Development of barley germplasm with tolerance to barley yellow dwarf virus (BYDV) and the effects of BYDV infection on isogenic barley cultivars
by James Mitchell Crosslin

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
Montana State University

Abstract:
Barley yellow dwarf (BYD) is a serious disease of barley (Hordeum vulqare L.) and causes major crop losses worldwide. Tolerance to barley yellow dwarf virus (BYDV), the causal agent, of B YD, offers a means of reducing losses to the disease. Genetic male sterility was used to facilitate the development of a six-row barley population with a high level of tolerance to BYDV. A large percentage of plants in the population exhibit no or only slight discoloration following inoculation with BYDV. The population was developed by crossing Composite Cross XXXIIIA and B, Composite Cross XLIII, and Sutter barley. Tolerance to the virus was gained by incorporating the Yd2 gene into the population. This gene moderates the development of BYD symptoms and reduces yield losses in BYDV infected barley. The genetic background of the population is very diverse.

In order to more fully understand the effects of BYDV on host plants, barley cultivars with and without the Yd2 gene were inoculated with BYDV in field trials and greenhouse experiments. Results indicate that leaf chlorosis and stunting of roots and shoots due to BYD are reduced in cultivars containing Yd2. Cultivars without Yd2 may suffer 90-95% yield reductions when infected with BYDV, whereas genetically similar cultivars with Yd2 show little, if any, yield reduction.

Electron microscopy of BYDV inoculated plants revealed the presence of BYDV particles in phloem cells of root tissue from barley cultivars with and without the Yd2 gene. However, virus particles were not observed in root tissue of the Yd2-containing cultivar Atlas 68.

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ABSTRACT

Barley yellow dwarf (BYD) is a serious disease of barley (Hordeum vulgare L.) and causes major crop losses worldwide. Tolerance to barley yellow dwarf virus (BYDV), the causal agent of BYD, offers a means of reducing losses to the disease. Genetic male sterility was used to facilitate the development of a six-row barley population with a high level of tolerance to BYDV. A large percentage of plants in the population exhibit no or only slight discoloration following inoculation with BYDV. The population was developed by crossing Composite Cross XXXIIIA and B, Composite Cross XLIII, and Sutter barley. Tolerance to the virus was gained by incorporating the Yd2 gene into the population. This gene moderates the development of BYD symptoms and reduces yield losses in BYDV infected barley. The genetic background of the population is very diverse.

In order to more fully understand the effects of BYDV on host plants, barley cultivars with and without the Yd2 gene were inoculated with BYDV in field trials and greenhouse experiments. Results indicate that leaf chlorosis and stunting of roots and shoots due to BYD are reduced in cultivars containing Yd2. Cultivars without Yd2 may suffer 90-95% yield reductions when infected with BYDV, whereas genetically similar cultivars with Yd2 show little, if any, yield reduction.

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CHAPTER 1

INTRODUCTION

Barley yellow dwarf (BYD) is a disease affecting many crop species including barley, oats, wheat, and corn. The disease-causing agent has been termed barley yellow dwarf virus (BYDV).

BYDV is not mechanically transmissible or seed transmitted, but it is transmitted (vectored) by aphids in a persistent manner. The virus is limited to phloem tissue and generally causes "yellows" type symptoms and dwarfing in host plants. The virus particles are isometric in shape and about 25-30nm in diameter (Rochow and Duffus, 1981; Matthews, 1982).

The designation Yd2 has been suggested for a gene in barley that conditions both reduced symptom expression and reduced yield loss in BYDV infected plants. The tolerance to BYDV conferred by Yd2 was noted in several Ethiopian barley lines (Rasmusson and Schaller, 1959).

Some researchers have found, however, a poor correlation between chlorosis development and reduced yield under field conditions. Catherall and Hayes (1966) and Gill et al. (1969) found that cultivars exhibiting the most leaf chlorosis did not always produce the lowest yields. However, these researchers found that increased

leaf yellowing generally denoted susceptibility and susceptible cultivars exhibit reduced yields (Bruehl, 1961). These findings suggest that selection of lines that exhibit little discoloration will, in most cases, also select for reduced yield loss from BYD.

Researchers studying the effects of incorporating Yd2 into European barley cultivars found that its expression varied with the source of the gene and with the background genotype into which it was placed (Catherall and Hayes, 1966). These observations indicated that one possible approach to obtaining agronomically acceptable BYDV tolerant lines would be to incorporate several sources of Yd2 into a diverse background containing agronomically acceptable genotypes. BYDV tolerant plants within this diverse "gene pool" could then be selected for regionally desirable characteristics (Qualset and Suneson, 1966).

Incorporation of genes for disease resistance into susceptible cultivars and repeated backcrossing has led to the development of "isogenic" barley cultivars (Weibe, 1968). Isogenic refers to two cultivars that are considered very similar in genetic make-up, with the exception of a single gene or perhaps a very few genes. In the case of Yd2, a cultivar containing the gene is

resistant to BYD whereas the isogenic without Yd2 is susceptible. The two cultivars are otherwise similar (Schaller and Chim; 1969a, 1969b).

The effects of BYDV on the growth and yield of cereals have been studied extensively over the last 20-30 years (Plumb, 1983). A few investigators have noted a decrease in root growth of infected plants. However, data quantifying the reduction in root growth through the use of isogenic barley cultivars are lacking.

Electron microscopic (EM) observations of ultrathin tissue sections from BYDV infected susceptible hosts have revealed the presence of virus particles in phloem tissue. However, to date little, if any, EM work has been conducted on BYDV resistant cultivars. Also, most EM work on BYDV infected plants has involved observation of oat leaf tissue while largely ignoring root tissue and other host plant genera, such as barley (Gill and Chong, 1979).

The four objectives of the research reported herein are listed below:

1. Develop a six-rowed barley population containing several sources of Yd2 in a diverse genetic background.

2. Study the effect of BYDV infection on the yield of isogenic barley cultivars that contain or lack Yd2.

3. Evaluate the effects of BYDV infection on root and shoot growth of isogenic barley cultivars.

4. Study the occurrence of BYDV particles in root tissue of isogenic barley cultivars using electron microscopy.

Fulfillment of the first objective would yield BYDV tolerant germplasm that could be exploited by barley researchers around the world due to wide genetic diversity within the barley population.

Achievement of the second and third objectives would allow appraisal of the effects of BYDV infections on yield and growth of isogenics and may show a correlation of reduction in growth with a reduction in yield.

Completion of the last objective would allow comparative analysis of virus particle occurrence in infected isogenic cultivars possibly revealing differences in the cytological and morphological responses of resistant versus susceptible plants.

This information will enable us to more fully understand the nature of BYDV infections and the effects of infection on host plants.

CHAPTER 2

LITERATURE REVIEW

History and importance of barley yellow dwarf

The viral nature of the disease barley yellow dwarf (BYD) has been assumed since Oswald and Houston (1951) reported the aphid transmission of a widespread disease affecting barley, oats, and wheat in California. However it was not until Rochow and Brakke (1964) established a relationship between the presence of small isometric particles and infectivity, that the viral origin of BYD was confirmed. Barley yellow dwarf virus (BYDV) was also found to be the causal agent of "oat red leaf disease" by Takeshita (1956). Work by Oswald and Houston (1953a) and Rochow (1961) has shown that BYDV is transmitted in a persistent manner by aphids and is neither mechanically nor seed transmitted.

Barley yellow dwarf can cause major economic losses, as was reported by Oswald and Houston (1953a), when an estimated 10% of the 1951 California barley crop was lost to this disease. Browning et al. (1959) reported that 12% of the oat crop in Iowa that year was lost due to yellow dwarf. This loss represented approximately 394,000 metric tons (Bruehl, 1961). Similar losses were reported in several other states between 1951 and

1960 (Bruehl, 1961). Recently, localized epiphytotics of BYD have occurred in Montana (Yount and Carroll, 1983) and Idaho (Forster and Rochow, 1983).

Losses due to BYD were also considerable in Canada in 1974. Gill (1975) reported a loss in oats of over 30,000 metric tons and in Conquest and Herta barleys an additional 42,000 were lost.

In addition to the United States and Canada, BYDV is known to cause losses in Britain (Doodson and Saunders, 1970), New Zealand (Smith, 1963b), and China (Zhang et al., 1983), to name a few areas of importance. Rochow and Duffus (1981) consider BYDV to be "the most common and widespread of all cereal viruses."

Host range and symptoms of BYDV

The most commonly observed symptoms of BYDV infection include yellowing and stunting. In barley the chlorosis typically appears first on the tips of older leaves, gradually moving down the blade until the entire leaf is bright yellow. Some barley cultivars may show a reddening of the leaves. There may be development of a noticeable green/yellow mottling, with yellow areas coalescing into a general bright yellow chlorosis. These symptoms are very similar to those of aster yellows in

barley (Banttari, 1965). The leaves often assume a stiff, erect posture and appear much thicker than healthy leaves. Some cultivars may develop serrated leaf margins. Experimental inoculations have shown that these symptoms begin to develop in 10-21 days, depending somewhat on the crop and cultivar (Oswald and Houston, 1953a; Bruehl, 1961; Rochow, 1961).

In oats, symptom development follows that of barley except that most oat cultivars exhibit varying degrees of reddening or purpling of the leaves (Takeshita, 1956). Also, water-soaked areas and streaks may appear in oats or barley (Rochow, 1961) and a sugary exudate may appear on the affected leaves (Esau, 1957b).

In addition to the leaf symptoms mentioned above, BYDV infection of susceptible cultivars results in marked stunting of roots and shoots (Bruehl, 1961; Kainz and Hendrix, 1981), reduction in number of seeds per spike in wheat and barley, blasting of florets in oats, and some cultivars show an increase in tillering (Oswald and Houston, 1953a; Rochow, 1961; Catherall and Hayes, 1966). The yield of a susceptible cultivar is dramatically reduced (Bruehl, 1961; Doodson and Saunders, 1970).

Host range studies of BYDV have shown that many members of the Gramineae are affected, including barley (Hordeum vulgare L.), oats (Avena sativa L.), wheat (Triticum aestivum L.), rye (Secale cereale L.), rice (Oryza sativa L.) and corn (Zea mays L.) (Bruehl, 1961).

Oswald and Houston (1953b) inoculated 55 grass species with the virus and found that 20 exhibited BYDV symptoms and from which aphids successfully recovered the virus. Sixteen species showed no symptoms, but aphids were able to recover the virus. The remaining 19 species failed to develop symptoms and virus could not be recovered. Notable symptomatic non-cereal hosts included Bromus inermis Leyss., Avena fatua L., and Festuca reflexa Buckl..

Rochow (1961) lists 84 species that have been found to be hosts of BYDV by one or more researchers. Some non-cereal grass hosts of possible epidemiological significance include Poa pratensis L., Agropyron intermedium Host., Lolium perenne L., and Phleum pratense L. (Bruehl, 1961). Recently, corn has been found to be an epidemiologically important host in Washington (Brown et al., 1984). Rochow and Duffus (1981) speculate that "since so many plant species do not develop symptoms of

BYDV infection, it would be unusual to find a grass species not susceptible to one or more isolates of BYDV."

According to Bruehl (1961), G. B. Orlob used English grain aphids to recover BYDV from dodder (Cuscuta campestris Yunck.) growing on diseased barley. In addition, Timian (1964) showed transmission of BYDV from diseased barley to healthy plants via dodder. Dodder however is not listed as a host of the virus by Bruehl (1961) or Rochow (1961).

Diagnosis of BYDV infections

As the symptoms of BYDV infection can be confused with other diseases or physiological disorders, diagnosis based solely on symptoms may be unreliable (Rochow, 1961).

Oswald and Houston (1951, 1953a, 1953b) used four species of aphids in studies on the transmission of BYDV. These researchers diagnosed BYD based on development of characteristic symptoms in susceptible cultivars following infestation of healthy plants by aphids that had fed on diseased plants. Symptoms normally appeared within two weeks of infestation with viruliferous aphids. Plants infested with aphids that had not fed on diseased plants remained symptom-free.

An improvement in the aphid transmission technique (Rochow, 1963) demonstrated that aphids could acquire the virus by feeding on detatched leaves or leaf pieces. In this technique virus free aphids were allowed to feed in the dark for 1-2 days on leaf pieces in humidified dishes. The aphids were then transferred to oat seedlings with a moistened camel's hair brush. After a 3-5 day inoculation period the plants were sprayed or fumigated to kill the aphids and observed for characteristic BYD symptom development.

Rochow (1960) also found that BYDV could be acquired from liquid extracts of infected plants by the English grain aphid (Macrosiphum avenae Fabr.) feeding through animal membranes. Extracts were made by grinding infected oat tissue in a 10% sucrose solution.

Some serological tests that have been used for BYDV diagnosis include neutralization of virus transmission in plant extracts used for membrane feedings, micro-agar double diffusion and latex agglutination (Rochow, 1970). More recently enzyme linked immunosorbent assay (ELISA) techniques have been applied to BYDV (Lister and Rochow, 1979). Tests comparing the efficiency and accuracy of biological techniques (vector studies) versus ELISA for

BYDV diagnosis have shown ELISA to be a reliable, rapid, and sensitive technique. (Rochow, 1979, 1982).

An additional technique for BYDV diagnosis involves electron microscopic (EM) observation of virus particles trapped on an EM grid by antibodies against BYDV (Paliwal, 1977). This technique is termed serologically specific electron microscopy (SSEM) or immunosorbent electron microscopy (ISEM), and has been recently applied to BYDV using monoclonal antibodies (Diaco et al., 1984).

Virus isolates and vector specificity

Bruehl and Toko (1957) tested two isolates of BYDV and noted some variation in host range between the two. For example, some Poa species were susceptible to one isolate and not the other. They also found that Bromus inermis was "immune" to the two isolates (from Washington) they used but was susceptible to the California isolate used by Oswald and Houston (1953b). Obviously differences among BYDV isolates exist.

The early work of Oswald and Houston (1951, 1953a, 1953b) involved a BYDV isolate that was vectored by all four of the aphid species tested. The species used were Rhopalosiphum maidis Fitch, the corn leaf aphid; R. prunifoliae Fitch (=R. padi L.), the apple-grain or oat

bird-cherry aphid; Macrosiphum granarium Kirby (=M. avenae Fabr.), the grain aphid; and M. dirhodum Walker, the grass aphid. These researchers noted that the four species varied in their transmission efficiences (1953a). Macrosiphum granarium was the most efficient vector (93.2%) and M. dirhodum the least efficient (26.8%).

A few years later, Allen (1957b) tested 43 isolates of BYDV for symptom development on Black Hulless, Rojo, and Atlas 46 barleys, and Coast Black oats (Avena byzantina K.Koch) using Rhopalosiphum prunifoliae Fitch as the vector. Allen divided the 43 isolates into seven "strain types" based on these tests and noted that most isolates produced severe discoloration and stunting on all the test species except Rojo barley. It was also noted that in plants simultaneously inoculated with mixtures of the strains, the separate strains could be later extracted in "pure" form.

Although Allen (1957b) noted that isolates of BYDV differed in their symptom expression, he did not attempt to differentiate these isolates based on vector specificities or lack thereof. Rochow (1959) tested 127 BYDV isolates for transmission by Rhopalosiphum fitchii Sand., M. avenae, R. maidis, and Toxoptera (=Schizaphis) graminum Rondani. BYDV was recovered from 68 of these

samples by M. avenae only, from 14 by R. fitchii only, and from 25 by both R. fitchii and M. avenae. The virus was also recovered from 12 samples by R. maidis only and from seven samples by R. maidis and other species. This work demonstrated that BYDV isolates differ markedly in their vector specificities.

Additional work by Rochow (1969) allowed hundreds of samples of BYDV to be classified into four groups based on differences in transmission by three aphid species. These groups consisted of PAV, vectored nonspecifically by R. padi (=R. fitchii in earlier work, Rochow, 1969) and M. avenae with a rare R. maidis transmission; RPV transmitted specifically by R. padi; MAV transmitted specifically by M. avenae; and RMV transmitted specifically by R. maidis. An additional isolate vectored specifically by Schizaphis graminum has been termed SGV (Johnson and Rochow, 1972).

Serological testing has shown that the RPV and RMV isolates are related , but are distinct from the PAV and MAV isolates, which are related. The SGV isolates appear to have some antigens in common with PAV (Rochow and Duffus, 1981).

Although these "types" of BYDV (PAV, MAV, SGV, RPV, and RMV) are widely recognized (Plumb, 1983), there are

no doubt others with different vector specificities and serological characteristics (Zhang et al., 1983).

Physical properties of the virus

Barley yellow dwarf virus (MAV type) was first purified by Rochow and Brakke (1964) and these workers were able to demonstrate a relationship between small (30nm) isometric particles and infectivity.

To date, BYDV isolates studied are composed of a single component of single-stranded RNA with a molecular weight of about 2.0×10^6d. A protein with a molecular weight of about 24,000d has been isolated from the MAV and RPV types. Estimates of the size of BYDV particles vary from 30nm for shadowed preparations to 22-26nm in ultrathin sections of infected tissue (Rochow and Duffus, 1981).

Location of BYDV particles in infected plants

Allen (1957a) found that in sections of leaf tissue on which R. padi had fed, over 77% of the feeding tracts ended in the phloem, less than 1% in the xylem, and about 22% elsewhere. This was a strong indication that BYDV was associated with the phloem tissue.

Electron microscopy of BYDV infected barley leaf and root tissue by Jensen (1969) showed isometric, densely

staining particles about 24nm in diameter in the phloem. Healthy tissue contained no such particles. However, Jensen was not able to identify the type of phloem cells which contained masses of virus particles due to the disrupted nature of the cell's internal structure. Generally only one or two cells in an entire vascular bundle contained virus particles.

Amici et al. (1979) studied the occurrence of BYDV particles in roots of "rice giallume" infected Leersia oryzoides (Br.) Sol., a perennial grassy weed. These researchers found small densely staining, isometric particles in phloem tissue of leaves and roots. Large crystalline arrays of virus particles were observed in one phloem parenchyma cell from root tissue.

Work by the various researchers cited above would indicate that BYDV particles are confined to phloem tissue. However, Gill and Chong (1981) noted that under conditions of double infection with MAV and RPV isolates, oat xylem tissue was found to contain virus particles.

Anatomical and cytological effects of BYDV infection

Investigations by Esau (1957a) conducted at the light microscope level indicated that BYDV had profound

effects on host phloem tissue. Esau noted that in BYDV infected leaves, necrosis of the first sieve elements occurred before comparable effects in healthy plants were observed. Parenchyma cells near these early sieve elements often underwent early necrosis as well. In infected root tissue Esau also noted that parenchyma cells of the pericycle and phloem began degeneration prior to that of healthy tissue. Sieve elements in roots were observed to accumulate a "gummous" material and collapse prematurely. The cells next to the sieve elements were observed to undergo the most degenerative changes.

At the electron microscope level Gill and Chong (1975) studied the effects of BYDV infection on oat leaf tissue. As early as four days after inoculation, inclusions consisting of virus-like particles, slender filaments and vesicles containing fibrils were observed in phloem parenchyma, companion cells, and sieve elements. These researchers noted that the nuclei of infected cells became distorted and heterochromatin appeared "clumped." Within 11 days inclusions were observed in nearly all phloem cells.

Later work by these investigators (Gill and Chong, 1979) provided evidence for division of BYDV isolates

into two subgroups based on cytopathology. Criteria used for division of the isolates included alterations in the nucleus, where in the cell virus particles were first observed, and the type of fibril-containing vesicles found in the cytoplasm. These effects were observed in sieve elements, companion cells and phloem parenchyma.

Subgroup 1, made up of PAV-, MAV-, and SGV-like isolates caused infections that showed: 1) distorted nuclear outlines, then aggregation of densely staining material, 2) virus particles first appearing in the cytoplasm, and 3) the presence of single-membraned vesicles containing fibrils in the cytoplasm.

Subgroup 2, consisting of the RMV- and RPV-like isolates caused infections that exhibited: 1) nuclei which remained normal in outline with a slow disintegration of heterochromatin, 2) virus particles usually appearing first around the nucleolus, and 3) fibril-containing vesicles enclosed within a second membrane.

It is interesting to note that each of these two subgroups (Gill and Chong, 1979) is composed of isolates that are considered serologically related (Rochow and Duffus, 1981).

Control of BYDV

Some possible control strategies for BYDV include insecticide applications to control the aphid vectors, removal of virus source plants, use of resistant crop cultivars, and avoidance of periods of high vector populations by adjusting planting dates (Rochow and Duffus, 1981).

The use of insecticides to control BYDV by destroying aphid vectors has been somewhat successful. Caldwell et al. (1959) used Dimethoate to control aphids in germplasm nurseries. Smith (1963b) achieved BYDV control in tests conducted in New Zealand by the use of systemic organo-phosphorous sprays to kill R. padi. Yield increases of 5 to 24 bushels of wheat per acre were noted when aphids were controlled. However, Smith (1963b) also noted that Metasystox sprays applied to oats in 1956-58 failed to control BYDV. This researcher hypothesized that since oats are more susceptible to BYDV than is wheat (Bruehl, 1961), a low population of aphids was sufficient to cause severe infection.

Regarding insecticides, Plumb (1983) is of the opinion that "at present chemicals seem unlikely to prevent primary infection and their effect on secondary

spread could be swamped by a continuous influx of infective vectors."

As many common grass species are known hosts of BYDV (Rochow, 1961), elimination of non-cereal hosts may not be practical. In New Zealand the widespread occurrence of perennial grass pastures near cereal fields virtually assures availability of both vector and virus (Smith, 1963b).

Since young plants are the most susceptible to BYD injury (Rochow and Duffus, 1981), adjusting planting dates to avoid periods of high aphid numbers can reduce losses from BYDV. Extremely early fall sown grains are apt to become infested and suffer disease loss (Bruehl, 1961). By avoiding early fall and late spring plantings, losses may be minimized (Plumb, 1983; Rochow and Duffus, 1981; Yount and Carroll, 1983).

If the disease cannot be avoided, the only alternative is to plant disease resistant cultivars. Oswald and Houston (1953a) noted varietal differences in wheat, oats, and barley to BYDV infection. Although most oat cultivars are very susceptible to BYDV infection, some resistant cultivars have been noted (Browning et al., 1959; Jedlinski and Brown, 1959). In wheat several sources of resistance have been found, but this

resistance appears to be quantitative in nature and does not impart complete freedom from BYD (Qualset et al., 1973). Although there seems to be some resistance to BYDV in certain cultivars of wheat and oats, it is in barley that the best resistance has been found.

Tolerance to BYDV in barley

Rasmusson and Schaller (1959) suggested the designation Yd2 for a gene that conditioned reduced symptom expression in barley. This gene, which was found in several barleys of Ethiopian origin, was studied by crossing the resistant lines C.I. 1227, 1237, 2376, and Abate (C.I. 14119) with susceptible lines or with other resistant material. Results from the F_3 indicated that the four cultivars tested contained the same gene. Later work by Damsteegt and Bruehl (1964) indicated that Yd2 was incompletely dominant in its action. However, as parents and progenies often differ in their apparent level of resistance to BYDV the presence of different alleles or modifying factors has been suggested (Schaller, 1964; Catherall et al., 1970). In addition, Catherall and Hayes (1966) noted that the expression of Yd2 varied with the background genotype into which it was placed.

One approach to studying the effects of BYDV infection has involved the use of barley cultivars that are "identical" in all respects except for resistance or susceptibility to BYDV (McLean, et al., 1983; Skaria, et al., 1984a, 1984b). These genetically similar cultivars are often developed by backcrossing and although it cannot be said with certainty that they differ only in regards to one gene, Yd2, they are considered isogenics (Weibe, 1968).

Over the years a number of BYD resistant barleys have been developed by backcrossing a good agronomic type with a Yd2 source. These include Atlas 68 and CM67 (Schaller and Chim, 1969a, 1969b) and Sutter (Schaller et al., 1973).

Barley composite cross populations

The first report of cultivation of a mixture of barley hybrids, or composite, is that of Harlan and Martini (1929). A number of years later, Suneson (1951) discussed using a recently found genetic male sterility factor (Suneson, 1940) to produce hybrid barley. Utilizing the male sterile plants as females eliminated the time consuming and laborious process of emasculation, thus enabling workers to make a previously unheard of

number of crosses in a very short time. In this way Suneson and Wiebe (1962) were able to cross virtually the entire American World Collection of spring barleys to produce Composite Cross (CC) XXI.

Since genetic male sterility in barley was controlled by a simply inherited recessive gene, this allowed an easy means of maintaining sterility in a population (Suneson, 1951). Crossing a homozygous male fertile (MsMs) plant with a homozygous male sterile (msms) plant will result in heterozygous male fertile (Msms) individuals. By selfing an Msms, the progeny will segregate approximately three fertiles (MsMs, Msms, msMs) to one sterile (msms) (Poehlman, 1979). As natural outcrossing of male sterile plants with nearby fertiles will occur (Suneson, 1951) the hybrid seed set in such a manner will be Msms, and male sterility can be maintained in a population by harvesting only male sterile plants and collecting the outcrossed seed.

Recurrent selection populations for disease resistance

Composite cross populations, facilitated by the use of male steriles, have been exploited in plant pathology. By making a large number of initial crosses, screening for resistant plants, and allowing these plants to

outcross, a system called male sterile facilitated recurrent selection has been developed.

The development of a male sterile facilitated recurrent selection population (MSFRSP, also referred to as RSP) begins with the establishment of a base population that is segregating for both male sterility and the character for which it is to be selected, such as disease resistance. This first step is accomplished by crossing agronomically acceptable, genetically diverse cultivars as males with male sterile plants as females. A number of different sources of male sterility have been identified (Hockett and Eslick, 1971). Ten to twenty cultivars are often included in these first crosses. The resulting seed is bulked and the F_1 grown, forming the recurrent selection population (RSP) base.

The next step is to cross male sterile plants in the RSP base as females with a number of lines containing sources of resistance to a particular disease. Typically at least 250 crosses will be made.

The F_1 is grown in isolation to prevent introduction of unwanted genotypes from outside the RSP. In this "recombination" cycle, male fertile plants will outcross with steriles. Male sterile plants only are harvested and the outcrossed seed collected.

A sample of the outcrossed seed, usually 500g or more, is space planted in a disease nursery. Normally the plant density is about 1 plant per 5-15cm of row with rows 30cm apart. This spacing facilitates individual plant evaluation, increases tillering, and simplifies the roguing process.

After inoculation with the disease in question, or the occurrence of natural infections, highly susceptible plants are rogued out. In the first selection cycle only 10-20% of the plants are discarded to avoid early narrowing of the germplasm. In later cycles up to 50% or more of the susceptible plants can be rogued out. Male sterile plants are also removed from the disease nursery. This is done to prevent susceptible plants from outcrossing. After removal of susceptibles and steriles the remaining plants are bulk harvested.

The population now undergoes a recombination cycle where only male steriles are harvested. The outcrossed seed is then space planted in a disease nursery as previously described. These selection-recombination cycles are repeated as long as an increase in disease resistance is observed (H. E. Bockelman and R. T. Ramage, personal communications).

Male sterile facilitated recurrent selection populations developed in the above manner include Composite Cross (CC) XLIII, developed for scald and net blotch resistance (Bockelman et al., 1983), and CC XXXIIIA and B, developed for BYDV resistance (Thompson and Craddock, 1979).

Use of terms

According to Cooper and Jones (1983) the British Mycological Society describes a resistant organism as "possessing qualities that hinder the development of a given disease." The same investigators state that the Terminology Sub-committee of the Federation of British Plant Pathologists substitutes the word "pathogen" for "disease" in the above definition.

Regarding viruses, Cooper and Jones (1983) have concluded from publications by the American Phytopathological Society, the British Mycological Society, and the Federation of British Plant Pathologists, that tolerant "is a term used to describe a host that a specific virus can infect and in which it can replicate and invade without causing severe symptoms or greatly diminishing the rate or amount of plant growth or marketable yield."

In contrast, Schaller and Chim (1969b), in the registration of CM67 barley state that this cultivar is "tolerant" to BYDV "having the Yd2 gene for resistance from C.I. 2376". The same authors (Schaller and Chim, 1973) in registering Sutter barley state that the cultivar is "highly tolerant to the BYDV, having the Yd2 gene for resistance from C.I. 1237."

Owing to the difference of opinion regarding use of the terms resistant, tolerant, sensitive, etc. (see Bos, 1983; Tavantzis, 1984) I will use the term "tolerant" to describe plants that exhibit reduced symptom expression. By comparison, the term "resistant" will be used to describe plants resistant to BYD disease as evidenced by their apparent ability to produce a near normal yield although they are infected by BYDV. "Sensitive" will be considered the opposite of tolerant and "susceptible" the opposite of resistant.

CHAPTER 3

DEVELOPMENT OF BYDV TOLERANT BARLEY GERMPLASM

Materials and Methods

1982 field season

In 1982 development of a 6-rowed barley male sterile facilitated recurrent selection population (RSP) with tolerance to BYDV was begun. In development of the population, tolerance to BYDV was judged by the lack of severe chlorosis development following BYDV inoculation. A preliminary report on this work has been published (Crosslin et al., 1984).

Parental lines for this population were chosen based on the need to incorporate BYD resistance, good agronomic qualities, and wide genetic diversity. One parent chosen was CC XXXIIIA and B (Thompson and Craddock, 1979), a 6-rowed RSP developed for BYD resistance having four sources of Yd2. The sources of Yd2 in CC XXXIII are C.I. 1227, 1237, 2376, and 14119 (formerly 3920-1). All of these are of Ethiopian origin (Oswald and Houston, 1953a). This population also contains male sterile genes (msg) 1 and msg 2. The second parent chosen was CC XLIII (Bockelman et al., 1983) a 6-rowed barley RSP developed for scald and net blotch resistance, but also contains

Yd2 from C.I. 14119 and 2376. CC XLIII is genetically very diverse and contains many good agronomic types in its background. This population also contains msgl0. The last parent chosen was a Manchuria (C.I. 2330) msgl0 x Sutter (C.I. 15475) F_2. Hereafter this hybrid will be referred to as M-S. Manchuria contributed msgl0 to facilitate crossing, and Sutter is BYD resistant, having gained Yd2 from C.I. 1237. Sutter also possesses some scald, net blotch, and powdery mildew resistance (Schaller et al., 1973).

A seed mixture of CC XXXIIIA and B was made by combining approximately 66g each of A and B from Pullman, WA with 22g each of A and B from Bozeman, MT disease nurseries. This resulted in about 175g of seed. More Pullman seed was included due to the presence of heavier BYD selection pressure there in 1979 (year the seed was harvested) than in Bozeman. To this 175g of CC XXXIII, 325g of CC XLIII was added. This will be referred to as "population mixture". To the 500g of population mixture, 2kg of killed wheat was added to facilitate space planting.

This seed was planted 24 May, 1982 at the Fort Ellis Research Farm near Bozeman, MT. The material was planted in two blocks, each consisting of 40 rows 21m long and

30cm apart. A mixture of 165g M-S and 660g of killed wheat was planted in six 12m rows between the two population mixture blocks. The entire planting was surrounded by 12 rows of spring wheat.

Approximately 30 days after planting, the stand was estimated by counting the number of plants in three rows and averaging over all rows. By this method an estimated 8,300 plants were present in the population mixture blocks and 450 in the M-S rows.

From 19 July, 1982 to 30 July, 1982 crosses were made by trimming the florets back on male sterile heads, covering with a glassine bag, and "twirling" a pollen-bearing head inside the bag. Male sterile plants in the population mixture were crossed with male fertile M-S plants and male fertiles in the population mixture were crossed with male sterile M-S. A total of approximately 350 crosses were made.

Crossed heads were harvested and threshed in September. The 264 harvested heads of population mixture females x M-S males yielded 183g of seed. The 53 M-S females x population mixture males yielded 30g of seed. Also, male sterile plants in both the population mixture and the M-S were harvested, and the outcrossed seed collected. Male sterile plants in the M-S and population

mixture yielded 55g and 571g of outcrossed seed respectively.

In the fall of 1982, three seed lots were assembled for shipment to Arizona, designated isolations 8, 9, and 10. Isolation 8 consisted of the 30g of M-S females x population mixture males. Isolation 9 was the 183g of population mixture females x M-S males. Isolation 10 consisted of a mixture of 55g of outcrossed M-S seed and 400g of outcrossed seed from the population mixture blocks. These three seed lots were planted 100m from other barleys by Western Plant Breeders near Chandler, Arizona on 3 January, 1983.

1983 field season

Male sterile plants in isolations 8, 9, and 10 were harvested in May, 1983, and the outcrossed seed collected. In addition about 100 heads from male fertile plants in isolations 8 and 9 were collected.

A seed mixture was made consisting of 500g of isolation 10, 75g each of isolations 8 and 9, and 25g each of seeds from fertile plants in isolations 8 and 9. This mixture will be referred to as RSP-BYDV 1983, and was planted at the Arthur H. Post Field Research Laboratory near Bozeman, MT on 26 May, 1983. Killed

wheat was added to the seed to facilitate space planting. Following emergence the plants were hand thinned to about 2-3 plants per 30cm of row. This was done to facilitate individual plant evaluation. After thinning, the plant stand was estimated at 10,600.

In addition to planting the block of RSP-BYDV 1983, a replicated trial was conducted to test the reaction of RSP-BYDV parental populations to BYDV infection. The design was a randomized complete block with four blocks. Each block consisted of three rows per entry. Entries were M-S, CC XXXIIIA and B (same mixture used in initial planting), CC XLIII, isolation 10, and RSP-BYDV 1983. Three rows of Coast Black oats (Avena byzantina K.Koch) were planted between the blocks to act as susceptible indicators of BYDV infection. Rows were 6m long and 30cm apart. All entries were space planted and thinned to 2-3 plants per 30cm of row. The entire experimental area was surrounded by 12 rows of spring wheat to act as a "barrier" for the inoculative aphids that would be introduced.

The main population block and the replicated trials suffered some damage from cattle grazing and hail. For this reason yield data were not collected from the rows of parental populations.

BYDV isolates used in 1983

Two PAV-like isolates of BYDV were used in field inoculations in 1983. One isolate, MT-PAV, was recovered from a BYDV symptomatic barley plant in the Montana State University greenhouse, Range 4, in early 1983. It should be mentioned that this is not the same "MT-PAV" referred to by Yount (1982). This isolate from the greenhouse was found to be highly virulent on Coast Black oats and was vectored effeciently by both R. padi and M. avenae (J. M. Crosslin, unpublished). The second isolate, termed WA-PAV, was a PAV-like isolate from Washington provided by S. D. Wyatt at Washington State University. This isolate was also vectored efficiently by R. padi and M. avenae. Both of these isolates were found to give positive results in ELISA tests with immunoglobulins against a New York PAV isolate (W. F. Rochow, personal communication).

Aphid rearing 1983

Virus-free R. padi and M. avenae, reared at the Montana State University insectary, were allowed to feed on detached leaves of Coast Black oats infected with MT-PAV and WA-PAV using the method of Rochow (1963). Following the two day acquisition period, leaf pieces infested with aphids were placed in 20cm pots containing

Coast Black oat and Klages barley seedlings. About 60 pots, each containing about 100 seedlings were infested. Aphids were allowed to colonize the seedlings and were left undisturbed. Plants were grown under fluorescent lighting and watered as necessary. The temperature was maintained at about 20-25 C. Within two weeks of infestation some of the plants began to exhibit chlorosis and reddening typical of BYDV infection. Large numbers of aphids were present on all plants within 2-3 weeks.

Aphids were collected by using a technique similar to that of Comeau (1976) and Yount (1982). The aphid infested plants were shaken over a large piece of heavy paper dusted with talcum powder. The talcum prevents the aphids from sticking together in large masses. The aphids were placed in plastic dishes with tight fitting lids for transport to the field. The plants were returned to the isolation room to allow the aphids to increase for subsequent harvests.

Field plot infestations 1983

Plants were infested with viruliferous aphids when they reached the 3-5 leaf stage (stage 13-15, Tottman and Makepeace, 1979).

The replicated trials involving parental lines were infested with the inoculative aphids by shaking a few aphids out of the containers directly onto plants in the center row of each three row replicate.

Very early in the aphid spreading procedure it was realized that there were insufficient aphids to infest every plant in the RSP-BYDV 1983 block. Due to the shortage of aphids every third or fourth row of the population was infested by sprinkling aphids onto each plant.

Aphids were spread three additional times as they became available over the course of about three weeks. Center rows of the replicated trials were reinfested as before. An attempt was made to evenly disperse aphids throughout the RSP-BYDV 1983 block.

Following development of BYDV symptoms in the population and parental lines, a 0-3 rating scale was devised as an aid in determining symptom severity. A zero rating indicated that leaf chlorosis was not observed. Plants receiving a one rating showed leaf tip chlorosis on one or a few leaves. A rating of two indicated that most or all of the leaves were affected, but entire leaves were not chlorotic or necrotic. The three disease rating indicated that virtually the entire

plant was severely affected and entire leaves were often chlorotic or necrotic. A few plants given a three rating had apparently been killed by BYD.

Dwarfing was not considered in assigning a disease rating because of the presence of dwarfing genes in the population. Most of the dwarf genotypes would have originated in CC XXXIIIA, members of which had been selected for short, stiff straw, and rapid early growth (Thompson and Craddock, 1979).

Following the harvest of RSP-BYDV 1983 (see Results and Discussion) 500g of seed was sent to Arizona for natural recombination. Outcrossed seed harvested from male sterile plants was returned to Bozeman in May of 1984.

1984 field season

In 1984 the population (now called RSP-BYDV 1984) was planted as in 1983. The parental lines were not planted. However, in an attempt to compare the reactions of the 1983 and 1984 populations to BYDV infection, replicated plots were established. Two plots, one to be infested with viruliferous aphids and one noninfested control, were space planted in a randomized complete block design in four blocks. The 1983 and 1984

populations were planted in three 6m rows per block. Rows of Coast Black oats were included as susceptible indicators.

Aphid rearing in 1984 was similar to that in 1983, except that about 120 20cm pots of Klages and Coast Black oat seedlings were infested. In 1984 only the MT-PAV isolate was used. Inoculative aphids were collected as in 1983.

Owing to the larger number of aphids available in 1984, the entire plot of RSP-BYDV 1984 was infested within a four day period, when plants were in the 3-5 leaf stage. All plants within the population, about 11,000, had aphids sprinkled directly onto them. The replicated trials of RSP-BYDV 1983 and 1984 were infested with inoculative aphids by sprinkling them directly onto each plant in the center row of each three row replicate.

The replicated plots which served as controls were not infested with virus-free aphids. Neither the aphid-infested nor the control plots were sprayed with insecticide at any time. Twelve rows of spring wheat planted between the infested and noninfested plots were to serve as a barrier to the spread of aphids into the noninfested plots. However, late in the season a few plants in the control plots were BYD symptomatic.

Whether these plants were infected as a result of natural aphid movements or "leakage" of aphids from the infested plots is not known.

Reaction of the 1983 and 1984 populations to MT-PAV were compared by measuring disease reaction type (0-3), total dry weight/row, straw weight/row, grain weight/row, 1000 kernel weight, hectoliter weight, germination, and seed protein.

The disease rating of each plant, except the first and last, in the center row of each three row replicate was recorded. The row was then harvested by cutting the plants at the soil line with a sickle. Plants were bundled and allowed to dry.

When dry, the bundle was weighed and then threshed. In this way it was possible to obtain total dry weight/row, straw weight/row, and grain weight/row. Thousand kernel weight for each row was obtained by weighing 1000 seeds which had been counted previously with an electronic seed counter. Hectoliter weight was extrapolated from the weight of 100cc of grain and was determined for each harvested row.

Germination tests were performed using 400 seeds from bulked seed of the four replicates of each population/treatment combination. The tests were

conducted by the Montana State Seed Laboratory at Montana State University according to the Rules of the Association of Official Seed Analysts (Copeland, 1981).

Protein determinations were made on two samples of bulked seed from the four replicates of each population/treatment combination. The analyses were performed at the Montana State University Cereal Quality Laboratory using the infra-red reflectance technique. The apparatus used was a Technicon InfraAlyzer 400 (Technicon Industrial Systems, Tarrytown, NY 10591).

The data obtained from measurement of the above parameters were analyzed using multi-factorial analysis of variance for a randomzed complete block design.

About 340 plants showing 0 or 1 reaction types and possessing desirable characteristics such as large heads with well-filled seed, erect stature, and many tillers, were tagged. Prior to harvest individual heads from these plants were removed for subsequent head-row evaluation.

An estimated 5-10% of the plants in RSP-BYDV 1984 possessed short, stiff straw, and early growth habit. Single heads from about 60 plants with these characteristics, as well as 0 or 1 reaction types, were harvested.

At maturity the population block was bulk harvested with a plot combine. The 340 selected heads were individually threshed and the seed placed in envelopes. The 60 heads from the short, early plants were threshed and bulked.

Results and Discussion

Reaction of RSP-BYDV 1983 to BYDV, Bozeman 1983

Within two weeks of initial infestation with viruliferous aphids, some plants within the population had developed chlorosis and mottling typical of BYDV infestion.

Although symptom development began at about the same time for many plants throughout the population, within three or four weeks obvious differences were observed among plants in the intensity of chlorosis. The 0-3 rating scale previously described was applied as an aid in determining how many plants should be rogued from the population.

Forty-eight days post-infestation, plants within ten random 2m x 2m areas of the population block were evaluated. By this method a total of 423 plants were rated. Of this number approximately 9% were rated as 0, 21% as 1, 53% as 2, and 17% were rated 3 (Table 1). This evaluation showed that the inoculation procedures were indeed very effective as less than 10% of the plants were symptom free. Of the symptomless plants some may have escaped inoculation and others could have been extremely tolerant of the virus and failed to develop

Table 1. Disease reaction of BYDV inoculated RSP-BYDV 1983 and RSP-BYDV 1984 main population blocks.[1/]

Population	% Plants with Reaction Type[2/] 0	1	2	3	# Plants Evaluated
RSP-BYDV 1983	9.2[3/]	21.3	52.7	16.8	423
RSP-BYDV 1984	9.9	29.7	20.8	39.5	595

[1/]RSP-BYDV 1983 was infested with Rhopalosiphum padi and Macrosiphum avenae carrying both MT-PAV and WA-PAV isolates of BYDV.

RSP-BYDV 1984 was infested with R. padi and M. avenae carrying MT-PAV.

Evaluations of ten random 2m x 2m areas of the population blocks were conducted 48 days after infestation in both years.

[2/]Plants with a 0 reaction type showed no leaf chlorosis. A rating of 1 indicated that one or a few leaves showed leaf tip chlorosis. A 2 rating indicated that most or all leaves were affected but entire leaves were not chlorotic or necrotic. The 3 rating indicated that the entire plant was severely affected and entire leaves were chlorotic or necrotic.

[3/]Statistical comparisons were not conducted on these data due to the use of different virus isolate mixtures in each of the years and the heavier early infestation of RSP-BYDV 1984 with inoculative aphids.

symptoms. Because the population would be subjected to BYDV inoculations in subsequent years, the presence of a few escapes was not considered important.

Noting that about 17% of the plants were severely affected and that 20% or fewer of the plants in an RSP should be discarded the first year (H. E. Bockelman, personal communication), it was decided to rogue all plants with a 3 reaction type. This was accomplished over the course of about ten days by walking down the rows and simply pulling the plants. The author was the only person involved in roguing in order to minimize differences in reading of symptoms. A running total of plants rogued was kept and at the end of the roguing process 1,697 plants showing a 3 reaction were removed. This was equivalent to about 16% of the plants in the population.

Some additional plants were rogued as well. Perhaps 100 smut infected plants and "black barleys" were removed. Approximately 500 male sterile plants were removed as well. Removing the male sterile plants reduced the amount of ergot harvested (most of the male steriles were heavily infected with ergot, Claviceps purpurea (Fr.)Tul.), and reduced the amount of outcrossed seed that would be harvested. Some of these outcrosses

could have been from the type 3 rogues or highly susceptible material planted nearby.

When the remaining plants were mature, they were bulk harvested with a plot combine. About 25kg of seed was obtained.

The seed was cleaned with an air blower so that the lightest 20% were removed. This was done to help select for BYDV tolerant types by removing small, shriveled seed set on the more sensitive, but unrogued, plants. One of the factors involved in yield loss due to BYDV is a reduction in kernel weight (Bruehl, 1961; Yount, 1982). Also, larger seeds may produce more vigorous plants (Kaufmann et al., 1963).

A 500g sample of the "cleaned" seed was sent to Arizona and planted near Chandler by personnel of Western Plant Breeders on 20 December, 1983. In late April of 1984 male sterile plants were harvested by personnel from Montana State University and the outcrossed seed returned to Bozeman.

Reaction of RSP-BYDV parental lines to BYDV, Bozeman 1983

Following inoculation with a mixture of MT-PAV and WA-PAV, the disease reactions of replicated rows of RSP-BYDV 1983 and the populations used as parents were determined. The rating scale used was the same as for

the main population block. Table 2 shows the reaction of the parentals and RSP-BYDV 1983 28 days after inoculation.

CC XXXIIIA and B showed the highest level of tolerance to BYDV. This result might be expected as CC XXXIIIA and B has undergone several cycles of selection for BYDV tolerance (Thompson and Craddock, 1979; and T. W. Carroll, personal communication).

The values for CC XLIII and RSP-BYDV 1983 are quite similar and this is not surprising as CC XLIII was the major parent. The relatively low tolerance of M-S is something of a surprise, however. Yount (1982) found Sutter to be highly tolerant to a BYDV isolate similar to MT-PAV. It is possible that crossing Manchuria with Sutter resulted in the loss of homozygosity of Yd2, and thus in a lower level of tolerance to BYDV. This reduction in the effectiveness of Yd2 in heterozygous plants was noted by Catherall and Hayes (1966) in crosses of resistant x susceptible cultivars.

Table 3 shows the reaction of the population and parental lines evaluated 38 days after infestation. The discoloration trends observed at 28 days remain. CC XXXIIIA and B still contained significantly more asymptomatic plants (0 reaction types) than the other

Table 2. Disease reaction of RSP-BYDV 1983 and parental populations 28 days after inoculation with a mixture of MT-PAV and WA-PAV isolates of BYDV, 1983. [1/]

Population	% Plants with Reaction Type[2/] 0	1	2	3	# Plants Evaluated
CC XXXIIIA,B	36c[3/]	24a	28a	12a	92
CC XLIII	2a	18a	63b	17a	100
Isolation 10	16b	16a	58b	10a	89
M-S F_2	10ab	15a	57b	18a	54
RSP-BYDV 1983	7ab	15a	59b	18a	67

[1/]Plants were infested with viruliferous Rhopalosiphum padi and Macrosiphum avenae in the 3-5 leaf stage.

Values are the means of all plants in four replicates of 6m, bordered rows.

[2/]Plants with a 0 reaction type showed no leaf chlorosis. A rating of 1 indicated that one or a few leaves showed leaf tip chlorosis. A 2 rating indicated that most or all leaves were affected but entire leaves were not chlorotic or necrotic. The 3 rating indicated that the entire plant was severely affected and entire leaves were chlorotic or necrotic.

[3/]Values followed by the same letter are not significantly different at P=0.05 using the LSD method. Statistical analyses were conducted prior to rounding.

Table 3. Disease reactions of RSP-BYDV 1983 and parental populations 38 days after inoculation with a mixture of MT-PAV and WA-PAV isolates of BYDV.[1/]

Population	% Plants with Reaction Type[2/] 0	1	2	3	# Plants Evaluated
CC XXXIIIA,B	22b[3/]	31a	21a	26a	92
CC XLIII	1a	6a	52b	40a	100
Isolation 10	6a	17a	49b	28a	89
M-S F_2	7a	9a	40b	45a	54
RSP-BYDV 1983	3a	10a	53b	35a	67

[1/]Plants were infested with viruliferous Rhopalosiphum padi and Macrosiphum avenae in the 3-5 leaf stage.

Values are the means of all plants in four replicates of 6m, bordered rows.

[2/]Plants with a 0 reaction type showed no leaf chlorosis. A rating of 1 indicated that one or a few leaves showed leaf tip chlorosis. A 2 rating indicated that most or all leaves were affected but entire leaves were not chlorotic or necrotic. The 3 rating indicated that the entire plant was severely affected and entire leaves were chlorotic or vecrotic.

[3/]Values followed by the same letter are not significantly different at P=0.05 using the LSD method. Statistical analyses were conducted prior to rounding.

lines, and over half of the plants were of reaction types 0 and 1. The number of severely affected plants (3 reaction type) had approximately doubled in all lines, indicating an increase in symptom severity with time.

Reaction of RSP-BYDV 1984 to BYDV, Bozeman, 1984

The outcrossed seed from Arizona 1983-1984, designated RSP-BYDV 1984, was planted and infested with R. padi and M. avenae carrying MT-PAV as previously described.

Within two weeks of infestation chlorosis typical of BYD began to develop. Symptom development appeared to be much more pronounced than in 1983. This was probably due to the fact that all of the plants in RSP-BYDV 1984 had been individually infested with viruliferous aphids, whereas those in RSP-BYDV 1983 had not. This difference in infestation probably meant that plants in the 1984 population had been inoculated at an earlier stage than in 1983. Generally the earlier that plants are inoculated with BYDV the more severe are the effects (Panayotou, 1979; Rochow and Duffus, 1981).

Although temperature plays an important role in BYD symptom development (Endo, 1957), the conditions in both 1983 and 1984 were conducive to symptom development. At