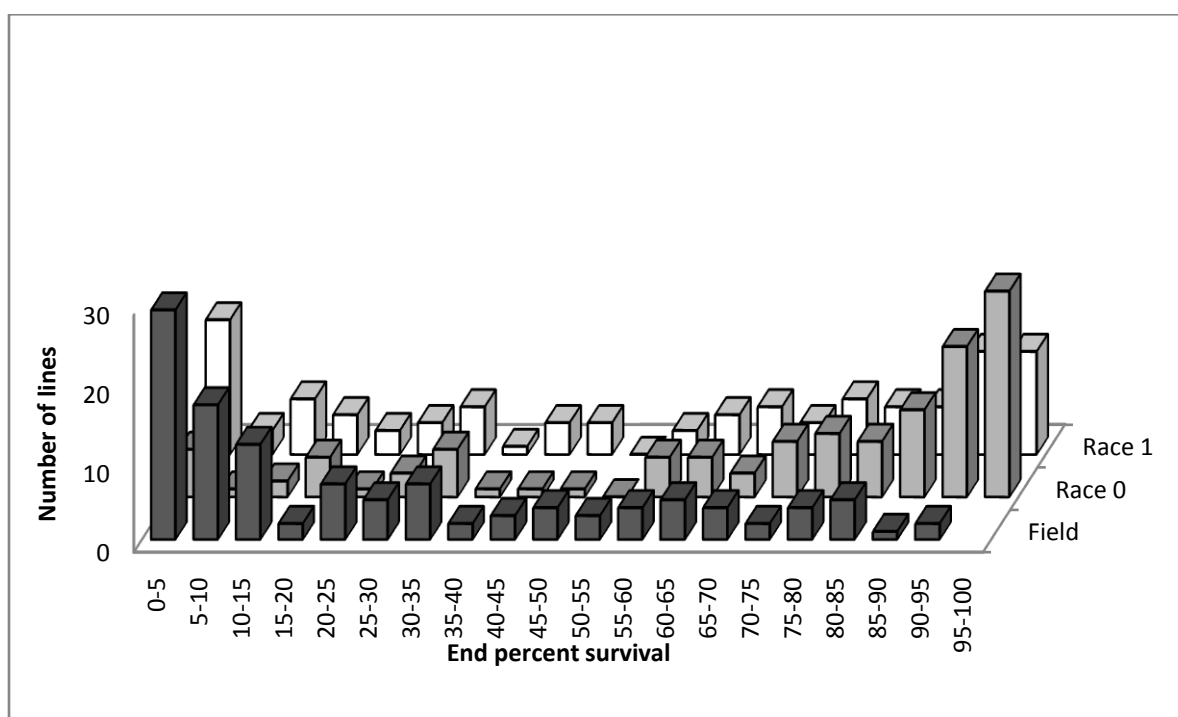


Figure 2. Frequency distribution for doubled haploid lines for end percent survival in field and growth chamber evaluations.

Multiple Interval Mapping (MIM)

As presented in Table 3, a total of three main QTLs (on linkage groups 4, 8, and 13) and one interaction effect were included in the model for explaining the phenotypic variation of the population inoculated with race 0. The QTL on linkage group 8, alone, was found to explain 52.9% of the total phenotypic variation. This model was found to explain 68.9% of the total phenotypic variation and 80.1% of the total genotypic variation.

A total of 5 main QTLs (on linkage groups 4, 8, 13, 15, and 23) and one interaction effect (between the QTLs on linkage groups 8 and 13) were included in the model to explain the variation in end percent survival in the race 1 evaluation (Table 3). The QTLs on linkage groups 4 and 8 together were estimated to explain 60.9% of the total phenotypic variation. This final model explained 74.8% of the total phenotypic variation and 82.2% of total genotypic variation.

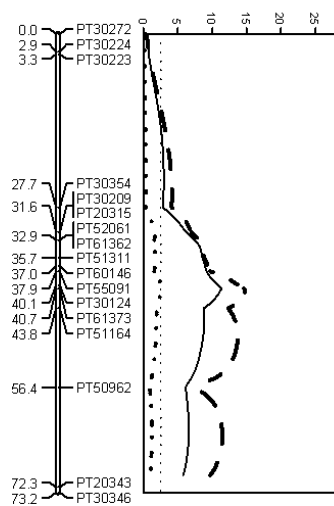
The QTL on linkage group 13, with the effect values of -10.92% and -11.74% end percent survival rate for race 0 and race 1 respectively, indicated that the favorable allele for this QTL was contributed by the susceptible parental line, Hicks. All the other QTL effects ranging from 5.85% to 24.4% end survival for race 0 and from 7.5% to 26.0% for race 1 evaluation indicated that the favorable alleles for those QTLs were contributed by Beinhart 1000.

The LOD scores calculated from MIM analysis of field (Chapter 3) and growth chamber data were plotted against the positions across the length of each linkage group as presented in Figure 3. The LOD score values for all the regions of interest are presented in Table 3. The QTL on linkage group 8, located between the markers PT30174 and PT61512, had consistently exhibited a high level of association with resistance by showing high LOD score peaks in all three cases. The QTL on linkage group 4, located between the markers PT61373 and PT51164, was found to have more influence on resistance against race 1 than it had on resistance against race 0. The QTL bordered by markers PT30480 and PT30417 on linkage group 13 was found to be associated with resistance for both race 0 and race 1. Additional QTLs on linkage groups 15 and 23 were found to be associated only with resistance against race 1.

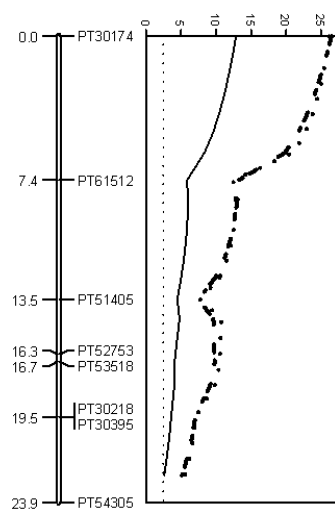
Table 3. Multiple interval mapping analysis for evaluation of doubled haploid lines in growth chamber experiments.

QTL	Linkage Group	Left Marker	Right Marker	Position (cM)	Effects (Percent survival)		LOD values		% Phenotypic variation explained	
					Race 0	Race 1	Race 0	Race 1	Race 0	Race 1
1	4	PT61373	PT51164	40.80	5.85	16.89	2.59	14.74	1.90	17.00
2	8	PT30174	PT61512	0.01	24.40	26.01	24.85	26.27	52.90	43.90
3	13	PT30480	PT30417	3.60	-10.92	-11.74	7.04	7.19	6.80	6.40
4	15	PT60114	PT1078	44.60	-	9.12	-	4.98	-	4.00
5	23	PT30257	PT30424	12.80	-	7.54	-	3.64	-	2.20
2 × 3					10.21	5.78	6.33	2.08	7.30	1.30

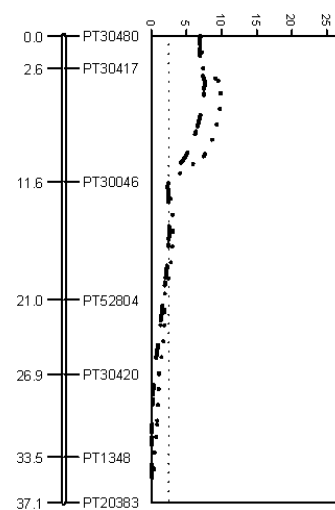
Linkage group 4



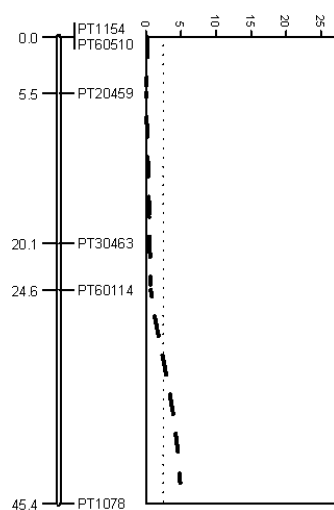
Linkage group 8



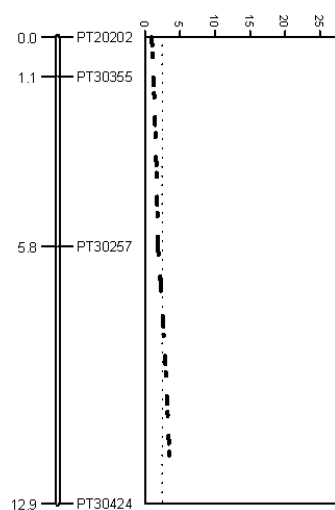
Linkage group 13



Linkage group 15



Linkage group 23



— Field
 Race 0
 - - - Race 1

Figure 3. LOD score peaks generated from multiple interval mapping analysis.

DISCUSSION

For reducing crop loss from a pathogen with multiple races, it is important to understand its population structure and deploy resistance genes accordingly to avoid the risk of losing effectiveness of available resistance mechanisms. It is also crucial to understand resistance sources for their effectiveness against specific races. Having identified six genomic regions associated with field resistance in Beinhart 1000 (Chapter 3), it was of interest to validate these QTLs for their specific effectiveness against the two prevalent races of the black shank pathogen, race 0 and race 1. In the present investigation, efforts were made to validate the identified QTLs and to study possible QTL \times race interactions by conducting growth chamber evaluation.

From the results of growth chamber evaluations, only two major QTL (out of the six QTLs reported in Chapter 3) that were present on linkage groups 4 and 8 were found to be significantly associated with resistance. The regions on linkage groups 2, 9, 11, and 14 with minor effects on field resistance could not be resolved by this growth chamber evaluation. Three new genomic regions on linkage groups 13, 15, and 23 were identified to possibly play some role in influencing resistance in the growth chamber evaluation. For the QTL on linkage group 13, it was observed that the favorable allele was contributed by the susceptible parent, Hicks.

The QTL on linkage group 8 explained 23.5% of the phenotypic variation in the field experiments, whereas, it explained 52.9% and 43.9% of the variation for end percent survival in the evaluations with race 0 and race 1, respectively. This QTL has also been

shown to be an important factor influencing black shank resistance in Florida 301 (Chapter 4). It can therefore be concluded that this region influences black shank resistance, in general, and acts in a race non-specific manner. As presented in Figure 3, this genomic region was not well mapped to precisely resolve this QTL, and there is therefore a need to put additional efforts into mapping this genomic region more densely.

In the growth chamber experiments, the QTL on linkage group 4 exhibited a major effect on resistance against race 1 and a smaller, but still significant effect, against race 0. This QTL was not significantly associated with resistance contained in Florida 301 (Chapter 4). As was observed in Chapter 2, this region in Beinhart 1000 also has significance in that it carries the genes responsible for the production of two leaf surface chemicals, β -methylvaleric acid-containing sucrose esters and *cis*-abienol. Florida 301 lacks the ability to produce these two components. As these leaf surface chemistries are synthesized by glandular trichomes that are also present on the stalk, it is plausible that they might play a contributing role in resisting infection at the base of the plant. The possible relationship between these leaf surface chemistry traits and black shank resistance need to be investigated.

Having characterized the genomic regions contributing to black shank resistance in Beinhart 1000 and Florida 301, it might also be valuable to know the genomic position of the *Ph* gene and its genetic location relative to these regions of interests. In the future, efforts should focus on identifying microsatellite markers associated with the *Ph* gene and on locating *Ph* on the available genetic linkage map.

Having successfully differentiated resistant and susceptible lines of the doubled haploid population, the growth chamber evaluation proved to be an efficient tool for black shank resistance evaluations. Even though the resistant lines in the field also proved to be resistant in the growth chamber, many lines that exhibited high susceptibility in the field exhibited resistance in the growth chamber experiment. However, considering the fact that this evaluation is easy and less expensive, these evaluations can serve as a powerful tool for confirming results obtained from the field evaluations.

CONCLUSIONS

The importance of the two major QTLs present on linkage groups 4 and 8 was confirmed in the present study. The objective of validating the method of evaluating the population in growth chambers has been achieved by confirming that the performance of the genotypes was highly correlated in the field and growth chambers. Other potential genomic regions on linkage groups 13, 15 and 23 were identified to be associated with black shank resistance using the growth chamber evaluation.

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APPENDIX I

**Leaf surface components and end percent survival mean values for
Beinhart 1000/Hicks doubled haploid lines in field and growth chamber
evaluations.**

Doubled Haploid Line	Leaf surface component ($\mu\text{g}/\text{cm}^2$)		Field end %survival	Growth chamber end %survival	
	<i>cis</i> -abienol	Group III-VI sucrose esters		Race 0	Race 1
DH05B 710-2	- ^a	-	2.78	83.33	50.00
DH05B 710-3	10.92	9.54	10.21	55.56	50.00
DH05B 710-6	-	-	3.70	38.89	33.33
DH05B 710-12	-	-	0.00	16.67	0.00
DH05B 710-14	13.98	38.21	60.41	100.00	83.33
DH05B 710-15	19.78	22.03	4.26	33.33	0.00
DH05B 710-28	-	-	0.00	11.11	0.00
DH05B 710-33	-	11.65	2.71	77.78	33.33
DH05B 710-40	-	-	0.00	16.67	5.56
DH05B 710-42	-	45.53	0.00	0.00	0.00
DH05B 710-47	27.52	33.12	29.19	88.89	66.67
DH05B 710-50	-	-	6.57	83.33	22.22
DH05B 710-51	N.A. ^b	N.A.	64.25	83.33	72.22
DH05B 710-53	30.73	-	41.98	100.00	100.00
DH05B 710-58	36.25	23.32	68.16	100.00	94.44
DH05B 710-59	13.55	21.57	58.05	100.00	94.44
DH05B 710-60	22.34	16.80	49.00	33.33	11.11
DH05B 710-65	49.49	47.91	11.43	27.78	27.78
DH05B 710-72	-	30.38	10.41	88.89	33.33
DH06B 162-30	-	-	0.00	16.67	0.00
DH05B 1252-52	-	23.52	52.07	88.89	55.56
DH05B 1252-60	-	-	5.65	94.44	61.11
DH05B 1252-84	16.69	23.30	6.41	88.89	55.56
DH05B 1252-95	-	-	22.16	77.78	38.89
DH05B 1252-100	50.97	55.87	81.97	100.00	100.00
DH05B 1252-101	-	-	11.11	94.44	94.44
DH05B 1252-116	20.85	25.99	81.53	100.00	94.44
DH05B 1252-118	16.15	23.02	6.48	5.56	11.11
DH05B 1252-133	20.01	22.44	7.26	0.00	0.00
DH05B 1252-137	29.54	42.65	39.60	94.44	72.22
DH05B 1252-148	39.05	36.70	55.29	100.00	100.00
DH05B 1252-175	-	-	59.56	100.00	77.78
DH05B 1252-184	-	27.72	24.26	94.44	66.67

(Continued....)

DH05B 1252-199	18.50	14.37	54.11	94.44	88.89
DH05B 1252-208	8.96	16.11	49.12	100.00	94.44
DH05B 1252-225	-	-	6.51	27.78	5.56
DH05B 1252-227	-	-	9.52	55.56	0.00
DH05B 1252-229	-	-	27.79	88.89	94.44
DH05B 1252-233	45.96	30.05	75.96	100.00	94.44
DH05B 1252-240	30.06	49.33	84.76	100.00	94.44
DH05B 1252-251	30.73	-	13.01	100.00	77.78
DH05B 1252-252	-	-	4.49	94.44	66.67
DH05B 1252-257	-	-	0.85	27.78	0.00
DH05B 1252-277	45.81	36.92	67.85	100.00	88.89
DH05B 1252-280	-	-	3.79	55.56	16.67
DH05B 1252-284	19.21	23.58	0.00	16.67	0.00
DH05B 1252-285	-	-	3.70	72.22	16.67
DH05B 1252-291	30.99	15.88	5.50	61.11	50.00
DH05B 1252-292	-	-	0.93	88.89	33.33
DH05B 1252-293	-	23.52	73.91	94.44	100.00
DH05B 1252-297	-	-	0.79	33.33	0.00
DH05B 1252-298	20.92	10.79	53.11	100.00	88.89
DH05B 1252-300	39.24	25.66	91.67	94.44	94.44
DH05B 1252-302	-	-	7.77	61.11	11.11
DH05B 1252-303	18.43	24.09	12.10	33.33	11.11
DH05B 1252-304	-	33.76	12.96	72.22	5.56
DH06B 173-2	-	29.07	48.77	88.89	83.33
DH06B 173-3	34.43	35.51	15.48	88.89	61.11
DH06B 173-4	18.80	24.83	63.25	94.44	83.33
DH06B 173-5	-	-	10.54	94.44	27.78
DH06B 173-7	-	-	16.31	83.33	44.44
DH06B 173-11	-	-	7.42	66.67	44.44
DH06B 173-13	-	-	0.00	55.56	0.00
DH06B 173-14	15.34	22.46	5.48	72.22	27.78
DH06B 173-16	40.04	35.54	21.72	66.67	72.22
DH06B 173-18	25.26	-	66.10	94.44	88.89
DH06B 173-20	47.92	43.57	75.24	100.00	100.00
DH06B 173-24	-	-	22.16	100.00	66.67

(Continued...)

DH06B 173-25	31.07	30.49	40.96	66.67	77.78
DH06B 173-26	40.11	33.81	0.00	0.00	0.00
DH06B 173-27	-	-	3.56	77.78	61.11
DH06B 173-30	-	-	27.05	94.44	61.11
DH06B 173-31	-	-	45.53	100.00	77.78
DH06B 173-35	-	-	14.84	83.33	27.78
DH06B 173-39	28.65	35.49	33.05	72.22	66.67
DH06B 173-40	-	28.81	4.56	72.22	16.67
DH06B 173-41	-	-	0.00	0.00	0.00
DH06B 173-49	24.75	15.06	82.28	100.00	94.44
DH06B 173-67	-	-	2.71	11.11	11.11
DH06B 173-68	19.30	19.77	20.61	94.44	16.67
DH06B 173-69	27.97	16.76	3.44	33.33	22.22
DH06B 173-81	21.60	21.02	8.58	100.00	94.44
DH06B 173-83	-	-	26.86	94.44	61.11
DH06B 173-85	50.53	48.49	30.34	88.89	83.33
DH06B 173-86	22.36	22.97	10.19	61.11	33.33
DH06B 173-87	15.31	18.91	76.87	100.00	100.00
DH06B 173-90	23.06	8.88	36.73	88.89	77.78
DH06B 173-101	43.58	30.52	6.27	61.11	77.78
DH06B 173-104	-	-	34.92	100.00	100.00
DH06B 173-114	29.34	26.37	0.00	16.67	0.00
DH06B 173-121	-	-	8.75	55.56	0.00
DH06B 173-130	33.15	35.49	64.39	94.44	83.33
DH06B 162-1	33.77	33.23	7.59	22.22	16.67
DH06B 162-2	30.26	29.22	26.43	83.33	66.67
DH06B 162-3	22.93	31.61	8.28	77.78	11.11
DH06B 162-4	51.77	58.56	58.67	100.00	100.00
DH06B 162-5	14.75	23.63	20.87	77.78	50.00
DH06B 162-8	18.63	36.50	77.71	88.89	94.44
DH06B 162-9	25.03	20.09	33.05	94.44	88.89
DH06B 162-11	-	-	4.28	50.00	44.44
DH06B 162-12	36.26	30.79	12.37	72.22	55.56
DH06B 162-13	25.49	54.49	0.00	0.00	0.00
DH06B 162-14	42.22	36.66	34.49	72.22	44.44
DH06B 162-16	23.08	27.85	90.74	100.00	100.00
DH06B 162-17	16.81	22.22	31.68	94.44	94.44

(Continued.....)

DH06B 162-18	15.44	21.84	1.78	44.44	33.33
DH06B 162-19	20.46	22.22	67.60	94.44	100.00
DH06B 162-20	43.92	46.57	3.17	0.00	0.00
DH06B 162-21	-	-	5.64	61.11	11.11
DH06B 162-26	-	-	61.11	100.00	100.00
DH06B 162-27	34.92	23.95	70.67	100.00	100.00
DH06B 162-32	27.93	20.66	24.66	77.78	22.22
DH06B 162-33	38.98	43.91	2.51	33.33	50.00
DH06B 173-46	27.35	31.69	89.08	100.00	88.89
DH06B 173-47	29.53	-	80.42	77.78	83.33
DH06B 173-52	18.98	14.81	31.60	77.78	100.00
DH06B 173-55	-	-	11.48	83.33	77.78
DH06B 173-102	-	-	42.09	94.44	72.22

^a ‘-’ indicates that the particular leaf surface component was undetectable.

^b ‘N.A.’ indicates that the doubled haploid line was not used for leaf surface chemistry evaluation.

APPENDIX II

Segregation distortion statistics for microsatellite markers in Beinhart 1000/Hicks doubled haploid population.

Marker	χ^2 -value	P-value
PT60557	2.21	0.1371
PT51539	0.62	0.4340
PT52260	1.22	0.2694
PT51013	0.03	0.8625
PT30351	0.04	0.8415
PT20391	0.21	0.6468
PT20189	0.03	0.8625
PT30485	0.14	0.7083
PT30327	0.70	0.4028
PT20163	1.22	0.2694
PT51878	0.31	0.5770
PT50736	0.14	0.7083
PT52633	0.14	0.7083
PT53697	0.01	0.9203
PT30242	0.03	0.8625
PT30114	0.08	0.7773
PT51624	0.00	-
PT30183	1.13	0.2878
PT30067	0.32	0.5716
PT50543	0.03	0.8625
PT61583	0.01	0.9203
PT60715	0.03	0.8625
PT53701	0.01	0.9203
PT40021	12.12	0.0005
PT20372	16.69	0.0000
PT30197	16.41	0.0001
PT1242	14.62	0.0001
PT20287	4.68	0.0305
PT30368	3.51	0.0610
PT30011	1.20	0.2733
PT30272	0.08	0.7773
PT30224	0.21	0.6468

(Continued....)

PT30223	0.04	0.8415
PT30354	16.08	0.0001
PT30209	6.23	0.0126
PT20315	6.88	0.0087
PT52061	6.76	0.0093
PT61362	8.04	0.0046
PT51311	7.63	0.0057
PT60146	4.52	0.0335
PT55091	4.68	0.0305
PT30124	2.51	0.1131
PT61373	2.51	0.1131
PT51164	1.07	0.3009
PT50962	12.67	0.0004
PT20343	0.55	0.4583
PT30346	0.69	0.4062
PT20275	0.42	0.5169
PT30316	3.19	0.0741
PT30337	1.11	0.2921
PT20196	3.45	0.0633
PT30302	2.47	0.1160
PT30296	2.32	0.1277
PT20234	2.51	0.1131
PT30087	2.79	0.0949
PT30245	4.68	0.0305
PT30184	2.06	0.1512
PT30468	6.34	0.0118
PT1199	0.60	0.4386
PT30169	1.11	0.2921
PT30477	0.93	0.3349
PT30393	1.03	0.3102
PT30138	0.54	0.4624
PT30394	0.23	0.6315

(Continued....)

PT30202	0.09	0.7642
PT30215	2.25	0.1336
PT30292	0.00	-
PT30419	0.01	0.9203
PT30452	1.33	0.2488
PT30174	1.66	0.1976
PT61512	3.45	0.0633
PT51405	2.51	0.1131
PT52753	4.17	0.0411
PT53518	3.83	0.0503
PT30218	5.83	0.0158
PT30395	4.17	0.0411
PT54305	1.44	0.2301
PT20291	2.51	0.1131
PT30470	0.43	0.5120
PT40015	0.42	0.5169
PT1140	0.03	0.8625
PT1399	0.03	0.8625
PT30107	0.72	0.3961
PT20235	0.86	0.3537
PT30416	1.22	0.2694
PT30140	1.92	0.1659
PT30213	2.51	0.1131
PT30421	2.21	0.1371
PT20149	0.31	0.5777
PT30077	1.22	0.2694
PT30250	0.70	0.4028
PT30408	1.81	0.1785
PT30132	0.55	0.4583
PT30372	0.46	0.4976
PT20445	1.05	0.3055
PT20192	0.55	0.4583

(Continued....)

PT30031	0.08	0.7773
PT30170	0.01	0.9203
PT53493	0.14	0.7083
PT51859	0.54	0.4624
PT30008	0.22	0.6390
PT30018	0.03	0.8625
PT1279	0.08	0.7773
PT20021	0.01	0.9203
PT30096	0.01	0.9203
PT30099	1.05	0.3055
PT20242	1.29	0.2560
PT60494	1.24	0.2655
PT20213	1.31	0.2524
PT50539	2.17	0.1407
PT55188	1.96	0.1615
PT30480	1.38	0.2401
PT30417	0.01	0.9203
PT30046	1.75	0.1859
PT52804	1.07	0.3009
PT30420	0.01	0.9203
PT1348	0.21	0.6468
PT20383	0.74	0.3897
PT30314	0.08	0.7773
PT30173	0.14	0.7083
PT55392	0.00	-
PT61011	0.31	0.5777
PT61337	0.03	0.8625
PT54419	0.22	0.6390
PT54081	0.85	0.3566
PT53287	0.85	0.3566
PT30462	1.69	0.1936
PT30403	0.45	0.5023

(Continued....)

PT1154	2.79	0.0949
PT60510	3.90	0.0483
PT20459	2.47	0.1160
PT30463	0.86	0.3537
PT60114	2.47	0.1160
PT1078	1.24	0.2655
PT52810	0.03	0.8625
PT50246	0.14	0.7083
PT30052	0.00	-
PT30063	0.08	0.7773
PT60053	0.31	0.5777
PT53576	0.15	0.6985
PT55360	0.33	0.5657
PT30053	0.54	0.4624
PT30156	0.01	0.9203
PT20376	0.77	0.3802
PT30274	0.14	0.7083
PT61499	0.08	0.7773
PT30339	0.21	0.6468
PT30111	22.92	0.0000
PT1193	22.23	0.0000
PT30285	20.52	0.0000
PT60050	18.88	0.0000
PT52752	11.17	0.0008
PT40005	6.88	0.0087
PT55103	5.24	0.0221
PT30094	0.14	0.7083
PT30005	0.04	0.8415
PT30248	0.03	0.8625
PT40009	0.70	0.4028
PT30165	0.00	-
PT30289	0.00	-

(Continued....)

PT30043	2.37	0.1237
PT30044	2.47	0.1160
PT20127	7.19	0.0073
PT50700	3.19	0.0741
PT30265	9.45	0.0021
PT20400	0.43	0.5120
PT30235	0.01	0.9203
PT30110	0.32	0.5716
PT60486	0.00	-
PT1194	0.24	0.6242
PT50392	0.01	0.9203
PT54729	0.03	0.8625
PT53424	0.08	0.7773
PT30028	0.00	-
PT61143	0.03	0.8625
PT1313	0.04	0.8415
PT30095	0.01	0.9203
PT1194-2	0.01	0.9203
PT30084	0.93	0.3349
PT30034	1.11	0.2921
PT30364	1.13	0.2878
PT30172	0.22	0.6390
PT20202	0.69	0.4062
PT30355	0.56	0.4543
PT30257	0.14	0.7083
PT30424	0.08	0.7773
PT30171	0.09	0.7642
PT1305	0.31	0.5777
PT30097	0.01	0.9203
PT52318	1.50	0.2207

APPENDIX III

Duncan's multiple range test for end percent survival of recombinant inbred lines derived from the Florida 301/Hicks cross.

Genotype	End percent survival	
GH07B-959	60.8	A
GH07B-966	57.54	AB
GH07B-987	46.35	BC
GH07B-948	36.79	CD
GH07B-1057	36.46	CD
GH07B-1018	32.99	DE
GH07B-1011	32.22	DEF
GH07B-964	31.62	DEFG
GH07B-1016	28.98	DEFGH
GH07B-1026	27.92	DEFGHI
GH07B-1064	27.35	DEFGHI
GH07B-1006	26.8	DEFGHI
GH07B-1056	24.4	DEFGHIJ
GH07B-1052	24.37	DEFGHIJ
GH07B-1020	23.61	DEFGHIJK
GH07B-1051	23.44	DEFGHIJK
GH07B-1033	22.67	DEFGHIJKL
GH07B-950	22.52	DEFGHIJKLM
GH07B-962	21.95	EFGHIJKLMN
GH07B-939	21.89	EFGHIJKLMNO
GH07B-1015	20.93	EFGHIJKLMNOP
GH07B-1027	20.87	EFGHIJKLMNOP
GH07B-1061	20.03	EFGHIJKLMNOPQ
GH07B-972	19.97	EFGHIJKLMNOPQ
GH07B-938	18.76	EFGHIJKLMNOPQR
GH07B-944	17.82	FGHIJKLMNQPORS
GH07B-1055	17.6	FGHIJKLMNQPORS
GH07B-1054	17.02	GHIJKLMNQPQRST
GH07B-945	16.75	HIJKLMNQPQRST
GH07B-1030	15.95	HIJKLMNQPQRSTU
GH07B-995	15.81	HIJKLMNQPQRSTU
GH07B-1023	15.75	HIJKLMNQPQRSTU

(Continued....)

GH07B-957	15.34	HIJKLMNOPQRSTUVWXYZ
GH07B-968	15.31	HIJKLMNOPQRSTUVWXYZ
GH07B-990	15.24	HIJKLMNOPQRSTUVWXYZ
GH07B-947	14.34	HIJKLMNOPQRSTUVWXYZ
GH07B-1034	13.32	IJKLMNOPQRSTUVWXYZ
GH07B-1003	11.66	JKLMNOPQRSTUVWXYZ
GH07B-969	11.05	JKLMNOPQRSTUVWXYZ
GH07B-1001	10.79	JKLMNOPQRSTUVWXYZ
GH07B-996	10.75	JKLMNOPQRSTUVWXYZ
GH07B-1037	10.61	JKLMNOPQRSTUVWXYZ
GH07B-1028	10.41	JKLMNOPQRSTUVWXYZ
GH07B-985	10.26	JKLMNOPQRSTUVWXYZ
GH07B-998	10.11	JKLMNOPQRSTUVWXYZ
GH07B-956	10.02	JKLMNOPQRSTUVWXYZ
GH07B-1013	9.8	JKLMNOPQRSTUVWXYZ
GH07B-981	9.79	JKLMNOPQRSTUVWXYZ
GH07B-1014	9.76	JKLMNOPQRSTUVWXYZ
GH07B-1066	9.66	JKLMNOPQRSTUVWXYZ
GH07B-1042	9.12	KLMNOPQRSTUVWXYZ
GH07B-1065	9.01	KLMNOPQRSTUVWXYZ
GH07B-1043	8.97	KLMNOPQRSTUVWXYZ
GH07B-1045	8.95	KLMNOPQRSTUVWXYZ
GH07B-940	8.84	KLMNOPQRSTUVWXYZ
GH07B-992	8.83	KLMNOPQRSTUVWXYZ
GH07B-951	8.12	LMNOPQRSTUVWXYZ
GH07B-1025	8.09	LMNOPQRSTUVWXYZ
GH07B-960	8.07	LMNOPQRSTUVWXYZ
GH07B-999	7.8	LMNOPQRSTUVWXYZ
GH07B-1236	7.69	LMNOPQRSTUVWXYZ
GH07B-1019	7.27	MNOPQRSTUVWXYZ
GH07B-984	7.21	MNOPQRSTUVWXYZ
GH07B-1058	7.07	NOPQRSTUVWXYZ
GH07B-943	6.89	NOPQRSTUVWXYZ

(Continued....)

GH07B-1010	6.88	NOPQRSTUVWXYZ
GH07B-1007	6.8	NOPQRSTUVWXYZ
GH07B-1053	6.73	NOPQRSTUVWXYZ
GH07B-1041	6.48	OPQRSTUVWXYZ
GH07B-1060	6.35	PQRSTUVWXYZ
GH07B-963	6.34	PQRSTUVWXYZ
GH07B-955	6.14	PQRSTUVWXYZ
GH07B-1062	6.13	PQRSTUVWXYZ
GH07B-1002	5.88	PQRSTUVWXYZ
GH07B-1044	5.46	PQRSTUVWXYZ
GH07B-971	5.13	QRSTUVWXYZ
GH07B-1039	5.07	QRSTUVWXYZ
GH07B-1050	5.01	QRSTUVWXYZ
GH07B-993	5.01	QRSTUVWXYZ
GH07B-980	4.95	QRSTUVWXYZ
GH07B-1035	4.68	QRSTUVWXYZ
GH07B-953	4.65	QRSTUVWXYZ
GH07B-983	4.34	RSTUV
GH07B-988	4.19	RSTUV
GH07B-974	4.16	RSTUV
GH07B-997	4.15	RSTUV
GH07B-970	4.15	RSTUV
GH07B-1021	3.97	RSTUV
GH07B-954	3.97	RSTUV
GH07B-989	3.97	RSTUV
GH07B-1059	3.97	RSTUV
GH07B-1063	3.42	RSTUV
GH07B-1238	3.24	RSTUV
GH07B-1048	3.17	RSTUV
GH07B-1040	3.03	STUV
GH07B-976	2.78	STUV
GH07B-1047	2.73	STUV
GH07B-977	2.64	STUV

(Continued....)

GH07B-942	2.57	STUV
GH07B-1000	2.5	STUV
GH07B-1038	2.44	STUV
GH07B-1008	2.38	STUV
GH07B-1024	1.85	TUV
GH07B-952	1.71	TUV
GH07B-1012	1.71	TUV
GH07B-1032	1.71	TUV
GH07B-1005	1.59	TUV
GH07B-1017	1.59	TUV
GH07B-967	1.48	TUV
GH07B-958	0.93	UV
GH07B-973	0.93	UV
GH07B-965	0.85	UV
GH07B-982	0.85	UV
GH07B-1031	0.85	UV
GH07B-986	0.85	UV
GH07B-1029	0	v
GH07B-1046	0	v
GH07B-1004	0	v
GH07B-994	0	v
GH07B-1036	0	v
GH07B-975	0	v
GH07B-978	0	v
GH07B-979	0	v
GH07B-941	0	v
GH07B-1022	0	v

Note: End percent survival values with the same letters do not differ statistically.