SELECTION AND DIFFERENTIATION OF TOBACCO MOSAIC VIRUS SUBSTRAINS BY CULTIVARS AND LINES OF LYCOPERSICON SPECIES

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ABSTRACT

SELECTION AND DIFFERENTIATION OF TOBACCO MOSAIC VIRUS SUBSTRAINS BY CULTIVARS AND LINES OF LYCOPERSICON SPECIES

by Thomas A. Zitter

The objectives of this investigation were (1) to determine the effect of different tomato (Lycopersicon spp.) hosts on the selection of variants within distinctive strains of tobacco mosaic virus (TMV) and (2) to attempt differentiation of the variants by mainly biological and to a limited extent by physical means. For this purpose, tomato cultivar Bonny Best (susceptible) and N32 (a derivative of Holmes' TMV-resistant P.I. 235673 X Spartan Red 8) were the primary hosts used. Four distinctive strains of TMV, (YA) yellow aucuba, (JSB-1) tomato internal browning, (S-IV) tomato mosaic and (HRG) a Holmes' ribgrass strain were serially passaged through the two lycopersicon hosts. The virus progenies were analyzed by studying the behavior of single lesion isolates. Two distinct forms were differentiated for three of the strains. Only with strain HRG did the virus remain apparently uniform in its response for both tomato hosts. Both forms of the virus multiplied and produced similar symptoms in Bonny Best, but only one

of these infected N32. Although initially both forms were considered as variants of a particular strain, additional experimental evidence suggested that the form specific for susceptible tomato was the parent and the form which infected both hosts was a substrain of the parent. The only means found for establishing the existence of these forms was through the use of resistant hosts.

Single lesion isolate studies of strain YA revealed that the parent form accounted for 94 and 98%, respectively, of the virus recovered from susceptible Bonny Best and White Burley tobacco sources. Virus obtained from resistant plants was exclusively a substrain of the parent form and designated substrain B. Results indicated that host selection was the means of obtaining substrain B. A 5-week delay in symptom expression in resistant plants, inoculated with virus from susceptible sources, was explained on the basis of gradual selection and subsequent increase of the substrain specific for resistant plants. Failure to infect all resistant plants initially with the original isolates was attributable to the apparently low amount of the substrain present in the inoculum. Other TMV-resistant breeding lines, with different sources of resistance, also selected and allowed only this substrain to multiply. The evidence strongly suggests that the same mechanism of selection was operative in these plants.

The parent and substrain of YA were not physically separable by sucrose or cesium chloride density gradients, nor biologically by local lesion symptomatology on several hosts. This research indicates that although the biological differences between the two viruses could be distinguished, their physical differences might be so slight as not to be detectable by the methods used.

Further attempts to differentiate between the parent strain and its substrain were limited to their systemic effects on susceptible tomato. The dominance of the parent strain in susceptible hosts appeared correlated with its faster rate of multiplication, movement and invasiveness. This increased pathogenicity was most evident in the severe stunting and occasional death of susceptible tomato.

Attempts to maintain the parent strain of YA in pure culture were unsuccessful in that none of the hosts tried selectively multiplied only the parent strain. This might possibly explain the occurrence of both parent and substrain in the original population.

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By

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INTRODUCTION

Replication of viruses almost invariably results in perfect copies. Under certain conditions, however, occasional variants which differ slightly but significantly from their parents may be produced. Recognition that variation exists within virus populations had its foundation in the work of Pasteur. By reinoculating a series of rabbits with the rabies agent, he observed decreased virulence of the virus for its original host, the dog (Pasteur et al., 1884). The resulting attenuated virus persisted and was designated as "fixed" or stable by Pasteur. Since then, many other animal viruses have been found which show similar biological variation. The concept that plant viruses may also vary was not established until some forty years after Pasteur's experiments. Since that time, numerous strains of plant viruses as well as mutants or variants of strains have been identified.

The plant virus resulting from a heritable change has been called a mutant or a variant, or more commonly a strain. The term "mutant" was used when a specific genetic relationship between parent and offspring is intended. "Strain" and "variant" are used herein in a more general sense. Both have been used separately or interchangeably

depending on the author. Strain implies close relationship to a particular group of plant viruses as determined by immunological and physicochemical tests. For example, Holmes' ribgrass virus is a strain of tobacco mosaic virus and thus belongs to the TMV group. Variant is a further subdivision of a strain and is synonymous with substrain. Variants of the ribgrass strain exist, but the similarities between them are more striking than their slight differences.

Customarily, viruses are transferred by inoculation from one host plant to another. Such transfers are commonly referred to as passages. The symptoms produced on the newly inoculated host are remarkably constant from one passage to another. As the virus particles multiply in the host cells, most of these particles are thought to be copies of their progenitors. Occasionally particles unlike their parents must appear. Certain conditions such as a different or unusual host or high temperatures may induce mutations as indicated by different symptoms.

This investigation was undertaken: (1) to determine the effect of different cultivars and lines of Lycopersicon esculentum Mill. on the selection of variants within distinctive strains of TMV and (2) to attempt differentiation of these variants by biological and physical means.

LITERATURE REVIEW

The first reported indication of change in virulence in plant viruses came from the work of Carsner and Stahl (1924, 1924a) and Carsner (1925). They found that passage of the sugar beet curly-top virus through a resistant plant, goosefoot (Chenopodium murale L.), resulted in the appearance of a mild strain of the virus as revealed upon transfer to healthy beets or other susceptible plants. Carsner and Lackey (1928) found that neither continued propagation in the original host nor repeated passage of the attenuated virus through a series of susceptible plants would restore its virulence. In later work (Lackey, 1929, 1929a) it was found that other resistant hosts, including resistant beets, would also attenuate the virus and that C. murale acted as a symptomless carrier. Lackey (1931, 1932, 1937) reported that the attenuated strain could be restored to approximately its original virulence by passage through chickweed, Stellaria media (L.) Cyrillo and several other susceptible hosts. All of these workers used the word attenuation to imply a lessening of the virulence or infective power and the failure to develop severe symptoms.

Giddings (1938) reexamined the question of sugar beet curly-top attenuation. He distinguished four different strains by their pathogenicity on different host plants. Later, he isolated six other strains (1944) and found that rate of spread in sugar beet fields depended on the strain of virus present (1946). Giddings finally concluded that attenuation was actually separation of virus strains and that the reported restoration of virulence was simply a reappearance of the more virulent strain in a dominant form after being latent in the infected plants. He contended that "the word attenuation should be used to denote a lessening of virulence by change and not a separation." In other papers (Giddings, 1940, 1941, 1950) he reported that none of the curly-top virus strains would immunize against other strains of the virus. In fact, one plant may be infected by at least three strains and that by proper choice of the host plants, separation of these strains could be accomplished. It was also found that when plants were inoculated with a virus mixture, they might develop symptoms of the less virulent strain, retain these symptoms for 2 or 3 weeks, and then develop severe symptoms characteristic of the more virulent strain.

Variation within TMV.--The majority of the work on plant virus strains and variants has centered on TMV since strains of this virus are easy to isolate, purify, and

transmit. Over 400 have been isolated or reported as occurring in nature (Kunkel, 1947).

McKinney (1926) first demonstrated that a large number of variants which caused yellowing and necrosis could be isolated from small, bright yellow spots that occasionally appeared on leaves of tobacco (Nicotiana tabacum L.) plants inoculated with ordinary TMV. He suspected that they might be due to contaminations. However, when he obtained 17 different isolates of TMV from various sources and again found that they gave yellow spots after inoculation to tobacco, he modified his view (McKinney, 1929). He suggested that "viruses may become altered locally in the plant, thus producing mutations."

Jensen (1933, 1936, 1937) greatly extended McKinney's study by isolation of yellow mosaic variants from plants infected with TMV. His purpose was threefold: to determine if the variant associated with the bright yellow spots was present in the original inoculum; occurred as the result of contamination; or arose in tissues invaded by the disease. By using the local lesion method of isolation (Holmes, 1929) and needle-puncture transfers (Jensen, 1936) he obtained 51 isolates which caused yellow mosaic. On the basis of variations in symptoms, rate of movement, and infectivity, he concluded that many, if not all, of these isolates were different from each other. He suggested that the yellow mosaic variants arose during the course of

multiplication of "green" TMV. In later work it was found that variants can also mutate, giving rise to submutants which show even wider variations, to the extent of causing necrosis of certain hosts (Jensen, 1936; McKinney, 1937; Norval, 1938).

Frequency of variation.--Although the existence of variants of different virus strains was recognized for some time, not much had been learned regarding the origin, nature, and frequency with which variants arise. Kunkel (1940) studied the frequency with which variants originate by "plating out" the sap from a TMV-infected tobacco plant onto leaves of N. glutinosa L. and then subinoculating from individual lesions to healthy tobacco plants. The term "plating out," which originated with Koch's method for counting bacteria, was used by early plant virologists who noted the similarity of local lesions on leaves to the production of bacterial colonies on agar plates. Kunkel found that about one lesion in 200 contained a variant if the tobacco plant had been diseased for a short time. The frequency was increased to one in 100 or one in 50 if the tobacco plant had been diseased for several months. Kunkel isolated 130 different variants based on their symptom expression on tobacco.

Kunkel also attempted to evaluate the differences in mutation rates of four strains of TMV by observing the

frequency of occurrence of yellow spots in mosaic-diseased leaves of Turkish tobacco. The four strains used were ordinary TMV, and variants of aucuba mosaic virus, Holmes' masked strain of TMV, and a masked strain of TMV obtained from petunia plants. The variants were acquired by separately passing each of the three strains in series through tobacco plants until it was replaced by a variant causing green mottling. It was found that the aucuba mosaic variant produced more yellow types than the other viruses, but all were capable of producing some.

Methods of obtaining variants of TMV.--Strains and variants of TMV have been obtained from segregated strains occurring in nature; by isolation from distinctive spots; by passage through hosts of different genera or through hosts with some resistance for TMV; and by holding diseased plants at high temperatures to induce mutation. My research involves only the latter two methods.

(a) Exposure to elevated temperatures.--Several workers have been successful in obtaining variants by holding infected plants at higher-than-normal temperatures, but below inactivating temperatures (Johnson, 1926, 1947; Holmes, 1934; Kunkel, 1934; Kassanis, 1957). In general, these workers found that when plants infected with the type strains of TMV are kept at about 36 C, variants that are

less virulent toward tobacco arise. Kunkel (1934) studied the effect of higher temperatures on yellow aucuba mosaic virus (YA). He found that when plants of N. sylvestris Spegaz. and Comes infected with YA were held at 35 C for 3 days, three very distinct variants of this virus could be recovered. At normal temperatures, YA produced primary necrotic lesions on N. sylvestris, but at elevated temperatures a systemic mottle resulted. These three variants were differentiated on the basis of the symptoms they produced on tobacco and tomato, and the appearance of local lesions or systemic mottle when reinoculated to N. sylvestris. The virus used in Kunkel's work should not be confused with yellow tomato aucuba mosaic virus (Y-TAMV) which was discovered by Miller in 1953. Kassanis (1957) used Y-TAMV in temperature studies and found it did not produce variants at high temperatures.

(b) Passage in different host plants.--Although virus strains are usually characterized by possession of a common host range, a strain which flourishes in one host may do poorly in another. Hence, if a given strain is cultured in a host in which it multiplies poorly, a variant or substrain which does better may eventually become dominant. One passage, or more often, several passages may be required to establish a substrain by this technique. Either the substrain was already present in small amounts

in the inoculum, or it arose by mutation of the first strain during multiplication in the different host.

McKinney (1943) found that the ordinary strain of TMV had greater invasive power in tobacco plants than any of its mutants. Thus, one would expect the parent strain to occur more frequently in tobacco than any substrain, since invasiveness of a virus provides it with a higher survival value. Valleau (1935), however, obtained mild substrains of TMV by passing the ordinary strain through N. tabacum cv. Ambalema, a resistant variety of tobacco. The mild substrains multiplied faster and were more invasive than the parent strain in this host. Johnson (1947) reported that bulk cultures (crude juice extract) of TMV were less virulent toward tobacco after passage through sea-holly (Eryngium aquaticum L.). By using single lesion isolates he showed that bulk cultures of TMV contained virulent and avirulent variants, and that the virulent one multiplied only locally in sea-holly. In contrast, the attenuated or mild variant moved systemically in the plant. Johnson found that in tomato the severe substrain predominated over the mild or attenuated strain.

Lister and Thresh (1955) isolated a virus causing a systemic disease in cowpea (Vigna ungiculata (L.) Walp.) in Nigeria which also produced a mosaic disease in tobacco similar to that due to TMV. The cowpea mosaic virus also caused systemic mosaic in many varieties of French bean

(Phaseolus vulgaris L.), a disease which was not previously associated with any known strain of TMV. Bawden (1956, 1958) confirmed and extended these observations with another virus. Suspecting that other reported diseases of leguminous plants might also be caused by strains of TMV, Bawden infected Prince bean and tobacco plants with a mosaic virus isolated from sunn hemp (Crotalaria juncea L.) (Capoor, 1950). The virus isolated from tobacco closely resembled type TMV. When isolated from bean however, its properties were similar to those of the cowpea virus from bean. Thus, an analogous situation seems to exist for the cowpea and sunn hemp strains of TMV. Briefly the findings of Bawden may be summarized as follows. When isolated from systemically-infected tobacco, the two strains closely resembled type TMV and shared many antigens with it. In systemically-infected French bean, they differed from TMV as much as any previously described strains. The different forms of the viruses shared few antigens, had different amino acid compositions, electrophoretic behavior and resistance to inactivation by ultraviolet radiation, and produced different kinds of local lesions in N. glutinosa. Since Johnson (1947) had shown that bulk cultures of a virus could change as a result of passage through a new host, Bawden's findings could be explained on this basis. However, when Bawden used single lesion isolates to infect plants, they behaved like the original bulk cultures.

Since these changes were reversible, it seemed unlikely to him that they occurred simply because different hosts selected different strains from existing mixtures. Although the "purity" of any culture is always questionable, Bawden felt it was unlikely that the solanaceous form of the cowpea virus was carried as such through local lesions produced by inoculations with the leguminous form. He concluded that the "two forms could change from one to the other by events equivalent to reversible mutations."

In recent years, several workers have shown that tomato plants carrying different genes for resistance to TMV are important in the identification and selection of strains and variants of TMV. The first reported case of the use of tomato lines for identification purposes was by McRitchie (1957). Using a tomato line, CStMW-18 developed by Walter (1956), McRitchie classified ten strains of TMV into three groups depending on the susceptibility, tolerance, or immunity of this line. The type of resistance in Walter's line has been described as non-symptomatic tolerance to TMV and was acquired from L. hirsutum Humb. and Bonpl., P.I. 126445. Later, McRitchie and Alexander (1963) differentiated by host-specificity on certain lycopersicon hosts, four strains of TMV which could not be distinguished by symptomatology on common varieties of tomato. Smith (1961) found a strain of TMV which could overcome the resistance in line H.E.S. 2603 (Tm_2 resistance, Clayberg

et al., 1960), but not that of Solanum pennellii, which is closely related to the genus Lycopersicon (Correll, 1958).

Several workers have used tolerant lines of tomato to select out an "adapted" type of TMV. Pecaut (1961) recovered a new strain from the tolerant Hawaiian line H.E.S. 5639-15. He found that when this strain was obtained from the tolerant line and again passaged through this line, it produced severe symptoms in these plants as well as in one of Walter's lines. This new strain also produced severe symptoms in Tm_1 plants (gene for resistance found in P.I. 235673, Clayberg et al., 1960), but not in Tm_2 plants, although there was limited multiplication in the tissues (Pecaut and Laterrot, 1963). Messiaen and Maison (1962) obtained similar results when they used another strain of TMV, aucuba mosaic virus. When this strain was reisolated from the tolerant line H.E.S. 5639-15 and used to inoculate a tolerant hybrid H.E.S. 5639-15 X Marmande, it produced much stronger symptoms and infected a greater percentage of the plants than in the first inoculation.

Dawson (1965, 1967) used a tomato mosaic virus isolate of greenhouse origin to study its multiplication in susceptible and resistant tomato plants. The susceptible variety used was Ailsa Craig and the resistant lines, with different types of resistance, were either CStMW-18 (Walter, 1956) or P.I. 235673 (Holmes, 1954, 1957). Dawson

found that when the virus was cultured in either of the resistant tomato lines, it could invade healthy plants of either line more readily than when the virus was grown in the susceptible variety. The "standard" form readily infected susceptible plants but infected resistant plants only with difficulty. He concluded that the resistant lines were able to select "adapted" forms of tomato mosaic virus. Dawson (1967) reported that the subsequent passage of the virus from resistant plants through susceptible plants did not cause the virus to revert to the "standard" form. When the "standard" and "adapted" forms of the virus were tested in six different species of plants, no differences in their biological activities were found.

Range of variability of TMV.--As noted before, the kinds of change most often reported for variants of viruses are pathogenicity and host range. The behavior of mutant strains from different sources shows that some characters may change while others do not. Holmes (1936) found that symptomatology of variants other than yellow mottling in tobacco depend on the origin of the variants. Those derived from an invasive line of TMV (distorting strain) had the ability to become systemic in tobacco, whereas those derived from a slow invader (masked strain) did not. The ability of a strain to cause yellowing in tobacco plants was independent of its ability to invade the plant.

Kunkel (1947) listed some of the factors in TMV mutants that vary independently of one another. These are: (1) green mottling in tobacco and localizing in N. sylvestris, (2) yellowing and invasiveness, (3) necrosis, localizing, yellowing and distorting, and (4) green mottling and the rate of production of bright yellow spots in tobacco. In contrast, the following pairs of characteristics appear to be linked: (1) rate of movement and rate of multiplication, and (2) infectivity and ability to move out of inoculated leaves. Holmes (1952) also found a linkage between infectivity and ability to spread systemically.

Physical and chemical properties of TMV strains.--Most physical studies have been made with distinctive strains of TMV which show wide differences in biological activities. Differences in electrophoretic mobilities have been found in the following cases: bean and tobacco strains of TMV (Bawden, 1958); U-1 and U-2 strains of TMV (Singer et al., 1951; Siegel and Wildman, 1954; Cohen et al., 1957; Streeter and Gordon, 1966); eight strains of TMV were classified into four groups on the basis of their mobilities (Siegel and Wildman, 1954). The property of buoyant densities has been used to distinguish U-1 and U-2 of TMV (Siegel and Hudson, 1959).

Publications dealing with the chemical composition of TMV strains are numerous. Two pertinent ones have been

selected for discussion. Rees and Short (1965) studied the amino acid composition of the tobacco and bean forms of the cowpea and sunn hemp mosaic viruses. When isolated from tobacco, the two strains had amino acid analyses that closely resembled the type strain of TMV. They showed approximately 23 amino acid replacements from their bean forms. They suggested that the changes in the amino acid composition when the cowpea and sunn hemp mosaic viruses were transferred from leguminous to solanaceous plants could be due to a reversible transformation or might reflect the selection of different variants in a new host.

Wang and Knight (1967) compared the protein components of 13 strains of tomato mosaic virus obtained from field-grown tomato. They found a close relationship among the strains in amino acid composition, C terminal amino acid, and general amino acid sequence. They concluded that the nature of the protein coat of a strain had little to do with its ability to infect tomato or any other host. It would seem more likely that natural selection of strains might be decided on the basis of some fundamental feature or structure of the viral nucleic acid rather than the protein coat.

MATERIALS AND METHODS

TMV strains.--Strains of TMV used were all received from various investigators: yellow aucuba mosaic (YA), from the Virus Laboratory at the University of California, Berkeley; a field isolate of TMV (S-IV) found in Ohio, from L. J. Alexander of the Ohio Agricultural Experiment Station at Wooster; a tomato internal browning isolate (JSB-1) from J. S. Boyle of Pennsylvania State University; and a rib-grass strain of TMV (HRG) from F. O. Holmes formerly of the Rockefeller Institute. All of these strains were usually maintained on White Burley tobacco but occasionally they were also maintained on Bonny Best tomato.

Description of tomato hosts.--In addition to the susceptible cultivar Bonny Best, a tomato breeding line used extensively in this study was N32, a derivative of P.I. 235673 which was developed by Holmes (1957) and described as a pure-breeding line carrying a single dominant gene for resistance to TMV. An early attempt by Holmes in breeding for resistance to TMV proved unsuccessful (Holmes, 1939). At that time he used L. chilense Dun. in a cross with L. esculentum, and found it to be characterized by a

tendency to escape infection (Holmes, 1943). However, he later found that the material derived from this cross was highly sterile and he was unable to determine the genetic nature of the resistance (Holmes, 1952). Later, Holmes (1954) obtained seeds of tomato lines showing resistance to TMV from W. A. Frazier of the Hawaiian Agricultural Experiment Station. These lines were described by Frazier and Dennett (1949) and Kikuta and Frazier (1947). Holmes found that these new lines proved to have a level of resistance to infection similar to that previously studied in derivatives of L. chilense. Knowledge of the original parentage for the Hawaiian lines is incomplete. It is believed to involve complex hybrids of L. chilense, L. hirsutum, L. peruvianum (L.) Mill., and L. pimpinellifolium Mill. Holmes (1954) states that "it may or may not be possible eventually to determine which of these species contributed the gene for resistance." Additional work (Holmes, 1957) culminated in the release of P.I. 235673. N32 is a result of a cross between P.I. 235673 and Spartan Red 8 which has desirable horticultural characteristics but is susceptible to TMV (Honma et al., 1961). Since N32 is a derivative of P.I. 235673 and in keeping with Holmes' description of its resistance, N32 will hereafter be referred to as TMV-resistant. Other tomato lines with different sources of resistance to TMV were also studied, but they are described under experimental results.

Culture and maintenance of plants.--Usually plants were grown in a greenhouse maintained near 22 C except during summer months when daytime temperatures sometimes reached 28 C. When the effects of higher temperatures were studied, plants were grown either in a house maintained continually at 28 C or in a chamber set at 35-36 C. Natural daylight was supplemented with fluorescent lights with an intensity of 450-700 ft c for a 16 hour day. Tomato and tobacco seeds were germinated in glass-covered clay saucers filled with either vermiculite or with a 1:1 mixture of steamed soil and vermiculite. Following germination, individual seedlings were transplanted into sterilized 4 inch clay pots filled with steamed soil. When a virus isolate was used to screen large numbers of different tomato lines, seedlings were grown in wooden flats containing steamed soil. In some cases assays were made on Scotia bean plants grown in 5 inch pots using a 1:1 mixture of steamed soil and vermiculite. After seeds germinated, the seedlings were thinned to five uniform plants per pot.

All plants were fertilized once a week with a commercial nutrient solution having a composition of 12, 31, and 14 percent N, P, and K, respectively. Insecticides were used periodically to control insects.

Preparation of inocula.--Various types of inocula were prepared and used. Crude sap was prepared with a mortar and pestle by macerating leaf tissue in a small

amount of 0.1 M, pH 7 potassium phosphate buffer. To obtain clarified plant sap, frozen tissue was homogenized in buffer (1:10, w/v) and the resulting slurry passed through four thicknesses of cheesecloth and then centrifuged at 12,000 g for 15 minutes. The supernatant was used as inoculum. Serial dilutions were made with buffer. Partially purified inoculum was obtained by sucrose density gradient centrifugation following the methods of Brakke (1953) and Corbett (1961). Briefly, the following procedure was used. A known weight of frozen tissue was macerated with buffer (0.01 M KCL, 0.005 M K2HPO4 and 0.005 M KH2PO4, pH 7.7) on a 1:2 w/v basis. Three ml of a clarified plant sap suspension was layered on top of three gradient columns which were prepared a day in advance and stored at 5 C. Gradients were prepared with sucrose dissolved in distilled water at the rate of 10, 20, 30 and 40 g per 100 ml; the respective solutions were layered (4, 7, 7 and 7 ml) in cellulose nitrate centrifuge tubes. Gradients were centrifuged for 1.5 hours at 23,000 rpm in the SW 25.1 rotor of a Spinco model L ultracentrifuge. After centrifugation the tubes were viewed in a darkened room with a concentrated beam of light placed directly over the tube. The visible zones were removed by puncturing the side of the tube with a syringe or by drawing off with a small pipette. Sucrose was removed by dialysis overnight against tap water and phosphate buffer. Virus concentrations were determined in

a Beckman-DB spectrophotometer following the method described by Takahashi (1951). Suitable dilutions were made with 0.1 M neutral buffer. Highly purified virus samples were prepared by the common methods of alternate low (10,000 g) and high (93,000 g) speed centrifugation. A chloroform-butanol mixture was used in the preliminary steps of purification (Steere, 1956). This aided in preparing a cleaner sample than that obtained by differential centrifugation alone.

Method of inoculation.--Bonny Best and N32 tomato seedlings were suitable for transplanting 10 to 14 days after sowing. Since physiologic condition of the seedlings was an important factor in the ease of infection and the subsequent development of symptoms, vigorously growing seedlings of uniform size were selected. Seedlings were inoculated 3-4 days after transplanting when they had recovered from the shock of transplanting and after the cotyledons had expanded fully. At this stage the first true leaves were just beginning to unfold.

Several inoculation methods were tried, but the following procedure, an adaption of the method of Webb and Porte (1962) gave consistently good results. The cotyledons were lightly dusted with 400 mesh Carborundum and a drop of inoculum was placed on each cotyledon with a small pipette. Using a glass slide as support, the

individual cotyledons were then rubbed several times with a small glass spatula. In most cases the stems were also inoculated by collecting a drop of inoculum on the spatula and gently rubbing the spatula up and down the stem several times. When older tomato plants were used, the same inoculation procedure was followed; however, the individual leaflets of 2 or 3 leaves nearest the growing point were inoculated. In order to minimize the amount of wilting and to insure quick recovery following the inoculations, plants were placed under greenhouse benches and sprayed with a fine mist of water. The following day they were placed in temperature controlled rooms maintained at either 22 or 28 C.

Method of assay.--The two species of plants used for most local lesion assays were N. tabacum cv. Xanthi-n.c. (Takahashi, 1956) and Scotia bean (Thornberry, 1935). For most routine assays Xanthi plants were used since they can be maintained in a rapidly growing state for a period of several weeks, and thus would be readily available. Two to 3 days prior to inoculation, the plants were topped to allow full expansion of the uppermost leaves. In most cases crude sap was used as the inoculum. Plants were first dusted with Carborundum, and then as a leaf was supported with a paper backing, the inoculum was applied with a 1 inch square polyurethane foam pad. Shortly after the inoculations were completed, the leaves were rinsed with

tap water. Local lesions were visible in 2-3 days and counts usually made 4-5 days after inoculation. This procedure was also followed to obtain single lesion isolates of TMV.

When Scotia bean plants were used, 10 or 11 day old plants with well expanded primary leaves and with the trifoliate leaves still folded were the most susceptible. The apical growing points were removed a few days before inoculating so as to increase the size and the sensitivity of the primary leaves. Thus, four nearly identical half leaves were available for inoculation and comparison on each plant. The half leaf method of Spencer and Price (1943) was followed so that treatments and controls appeared on two half leaves per plant. The order of treatments was reversed on each of the two leaves. Inoculum consisted of clarified plant sap properly diluted with buffer. Bean leaves dusted with Carborundum were supported with polyurethane foam pads. A drop of inoculum was placed on each half leaf, and was applied over the leaf surface with a glass spatula. Lesions were suitable for counting 4-5 days after inoculation. The pads and glass spatulas were washed and steam sterilized between use.

EXPERIMENTAL RESULTS

Symptomatology of TMV strains.--Bulk cultures of the four strains of TMV, obtained from systemically infected White Burley tobacco or Bonny Best tomato plants readily produced symptoms upon inoculation to Bonny Best plants. Symptoms were apparent in less than 2 weeks on the new growth of plants maintained at 22 C. Strain YA produced distinctive bright yellow mosaic symptoms. The other strains produced a general green mottling. In addition, JSB-1 and S-IV caused extensive leaf deformity.

When similar cultures of the four strains were inoculated to N32 tomato, in most cases the plants failed to develop definite symptoms. Strain YA was an exception, since 7 weeks after inoculation, faint yellow spots were observed on the new growth. Although N32 plants appeared normal, virus was recovered when these plants were indexed on Xanthi tobacco. One-gram samples were harvested from N32 plants 3, 5, and 7 weeks after inoculation (Table 1). Similar samples were obtained from infected Bonny Best plants. In most cases virus titers in N32 reached a peak at 5 weeks and then decreased; however, with strain YA this occurred at 7 weeks, corresponding with the time of

symptom appearance. Virus titers in Bonny Best were 3 to 4 times greater, and since additional dilutions were necessary, their lesion counts were not directly comparable with those from N32.

Table 1.--Recovery of four strains of TMV from N32 tomato plants after different periods of incubation.

TMV strain	Average number of local lesions on six half leaves[a]		
	3 weeks	5 weeks	7 weeks
YA	1	5	92
JSB-1	1	25	1
S-IV	269	100	48
HRG	0	442	251

[a]Samples were diluted 1-10 with phosphate buffer for assay on Xanthi tobacco.

Continuous passage through Bonny Best or N32.--The sources of virus inoculum used were White Burley tobacco, Bonny Best and N32 tomato plants, with the latter two also serving as the test hosts. Strains YA and S-IV were used in most of these studies. Inoculum was prepared by sucrose density gradient centrifugation. This method yielded maximum amounts of relatively pure virus considering that only 1-3 gram samples of infected tissues were used. Virus concentrations were adjusted to 20-25 µg/ml to insure that equal amounts of virus were applied.

A virus was considered passaged once after the virus had been used to inoculate a plant, incubated and then later reisolated from this host. In this manner numerous passages of the viruses through Bonny Best or N32 were performed. Material was harvested 5 weeks after inoculation and kept frozen until it was prepared for the next passage. Plants were observed for symptoms for at least 7 weeks. Results of continuous passage of strain YA through the two test hosts are summarized in Table 2.

All Bonny Best plants developed symptoms in less than 2 weeks; however, the degree of infection depended on the source of inoculum. When the virus obtained from Bonny Best was used to inoculate healthy Bonny Best plants, severe stunting and considerable malformation of leaves resulted. Higher temperatures (28 C and above) greatly accelerated symptom development resulting in killing of young plants and causing necrosis of leaves and flowers on older plants. When N32 plants served as the source of inoculum, comparable Bonny Best plants were less severely infected, and although these symptoms were intensified at higher temperatures, the plants were never killed. Another difference between the virus from the two sources was the time required for symptoms to appear. Symptoms consistently appeared one or more days sooner when susceptible tomato served as both the source and test plants.

Table 2.--Effects of source, test host and continuous passage of TMV-YA on infectivity to tomato.

Source of virus	Test[a] host	Number inoculated	% with symptoms[b]	Incubation time (days)
1)[c] Tobacco	S	5	100	10
	R	10	0	59
Bonny Best				
1) S-S[d]	S	5	100	8
	R	15	0	54
2) S-S-S	S	5	100	8
	R	25	0	--[e]
3) S-S-S-S	S	5	100	6
	R	5	0	--
4) S-S-S-S-S	S	5	100	5
	R	5	0	--
N32				
1) S-R	S	10	100	12
	R	10	50	15
2) S-R-R	S	10	100	10
	R	40	90	12
3) S-R-R-R	S	5	100	6
	R	10	90	7
4) S-R-R-R-R	S	10	100	6
	R	10	100	6

[a]S = susceptible Bonny Best.

R = resistant N32.

[b]Symptoms in 3 weeks.

[c]Numbers indicate those inoculations made at the same time.

[d]Sequence of passages through tomato with the last indicated host serving as source of inoculum.

[e]No symptoms appeared during length of experiment.

The picture was different when the two sources of virus were tested on N32 tomato. Symptoms appeared on the resistant tomato when the source was White Burley or after two passages through Bonny Best, but only after many weeks. As these passages through Bonny Best were continued, the inoculum failed to produce symptoms when tested on N32. When symptomless plants were indexed on Xanthi, the results were seldom positive. In contrast, when N32 was the source host, symptoms were readily produced on a large percentage of the inoculated N32 plants and virus was easily recovered.

Since serial passages were conducted and assayed in succession, all plants (Table 2) could not be inoculated at the same time. Plants were grown in a temperature-regulated greenhouse, but during summer months it was impossible to maintain temperatures at 22 C when the later passages were made. Thus, the reduction in YA incubation period on the two hosts could reflect the effect of higher temperatures or an increase in virulence of the virus. Consequently, the same experiment was repeated the following spring so that all plants could be inoculated on the same day and the effect of two temperatures could be compared. Ten plants of both Bonny Best and N32 were again inoculated with materials from the three sources which were the frozen purified samples used in the previous experiment. Concentration of TMV was adjusted to 25 µg/ml in all samples. Half of the plants after inoculation were held at 22 and the remainder at 28 C.

Similar results were obtained with respect to the effect of source of inoculum on the percentage of plants developing symptoms (Table 3). Since all inoculations were made on the same day, it is apparent that the previous reduction in time for symptom expression was undoubtedly due to the different temperature conditions that existed when the inoculations were made. However, the average incubation period in Bonny Best was still dependent on the source of the inoculum, since at either temperature, symptoms appeared 1 to 3 days sooner when Bonny Best was the source of the inoculum.

At the same time that the initial experiments with YA were conducted, similar passages were made using S-IV. Results of these experiments (Table 4) indicate that S-IV resembles YA with respect to the effect of passage through the two tomato hosts. Continuous passage of the virus through susceptible Bonny Best did not affect its ability to produce severe symptoms on this host, but did prevent symptoms from appearing on N32. Indexing revealed that 80% of the N32 plants had virus present when inoculated with material passaged two times through Bonny Best; however, with additional passages through the susceptible host, the virus was not recovered from N32. When N32 plants served as the source host, not all Bonny Best and N32 test plants developed symptoms in the initial passages. This may be due to the low amount of virus present in N32 in the early

Table 3.--Effects of source of virus and temperature on infectivity and incubation period of TMV-YA.

Source of virus	Test host[a]	Plants infected/incubation time			
		22 C.		28 C	
		%	/ days	%	/ days
Tobacco	S	100	7	100	7
	R	0	-[b]	20	18
Bonny Best					
S-S[c]	S	100	7	100	4
	R	0	-	20	29
S-S-S	S	80	6	100	4
	R	0	-	0	-
S-S-S-S	S	100	7	100	4
	R	0	-	0	-
S-S-S-S-S	S	100	5	100	4
	R	0	-	0	-
N32					
S-R	S	100	8	80	7
	R	40	9	80	6
S-R-R	S	100	7	100	6
	R	100	8	100	6
S-R-R-R	S	60	7	80	5
	R	80	10	80	7
S-R-R-R-R	S	60	7	100	5
	R	80	10	80	6

[a]S = susceptible Bonny Best.
R = resistant N32.

[b]No symptoms appeared during length of experiment.

[c]Sequence of passages through tomato with the last indicated host serving as source of inoculum.

Table 4.--Effects of source, test host, and continuous passage of TMV strain S-IV on infectivity to tomato.

Source of virus	Test host[a]	Number inoculated	% with symptoms[b]	Incubation time (days)
1)[c] Tobacco	S	5	100	8
	R	10	0	59
Bonny Best				
1) S-S[d]	S	10	100	12
	R	15	0	59
2) S-S-S	S	5	100	10
	R	10	0	--[e]
3) S-S-S-S	S	5	100	7
	R	5	0	--
N32				
1) S-R	S	10	20	21
	R	10	40	21
2) S-R-R	S	10	80	13
	R	10	70	13
3) S-R-R-R	S	5	100	9
	R	10	100	8

[a]S = susceptible Bonny Best.
R = resistant N32.

[b]Symptoms in 3 weeks.

[c]Numbers indicate those inoculations made at the same time.

[d]Sequence of passages through tomato with the last indicated host serving as source of inoculum.

[e]No symptoms appeared during length of experiment.

stages. However, all plants developed mild symptoms when inoculated with virus passaged three times through N32. The reduction in incubation time may again be attributed to the different growing conditions when the inoculations were made.

Virus strain JSB-1 was more difficult to separate by host plant inoculations. After five passages through Bonny Best, single lesion isolates of JSB-1 produced symptoms on Bonny Best plants but not on the resistant line. Indexing confirmed that no virus was present in N32. In contrast, when the same virus was passaged four times through N32, single lesion isolates produced symptons on both resistant and susceptible plants.

Similar attempts to separate strain HRG into two types of progeny on the basis of source were unsuccessful. After several passages through either Bonny Best or N32 the virus still behaved as did the original isolate. Both the original and the derived virus were recovered from symptomless N32 plants. Thus, this isolate would appear to behave more like the viruses recovered from the resistant source plants.

Results from the continuous passage experiments with YA, S-IV, and JSB-1 suggested that resistant N32 plants might be able to select a specific substrain or variant from the original population. At the time it was not possible to conclude whether both types of progeny were variants or

substrains of the original virus, or if they should be considered as the parent strain and one substrain. In order to distinguish between the two possibilities, further experiments were made. The virus which predominated in Bonny Best after repeated passages was designated "variant A," whereas the virus which was specific for N32 was called "variant B."

Composition of virus populations as determined by single lesion isolates.--Strain YA was selected for additional studies because of its ease of separation into "variants," its relatively fast multiplication rate in N32, and its characteristic symptoms which permitted easy detection without indexing. Since high temperatures were previously found to increase the speed and severity of symptom development as well as the rate of virus multiplication, most of the following experiments were performed in a greenhouse maintained near 28 C.

Bulk cultures of the virus from three sources were used in previous experiments. Results suggested that the two tomato cultivars were capable of selecting specific "variants" from the original population because each host favored one variant over another. However, the possibility existed that one of the hosts selected a variant which arose naturally in that host or that there was a host-induced mutation. In order to answer some of these questions, the composition of virus populations from three sources was

analyzed by the use of single lesion isolates. Xanthi tobacco plants were inoculated with virus from the original source on White Burley tobacco and with the same virus after it had been passaged four times through either Bonny Best or N32 tomato. Local lesions were subinoculated to tomato seedlings two days after they appeared on Xanthi plants. A single local lesion was cut out, macerated in buffer and used to inoculate both a Bonny Best and an N32 seedling as previously described. In several experiments, this inoculum was further tested on Xanthi leaves, as a check on the viability of such local lesions. Since both "variants" of YA produced symptoms on Bonny Best, "variant B" could only be identified when a local lesion isolate evoked symptoms on N32. Data were kept on the size and general appearance of the local lesions selected to determine its effect on the results obtained.

"Variant A" predominated in the original source as well as in Bonny Best when YA was continually passaged through this host (Table 5). The fact that "variant B" was recovered from these two sources indicated that this variant was inherently present, but in very small amounts. When the virus was passaged through N32, "variant B" was obviously favored, since local lesions from this source produced symptoms on a large percentage of N32 plants. Failure to infect all Bonny Best plants and not all of the N32 seedlings when inoculated with local lesion isolates

from the resistant source was probably due to inoculation techniques. In cases where infectivity of local lesion isolates was checked on Xanthi, all were infectious in varying degrees. The limiting factor for this technique may be the small amount of extractable virus present in local lesions. No correlation was found between the size and appearance of local lesions selected and the results obtained on the test hosts. The findings that both the original and Bonny Best sources consisted of a mixture of the two viruses and that "variant A" predominated in these sources suggested a possible answer to the question of origin and terminology of the two forms of YA. The results support the hypothesis that "variant A" was the parent and that "variant B" was substrain B.

Table 5.--Composition of three sources of TMV-YA based on single lesion isolates.

Source of virus	Test host	Number inoculated[a]	% of plants with symptoms
White Burley tobacco	Bonny Best	100	72
	N32	100	2
Susceptible Bonny Best tomato	Bonny Best	50	100
	N32	50	6
Resistant N32 tomato	Bonny Best	50	84
	N32	50	82

[a]Number of local lesions tested on Bonny Best and N32 tomato plants.

Attempt to identify parent strain YA and its substrain by local lesion hosts.--In attempting to find a fast and simple means of distinguishing between parent and substrain, numerous local lesion hosts were inoculated, and the size and appearance of the resulting local lesions were compared. Crude sap from Bonny Best or N32 plants, infected respectively with single lesion isolates of the parent strain or with substrain B, was used to inoculate these plants. The viruses were compared on opposite halves of several leaves of the following hosts: N. sylvestris, N. rustica L., N. repanda Welld., N. glutinosa and Scotia bean. No differences between the two forms of YA were discernible in terms of appearance, size, or color of the local lesions produced.

N. sylvestris was also used to study the effect of high temperatures on symptom expression. Since TMV will become systemic when local lesion hosts are held at high temperatures, the parent and substrain were inoculated separately, and a day later were placed in a chamber maintained near 36 C. Both viruses produced local lesions on inoculated leaves before becoming systemic. Symptoms caused by the parent strain were very severe and plants were killed in 4 weeks, whereas a similar occurrence with substrain B required 6 weeks. Controls showed no symptoms. Thus, the difference in severity of the two viruses on N. sylvestris was similar to that with Bonny Best.

Reaction of additional tomato cultivars and breeding lines to the parent YA and its substrain.--Tomato cultivars as well as accessions of Plant Introductions and breeding lines with different sources of resistance to TMV were used to determine their response to parental YA and its substrain B. In most cases, tomato seedlings were transplanted into wooden flats and half the plants in each flat were inoculated with the parent strain while the other half received substrain B. Inoculum usually consisted of partially purified virus. Following inoculation, the plants were held at 28 C and observed for at least 7 weeks. Most of the breeding lines were previously tested by Murakishi and Honma (1963), and their code numbers will be incorporated here. Descriptions of breeding lines and introductions are summarized in Table 6.

Susceptible tomato cultivars had the same response as Bonny Best to virus strain YA and its substrain (Table 7). The parent strain caused severe stunting and malformation of plants, whereas substrain B caused less severe symptoms. Resistant lines and introductions all had the same general response described for N32 tomato; there was no infection by the parent strain, but usually 100% infection by substrain B except for P.I. 128650. This was true despite the fact that these breeding lines carried three different kinds of resistance to TMV. Test hosts P.I. 235673, WN32, N32, and Moto-red (Honma et al., 1968) have

Table 6.--Tomato breeding lines and introductions[a] used in this study.

Code[b] or accession number	Designation, description and developer of breeding lines
P.I. 235673	True breeding for TMV resistance (Holmes)
WN32	Derivative of Walter's STEP 305, Holmes' P.I. 235673, and Spartan Red 8 cross (Honma)
Moto-red	Released variety derived from N32 (Honma, Murakishi, and Wittwer)
CStMW-18-15	Single plant selection of a TMV resistant line (Walter)
W-1	STEP 305, advanced TMV resistant line (Walter)
W-2	7-4-3-1-CH-BK ACEMStW, combined resistance to TMV and tobacco etch viruses (Walter)
W-10	262-2-1-2 CAStMW, TMV resistant (Walter)
W-11	STEP 390, TMV resistant (Walter)
S-1	Single dominant gene for TMV resistance (Tm_2) (Soost)
P.I. 128650	L. peruvianum introduction, used as source of TMV resistance (Alexander)

[a]Supplied by North Central Regional Plant Introduction Station, Ames, Iowa.

[b]Code used in part by Murakishi and Honma (1963).

Table 7.--Reaction of several cultivars, breeding lines and introductions to virus strain YA and its substrain.

Test host	Parent strain	Substrain B
Susceptible cultivars		
Fireball	5/5[a]	5/5
Homestead-24	5/5	5/5
Rutgers	5/5	5/5
Resistant lines and introductions		
P.I. 235673	0/20	20/20
WN32	0/20	19/19
Moto-red	---[b]	60/60
CStMW-18-15	---	12/12
W-1	---	5/5
W-2	0/25	25/25
W-10	23/23	24/24
W-11	0/23	24/24
S-1	---	3/3
P.I. 128650	---	4/120

[a]Number of plants showing symptoms/number of plants inoculated.

[b]Not determined.

the same single dominant gene (Tm_1) for resistance. These plants had the same reaction to virus inoculation, as described for N32 tomato. Lines CStMW-18-15, W-1, W-2, W-10, and W-11 were developed by Walter and have three recessive genes for resistance. One hundred percent infection occurred when these lines were inoculated with substrain B. Line W-10 developed symptoms when inoculated with the parent strain and would appear to differ from the others. However, this line was previously found to be susceptible to another strain of TMV (Murakishi and Honma, 1963). Line S-1, containing a single dominant gene for resistance (Tm_2) was susceptible to substrain B, although only a limited number of plants were available for testing. P.I. 128650, an introduction used in breeding work by Alexander (1963), was resistant to substrain B, the only virus tested. The same virus parental strain preparation was used to test P.I. 235673, WN32, W-2, W-10, and W-11. The results indicated that all except W-10 were resistant to the parent strain (Table 7). Pure parent strain was not available for testing the remaining tomato lines. The difficulties of maintaining the virus parent strain in pure form will be discussed later.

Effect of virus strain YA and its substrain on growth of tomato at several temperatures.--Previous experiments had shown a distinct difference between the two forms of YA in incubation period, severity of symptoms and relationship to temperature. The effects on growth of tomato were compared

in further experiments. Bonny Best seedlings, inoculated separately with the parent virus and its substrain, and N32 tomato plants inoculated with substrain B were grown in greenhouses maintained at 22 and 28 C. Comparable control seedlings were inoculated with water rather than virus suspensions. The growth of plants was followed at weekly intervals for 7 weeks by arbitrarily measuring the distance between the cotyledonary node and the growing point in mm. After 7 weeks, plants were excised at the cotyledons and weighed.

Healthy N32 tomato plants were slightly larger at 22 than at 28 C (5% level) at the end of 7 weeks (Fig. 1). Substrain B-infected plants held at 22 C were reduced in height (5% level) when compared with the control. When infected plants were grown at 28 C the disease severity was more pronounced (1% level). After 7 weeks these diseased plants averaged 158 mm in height compared to 219 mm for the control. Symptoms appeared 3 days sooner on infected plants at 28 than at 22 C.

Healthy Bonny Best tomato plants also grew better at 22 than at 28 C (5% level)(Fig. 2). Plants infected with the parental and the substrain virus did not differ significantly in height at 22 C. Parental virus and its substrain caused symptoms 9 days after inoculation. At 28 C, Bonny Best plants infected with either the parent strain or its substrain were significantly reduced in height (5% level)

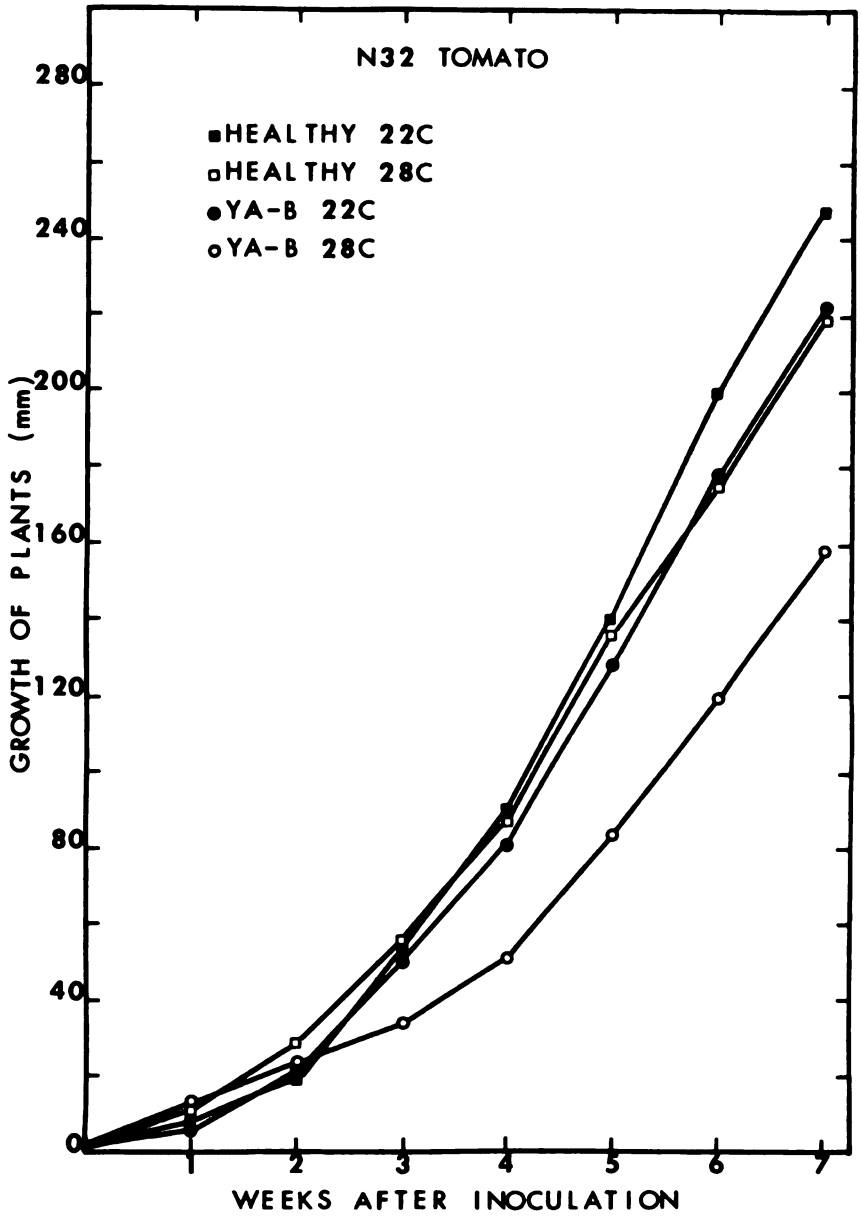

Figure 1.--Effect of temperature on growth of N32 tomato infected with virus substrain B. The parental strain (YA-A) is not infectious to N32 tomato; inoculated and non-inoculated plants made equal growth.

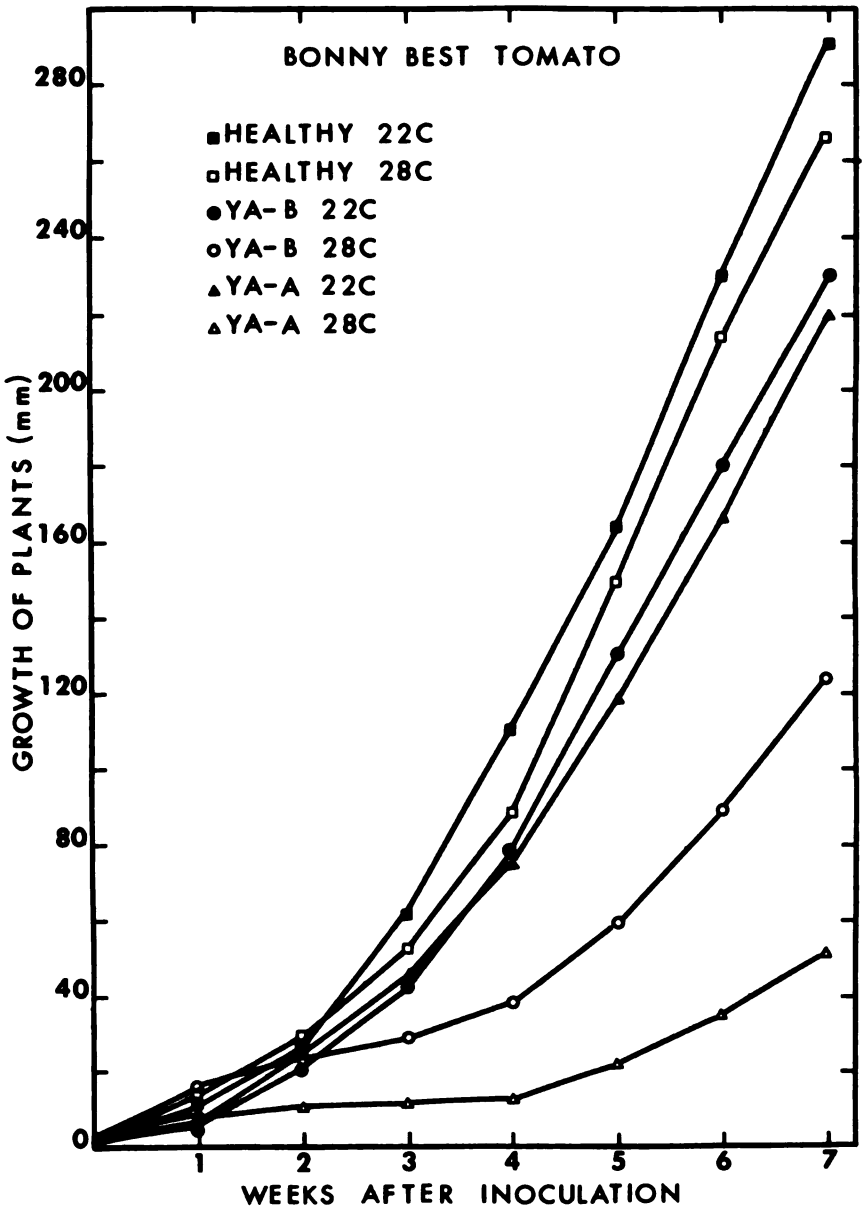

Figure 2.--Effect of temperature on growth of Bonny Best tomato infected with parental virus (YA-A) or substrain B (YA-B).

when compared with controls. In addition, the difference between the 2-fold reduction in growth caused by substrain B and the 5-fold reduction caused by the parent strain was also significant (5% level). At 28 C, substrain B caused symptoms in 4 days while the parental strain caused symptoms in 3 days. A further indication of the severity of the parent strain upon susceptible plants at 28 C was apparent when several plants were killed 2 weeks after inoculation. Further losses were prevented by placing the remaining plants at 22 C for several days before returning them to 28 C. The effects of the various temperature-virus combinations are shown in Figure 3.

The weight of all plants was reduced when compared with healthy controls grown at the same temperatures (Table 8). In addition, comparison of the weight of Bonny Best plants infected with either the parent or substrain and grown at the same temperature were also significantly different (5% level). Thus, through the use of different temperatures it was possible to differentiate virus strain YA from its substrain. There were differences in length of incubation period and in size and weight of infected plants.

Bioassay of parent and substrain of YA in tomato.--The relative concentration of virus was determined by infectivity assay of clarified juice from Bonny Best and N32 plants. This also served to give a measure of the effect of source of inoculum. Bonny Best and N32 seedlings

Figure 3.--Symptoms of the parent and substrain of YA on two tomato hosts as influenced by temperature. A,B) Control and infected Bonny Best plants held at 22 and 28 C, respectively; left to right: healthy, substrain B, parent strain. C) Control and infected N32 plants held at 22 and 28, respectively; left to right: healthy and substrain B at 22, healthy and substrain B at 28. Photograph was taken 7 weeks after inoculation.

Table 8.--Effects of temperature and infection with parent or substrain of TMV-YA on growth of tomato plants.

Tomato cv.	Temperature	Average[a] fresh weight in grams		
		Control	Parent	Substrain
N32	22	73	---[d]	66[b]
	28	68	---	44[b]
Bonny Best	22	71	47[b]	55[c]
	28	69	6.5[b]	25[b]

[a]Average of seven plants in most cases.

[b]Significant at 1% level.

[c]Significant at 5% level.

[d]Not determined.

were inoculated with virus from either the original source on tobacco (mixture of parent and substrain) or with virus passaged through susceptible Bonny Best five times (5S, mixture of parent and substrain) or through resistant N32 four times (4R, exclusively substrain B). All tomato plants were grown at 22 C. Pooled, one-gram samples from three plants of each series were harvested 3, 5, and 7 weeks after inoculation and the virus titer determined by bioassay on half leaves of Scotia bean as described. Control inoculum consisted of virus recovered from Bonny Best plants inoculated with virus from the tobacco source and harvested at the same times as the other samples.

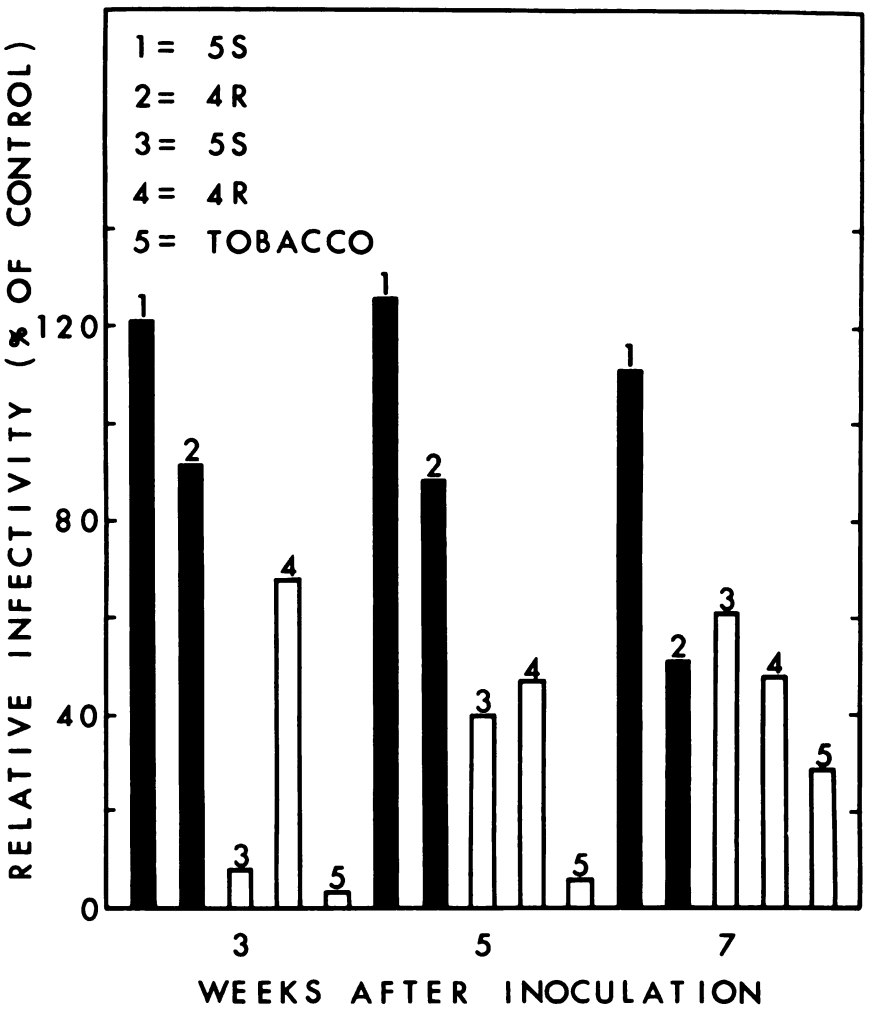

Figure 4.--Relative infectivity (local lesion assay on Scotia bean) of YA-infected Bonny Best ■ and N32 □ plants. Plants were inoculated with the following sources of virus: (A) five passages through Bonny Best (5S, columns 1 and 3); (B) four passages through N32 (4R, columns 2 and 4); and (C) passage through tobacco (tobacco source, column 5). Bonny Best plants infected with the tobacco source served as control.

Virus titers from Bonny Best plants infected with the 5S inoculum (column 1) were always greater than similar plants inoculated with the 4R inoculum (column 2)(Fig. 4). This was most pronounced for the 7 week harvest. In Bonny Best, the parent strain was able to multiply more readily than its substrain. It is possible that these differences would have been more striking if the plants had been grown at 28 C. The fact that the 5S inoculum was always more virulent than the control inoculum may indicate greater infectivity after passage through Bonny Best. Column 3 represents 5S inoculum tested in N32, and indicates the gradual selection and multiplication of substrain B. The titer of substrain B in N32 decreased as the plants matured (column 4), with only faint symptoms present at 7 weeks. Column 5 indicates N32 plants infected with virus from the original source, showing the gradual increase in substrain B. Samples which produced at least 45% the number of local lesions as did the control were found to be from plants showing virus symptoms at time of sampling.

Stability of YA viruses.--At the same time that continuous passage of strain YA was conducted, samples were available to test the effect of alternating the passage of the two forms of YA between susceptible and resistant tomato. Inoculum was prepared and plants were inoculated as described for the continuous passage experiments.

Alternating the virus passages between the two tomato hosts had no effect on the ability to infect Bonny Best, since 100% infection occurred in all cases (Table 9). This was expected because both the parent and substrain of YA are infectious to susceptible tomato. The fact that all of the virus samples produced symptoms on 50% or more of the N32 plants, indicated that once substrain B was increased in N32, further passages through Bonny Best had no apparent affect on the substrain. Continuous maintenance of substrain B in Bonny Best or White Burley tobacco similarly did not produce any apparent change in this virus. Crude sap obtained from these sources infected N32 as readily as substrain B isolated directly from the resistant source. The same results were obtained when isolates of substrain B were passaged through Bonny Best three times before testing in N32.

The stability of the parent strain in susceptible Bonny Best was also studied. Local lesion transfers of the parent strain were made 3 to 7 times through Xanthi tobacco plants before being subinoculated to Bonny Best and N32 seedlings. Following inoculations all plants were kept at 28 C. Susceptible plants developed symptoms in a week or less, while the resistant plants remained symptomless and yielded no virus upon indexing. To insure that the single lesion isolates were pure parent strain, the same N32 plants were reinoculated a second and a third time with crude sap

Table 9.--Effect of alternating the passage of TMV-VA through susceptible and resistant tomato on its infectivity to tomato seedlings.

Source of virus	Test host[a]	Number inoculated	% with symptoms[b]
Exp. 1[c]			
S-R-S[d]	S	5	100
	R	10	50
S-S-R	S	10	100
	R	10	80
Exp. 2			
S-R-S-R	S	5	100
	R	10	80
S-S-R-R	S	5	100
	R	10	90
S-S-R-S	S	5	100
	R	10	90
S-R-S-S	S	5	100
	R	10	90
S-R-R-S	S	5	100
	R	10	90

[a]S = susceptible Bonny Best.
R = resistant N32.

[b]Symptoms in 7 weeks.

[c]The two experiments were performed at different times.

[d]Sequence of passages through tomato with the last indicated host serving as source of inoculum.

from the corresponding systemically-infected Bonny Best plants. In addition, other N32 plants in different stages of development were also inoculated with crude sap from the same Bonny Best plants infected with the parent strain.

Results of three experiments in which 28 individual isolates of the parent strain were studied showed that out of 147 inoculations made to 102 N32 plants, 37 of the plants developed yellow mosaic symptoms. When virus from the latter was further tested in healthy N32 seedlings, all developed symptoms about the same time as control N32 plants inoculated with known substrain B. Results indicate that substrain B can be recovered from the Bonny Best plants at any time after 3 weeks (Table 10). However, if inoculum was from Bonny Best plants which had been inoculated less than 3 weeks previously with the parent strain, no symptoms developed on N32 test plants. If we assume that the original single lesion isolates were pure parent strain, then apparently the parent strain can mutate to substrain B while it is maintained in Bonny Best. These results further support the hypothesis that virus designated as substrain B seems to have originated from the parent strain.

Attempted differentiation of parent and substrain by physical means.--Discontinuous sucrose density gradients consisting of 50, 40, 30 and 20% sucrose concentrations were prepared as described. One ml aliquots of purified parent and substrain B of YA of equal concentrations were layered

Table 10.--Recovery of substrain B from Bonny Best plants originally inoculated with the parent strain of YA.

Age of source plant (weeks)	N32 test plants	
	Number inoculated	% with symptoms
0[a]	28	0
1	6	0
2	21	0
3	25	44
4	35	37
5	12	66
6	7	71

[a]Inoculum was from single lesions caused by the parent strain.

separately or in a mixture on top of three gradients. Gradients were centrifuged for 1.5 hours at 23,000 rpm in the SW 39L rotor of a Spinco model L ultracentrifuge. After centrifugation the bottoms of the tubes were punctured and 2 drop fractions were collected in tubes containing 1.5 ml of neutral phosphate buffer. Optical densities were determined spectrophotometrically. No differences were found in the location of the two viruses in the tubes.

Similar procedures were followed when cesium chloride gradients were used. A saturated solution of

cesium chloride was diluted with distilled water and 4 ml were added to each tube. One ml samples of the two viruses separately or in a mixture were layered on top. After centrifuging for 22 hours at 30,000 rpm in the SW 39L rotor, fractions were again collected. Location of the virus peaks in all 3 gradients were nearly identical.

DISCUSSION

Reviewers have mentioned the effects of passaging TMV through hosts of different genera or through resistant plants (Knight, 1959; Price, 1964). Only recently has the change in virulence within TMV populations been shown through the use of mosaic-resistant tomato lines (Pecaut, 1961; Messiaen and Maison, 1962; Pecaut and Laterrot, 1963; Dawson, 1965, 1967; Zitter and Murakishi, 1967). Recognition of the variability of TMV strains may also serve to explain some of the discrepancies associated with breeding for resistance to this disease (Pelham, 1966).

Results from this study indicate that variation of TMV strains affecting tomato may be quite common, since three of the four strains studied had the ability to produce two biologically distinct types of progeny. Only by passaging the original virus population through resistant tomato lines was it possible to show the heterogeneity of the original population. Collectively, the results suggest that TMV-resistant tomato lines are able to select a specific form of the virus mixture in susceptible plants. The delay in symptom expression on resistant lines inoculated with the original virus population can be explained

by the gradual selection and subsequent increase of the substrain specific for the resistant plants. Hooker and Benson (1960) reported that low potato virus X concentrations can delay the time of symptom appearance and a suggestion of a similar response was obtained at 28 C (Table 3) with inoculum of substrain B in a mixture with the parent strain. Failure to infect all resistant plants initially with the original TMV isolate appears to be due to the low amount of the substrain present in the inoculum. Once the titer of substrain B was sufficiently increased in N32, it was possible to alternate the passage of the virus between susceptible and resistant plants without affecting the ability of the virus to reinfect N32. Although the parent strain does not move systemically within resistant plants, it is not known if the virus can multiply in the inoculated leaf.

Although both types of progeny were considered originally as "variants" of a particular strain, later evidence suggested that "variant A" should probably be considered as the parent strain with "variant B" as one substrain. The basis for this interpretation is 1) no other substrains were detected, 2) progeny designated as A always predominated in a mixture in susceptible hosts, and 3) "variant A" apparently can mutate to form substrain B during the course of multiplication in Bonny Best. Although the evidence suggests that this classification is

correct, one should not exclude the possibility that substrain B may be the parent virus and that what is considered as the parent strain could be the substrain. This is possible if we consider that the less virulent virus (substrain B) would have greater survival value.

The three strains which produce substrains were first isolated in nature from tomato: YA (Smith, 1928); JSB-1 (Boyle and Wharton, 1957); and S-IV (McRitchie and Alexander, 1963). The substrains of both YA and S-IV were easily separated from their parent strains but a similar occurrence with strain JSB-1 required many passages through N32. The fourth strain, HRG, was uniform in its response for Bonny Best and N32 tomato. This strain was originally isolated from ribgrass (Plantago lanceolate L.) and although substrains were discovered, these were not detectable on tomato (Holmes, 1941). Later studies showed that of the several strains which could be isolated from Plantago species, none produced mosaic symptoms on tomato lines whose resistance now appears in P.I. 235673 (Holmes, 1950). Similar results were found here since N32, whose source of resistance was derived from P.I. 235673, exhibited non-symptomatic tolerance to strain HRG.

Although other workers have observed the role that a source of inoculum from resistant plants can have on subsequent infection of resistant lines, this effect has been interpreted in different ways. Pecaut (1961) considered

that symptoms which developed on an old plant of line H.E.S. 5639-15 were due to the recovery of a new strain of TMV from the original isolate. Although it is possible that the original strain was contaminated with this new strain, it is conceivable that line H.E.S. 5639-15 selected a substrain of the original virus. Both Messiaen and Maison (1962) and Dawson (1967) referred to that portion of the virus population which could readily infect, multiply and produce symptoms on resistant tomato lines as an "adapted" form of the original isolate. Dawson considered that the "adapted" form arose as a result of "change" in the virus population during the course of multiplication in resistant plants. He reported that when the "standard" form of the virus was obtained from susceptible Ailsa Craig, it readily infected susceptible plants within 2 weeks, but infected resistant plants only with difficulty. By 5-7 weeks after inoculation only about a third of the resistant plants were systemically infected, whereas complete infection of these lines with the "adapted" form required only 16 days. Dawson failed to study the original population through the use of single lesion isolates. Since these and other findings of Dawson are in general agreement with data presented here, it would appear that selection from a mixed population rather than a "change" in the virus population can adequately explain his results. The "adapted" form should more appropriately be called a substrain and what Dawson

refers to as the "standard" form should be considered the parent strain. It is possible that if Dawson had used a single lesion isolate of his "standard" form, he would have been unsuccessful in attempts to infect the resistant plants.

The mechanism by which substrains are selected seems to depend on tomato hosts having some form of resistance to TMV. Of the four distinct and widely used sources of resistance to TMV listed by Pelham (1966), all have been shown by various workers at some time to be susceptible to TMV strains. These resistant sources are: 1) Tm_1 gene found in Holmes' P.I. 235673 (Clayberg et al., 1960) and also reported in Hawaiian line H.E.S. 5639-15 (Pecaut, 1964); 2) a gene found in line H.E.S. 2603 by Soost (1963) and given the symbol Tm_2 (Clayberg et al., 1960); 3) resistance isolated by Walter (1956) from L. hirsutum, P.I. 126445; and 4) resistance derived from Solanum pennellii Correll. In this study, tomato lines with genes Tm_1 and Tm_2 and Walter's lines with resistance derived from P.I. 126445 were susceptible to substrain B of strain YA. Similar findings for Tm_1 plants were found by Pecaut (1961), Pecaut and Laterrot (1963) and Dawson (1965, 1967). Tm_2 plants were found to be susceptible by Smith (1961), but allowed only limited multiplication for Pecaut's TMV-isolate (Pecaut and Laterrot, 1963). Walter's source of resistance was found to be susceptible by Pecaut (1961) and by Dawson

(1965, 1967). Although Smith (1961) found S. pennellii to be resistant to his strain, Pecaut reported it was susceptible (Pecaut, 1964). Other forms of resistance have been reported but these appear to be similar to one of the four previously mentioned. For example, Alexander's line with resistance derived from L. peruvianum, P.I. 128650 has been shown to be allelic with Tm_2 (Pecaut, 1965). Pecaut's findings that his new strain of TMV multiplied very little in Tm_2 plants (Pecaut and Laterrot, 1963) as well as in Alexander's line (Pecaut, 1964) could suggest such allelism. However, Alexander found that although lines derived from P.I. 128650 were resistant to four strains of TMV (Alexander, 1963; Alexander and Cirulli, 1966), a line carrying Tm_2 resistance proved susceptible to S-IV (Pelham, 1966). Results here are in agreement since Tm_2 plants were very susceptible to substrain B of YA but accession P.I. 128650 was highly resistant. It is obvious that unless different forms of resistance are tested under identical conditions with the same isolate of TMV, literature on resistance in tomato to TMV will remain very confusing. This study also indicates that we must be aware of the potential danger that virus progenies such as substrain B pose to plant breeders and pathologists.

Knowledge that substrains capable of multiplying in resistant hosts are present in small amounts in susceptible sources, indicates the importance that the source host can

have on the infectivity of TMV isolates. A previous study by Lindner, Kirkpatrick and Weeks (1961) suggested that substrains may be important in this respect. The effect of virus source also appears important in studies made to determine the inheritance of resistance to TMV in tomato. Phillip et al. (1965) used strain JSB-1 to study the inheritance of resistance from source P.I. 235673. It was found that symptoms developed in some of these plants about 5 weeks after inoculation while other plants failed to show mosaic symptoms after 9 weeks. Since strain JSB-1 can produce a substrain specific for this type of resistance, these findings can be explained on the basis of selection of this substrain.

Differences in host range and pathogenicity have been noted before as the most striking ways in which TMV substrains differ from the parent strain (Kunkel, 1947; Knight, 1959). The results of this study tend to substantiate this fact. Although both parent and offspring could multiply in Bonny Best, only substrain B was capable of infecting resistant lines of tomato. The only other distinguishing feature between the parent strain and its substrain was the greater severity of symptoms produced by the parent in Bonny Best. Studies made with YA showed that this increased severity of the parent strain correlated with its faster rate of multiplication, movement and greater invasiveness. Symptoms produced by the parent strain

consistently appeared 1 to 3 days sooner than those caused by its substrain. Although single lesion isolates of the parent strain tended to be more infective than those of the substrain, it is not known to what degree obtaining the substrain from the resistant source may have influenced this effect. Infectivity studies based on bioassay on Scotia bean showed that the parent strain of YA always yielded higher titers than its substrain when both were cultured in Bonny Best. Similar results were reported by Dawson (1967). However, after 26 weeks he found no difference in infectivity between his "standard" and "adapted" forms in susceptible tomato. Contrary to Dawson's finding that infectivity of the "adapted" virus gradually increased in line CStMW-18 over a period of 26 weeks, the infectivity of substrain B was found to decrease in N32 by the end of 7 weeks. Only when N32 selected substrain B from a mixture did a gradual increase in infectivity occur in N32.

The effects of two temperatures on the growth of tomato plants infected with either the parent or substrain of YA again showed the marked difference between the two types. This was especially true at 28 C where Bonny Best plants infected with the parent strain were significantly reduced in both height and weight. In addition, Bonny Best seedlings were killed and older plants failed to set fruit due to the severity of infection by the parent strain. This did not occur on similar plants infected with substrain B.

Failure in attempts to separate the parent and substrain of YA on the basis of size and time of appearance of local lesions on several hosts agrees with the observation of Dawson (1967). This approach has been used to differentiate between U-1 and U-2 strains of TMV (Wildman and Ford, 1960) and the bean and tobacco forms studied by Bawden (1958). Although it was not possible to separate the parent and substrain of YA by sucrose or cesium chloride gradients, other cesium salts such as cesium bromide and sulfate were not tried (Magdoff-Fairchild, 1967). "Spherical" viruses which sedimented as a single component during centrifugation have been found to separate into several components on the basis of their electrophoretic mobilities (Day and Venables, 1960; Bancroft, 1962; Magdoff-Fairchild, 1967). Electrophoresis has also been used successfully for the separation of TMV strains such as U-1 and U-2 and the bean and tobacco forms. Although electrophoresis was not tried, it is possible that this technique could differentiate between the parent and substrain. Analyses of protein components of tomato strains of TMV were very similar and it was thought that natural selection of strains might be decided on some fundamental feature of viral nucleic acid structure (Wang and Knight, 1967). Reddi (1957) showed that there were significant differences in cytidylic and uridylic acids in strains of TMV.

Another area which was not investigated but which appears promising is serology. Henderson et al. (1967) showed host passage selection of antigenically different subpopulations of strains of arbovirus using a chick tissue culture system.

SUMMARY

Tobacco mosaic virus strains YA, S-IV, and JSB-1 were separated into substrains by continuous passage through resistant or susceptible tomato cultivars. Both forms multiplied and produced similar symptoms in susceptible tomato (Bonny Best) but only one of these could infect and elicit symptoms in resistant tomato (N32).

Single lesion isolate studies of strain YA inoculum from Bonny Best and tobacco plants revealed that both sources consisted of a mixture of the two virus forms. Virus specific for susceptible tomato plants was called the parent strain while the virus which infected both susceptible and resistant plants was designated as a substrain. The latter was isolated as an apparently homogeneous population.

The fact that other TMV-resistant breeding lines, with different sources of resistance also selected and allowed only the substrain to multiply, suggested that the same mechanism of selection was operative in these plants.

Dominance of the parent strain in all susceptible sources appeared attributable to its faster rate of multiplication, movement and invasiveness. This increased

pathogenicity was most apparent in susceptible Bonny Best where seedlings were killed and older plants were greatly stunted and failed to set fruit. Similar plants infected with the substrain were only mildly affected.

Other ways to differentiate between the parent and substrain of YA were unsuccessful. The two viruses were not physically separable by sucrose or cesium chloride density gradients, nor by appearance of local lesions on several hosts. This suggested that the physical differences were too slight to be detectable by these methods.

The apparent ability of the parental strain of YA to mutate to form its substrain may explain the occurrence of both forms in the original population. It further suggests that the parent-substrain classification is valid.

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