Relationship between Beet Ringspot, Potato Bouquet and Tomato Black Ring Viruses

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SUMMARY: Serological and plant-protection experiments provided evidence that potato bouquet and beet ringspot viruses are strains of tomato black ring virus. Tomato black ring and potato bouquet viruses are more closely related to each other than either is to beet ringspot virus. The three produce similar symptoms and have a similar host range. There was no evidence that they are related to tobacco ring spot or peach yellow bud mosaic viruses. In the plant-protection tests, protection between serologically related virus strains often was not reciprocal; when protection was incomplete, the symptoms produced by the second virus were milder than those in plants infected by it alone. Strains of tomato black ring virus could be arranged in order of their ability to cause symptoms in plants already infected with other strains. Although results of the plant-protection and serological tests agreed broadly, the correlation was not complete, suggesting that the tests measure different properties of the virus particles. The degree of difference between strains of tomato black ring virus seems to reflect their geographical separation, something that is perhaps to be more expected with soil-borne than with other viruses.

Several distinct plant viruses cause similar symptoms of the ringspot type in tobacco and other host plants, so that symptomatology is not a reliable guide to identity. Two kinds of tests group such virus isolates as related strains or distinguish them as different viruses. First, related virus strains react with one another's antisera, and secondly, infection with one strain protects plants from infection with another. Although there are examples of serologically related viruses which do not protect plants from one another, and of plants infected with one virus which are refractory to a second apparently unrelated one (Bawden & Kassanis, 1945, 1951), the plant-protection test has usually worked well with ringspot viruses (Tall, Price & Wertman, 1949).

It was mainly similarity of symptoms which led Köhler (1950) to suppose that a virus isolated in Germany from potatoes with bouquet disease was a strain of the North American tobacco ring spot virus, but the serological and plant-protection tests done by Bercks & Gehring (1956) show little or no relationship between the two. Potato bouquet is very like the potato disease caused by the soil-borne beet ringspot virus found in Scotland (Harrison, 1957*a*). Beet ringspot virus also infects plants of many other species (Harrison, 1957*a, b*), and the symptoms produced in peach resemble those caused by peach yellow bud mosaic virus which is soil-borne in California (Thomas, Scott, Wilson & Freitag, 1944; Wagnon & Breece, 1955); in some other species the symptoms caused by beet ringspot virus resemble those produced by tomato black ring virus, described in England (Smith, 1946). Because tobacco

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plants infected with beet ringspot virus were not immune from potato bouquet virus and because beet ringspot virus antiserum when diluted 1/35 precipitated beet ringspot but not potato bouquet virus, these two were considered distinct (Harrison, 1957*a*). However, further experiments showed that tobacco plants infected with potato bouquet virus were immune from beet ringspot virus, and suggested that a more detailed study of the relationships between the viruses mentioned above would be worthwhile. The results are reported in this paper.

METHODS

All the viruses were propagated in tobacco plants (*Nicotiana tabacum* L. var. White Burley). The two isolates of beet ringspot virus usually used were originally isolated from potato and burdock (*Arctium lappa* L.); no differences between them were detected in the plant-protection and serological tests. Potato bouquet virus was kindly supplied by Dr R. Bereks, Brunswick, Germany, and peach yellow bud mosaic virus by Dr C. E. Yarwood, Berkeley, California. Two isolates of tomato black ring virus, originally obtained from tomato and lettuce respectively, and also tobacco ring spot virus were kindly supplied by Dr K. M. Smith, Cambridge.

When these viruses were transmitted by rubbing leaves with infected sap, 'Super-floss celite' powder (Johns-Manville Ltd.) was mixed in the inoculum to increase the number of infections. Preparations of sap for serological tests were obtained by macerating either inoculated leaves covered with local lesions or systemically infected leaves. Such sap was stored frozen, and when required was thawed and clarified by centrifugation, 9000 *g* for 3 min., immediately before use.

Tobacco and *Petunia hybrida* Vilm. plants show severe symptoms when first infected, but later produce leaves that look normal although they contain virus. Such infected but normal-looking leaves were inoculated for the plant-protection tests. Plants are more susceptible in winter than in summer, and to keep them susceptible in summer they were kept well watered and shaded. All plants were grown at a mean temperature of 20°. For each protection test in tobacco, several plants were used; the left half of each normal-looking tobacco leaf was inoculated with the virus it already contained and the right halves with the virus under test. For protection tests in *P. hybrida*, each virus was inoculated on all the normal-looking leaves of several different plants.

In the serological tests, 1 ml. of suitably diluted virus preparation was mixed, in tubes of 8 mm. diameter, with 1 ml. of suitably diluted virus antiserum. Control mixtures containing normal tobacco sap or normal rabbit serum, or both, were always made up. The amount of precipitation after 5 hr. incubation at 37° was recorded. A few tests were made with an antiserum to potato bouquet virus kindly supplied by Dr R. Bereks, but most of the tests were done with antisera made by the method already described (Harrison, 1957*a*) to local-lesion isolates of beet ringspot and potato bouquet viruses. The antisera were either stored frozen or diluted with an equal volume of glycerol and kept at 2°.

RESULTS

Symptoms and host range

The symptoms caused by beet ringspot virus in tobacco, *Nicotiana rustica* L., French bean (*Phaseolus vulgaris* L. var. Prince), *Petunia hybrida* and *Chenopodium amaranticolor* Coste & Reyn. were thought to be characteristic (Harrison, 1957a), but potato bouquet and tomato black ring viruses also produce symptoms in all these plants very like those caused by beet ringspot virus, except that those caused by the latter are somewhat more severe in tobacco, French bean and *C. amaranticolor*. Tobacco ring spot virus and peach yellow bud mosaic virus cannot be readily distinguished from the other viruses in *P. hybrida* and French bean, but in contrast to beet ringspot virus they rarely become systemic in *C. amaranticolor* although they produce local lesions in inoculated leaves. In tobacco, peach yellow bud mosaic virus produces local lesions but, unlike beet ringspot virus, does not become systemic: although tobacco ring spot virus produces local lesions and usually becomes systemic in tobacco, the young leaves on 'recovered' plants are mottled, whereas those on similar plants infected with beet ringspot virus are not. Tobacco ring spot virus causes a more severe systemic disease than beet ringspot virus in *N. rustica*. The symptoms produced by beet ringspot virus in cucumber (*Cucumis sativus* L. var. Lockie's Perfection) and tomato (*Lycopersicon esculentum* Mill. var. Kondine Red) are like those caused by tomato black ring virus (Smith, 1946): the symptoms on cucumber include the large enations on the undersides of systemically infected leaves.

Although beet ringspot virus occurs naturally in potato and sugar beet, and potato bouquet virus occurs naturally in potato, Smith (1946) states that these two species are immune from tomato black ring virus. In my tests, all three viruses were readily transmitted to sugar beet (*Beta vulgaris* L. var. Kleinwanzleben E) by mechanical inoculation and all caused systemic infection. Potato plants are not easily infected with these viruses by mechanical inoculation, and in tests with the varieties Kerr's Pink and King Edward, beet ringspot and potato bouquet viruses infected some plants whereas tomato black ring virus (tomato and lettuce isolates) infected none. All the isolates except tomato black ring virus (tomato) infected plants of the variety Chancellor systemically. The number of plants which developed local lesions and the number which became systemically infected respectively, were 4/4 and 4/4 for beet ringspot, 4/4 and 2/4 for potato bouquet, 7/7 and 6/7 for tomato black ring (lettuce) and 4/7 and 0/7 for tomato black ring (tomato) viruses. Thus although tomato black ring virus (tomato), which is derived from Smith's original isolate of the virus, failed to infect potato systemically, plants of the variety Chancellor did become systemically infected with tomato black ring virus (lettuce).

Comparison of the lists of species found naturally infected with beet ringspot, potato bouquet and tomato black ring viruses shows that, with one exception, each virus has been found in different species. Beet ringspot virus has been isolated from sugar beet, potato, turnip, strawberry, *Crocus* and

several weed species, potato bouquet virus from potato, bean and a weed, and tomato black ring virus from tomato and lettuce. Although these lists show that beet ringspot virus has many natural hosts, further work indicated that even the list for it was far from complete. Beet ringspot virus is soil-borne, and it was found to infect systemically plants of wheat, oat, French bean, peach and the cherry rootstock F 12/1 when these were grown in soil collected from a field where plants infected with beet ringspot virus were common: of the species used only barley did not become infected. It therefore seems probable that our knowledge of the natural host range of all three of these viruses has been restricted by the small number of attempts made to detect them in different species. Tobacco ring spot virus has also been found in many species (see Smith, 1957); peach yellow bud mosaic virus occurs naturally in peach (Thomas *et al.* 1944) and probably in Himalaya blackberry (Alcorn, Wilhelm & Thomas, 1955).

The thermal inactivation points of beet ringspot, potato bouquet and tomato black ring viruses have been given as 58–63°: that for tobacco ring spot virus is usually given as 63–68°.

Plant-protection tests

Thirty-one isolates of beet ringspot virus originally obtained from 15 different species growing on five farms within 40 miles from Dundee, Scotland, produced no local or systemic symptoms when inoculated to tobacco plants which were already infected with either of the stock isolates of beet ringspot virus. Similarly, the two stock isolates caused no symptoms when inoculated to plants infected with these 31 isolates. No variations among isolates of beet ringspot virus from Scotland were revealed in these tests.

Seven isolates of beet ringspot virus caused no symptoms in plants infected with potato bouquet virus but, by contrast, beet ringspot virus did not protect plants from infection by potato bouquet virus. Many chlorotic or necrotic lesions developed especially in the youngest inoculated leaves of these plants (Harrison, 1957*a*, fig. 9) and systemic symptoms appeared later. Neither the lesions in the inoculated leaves nor the systemic symptoms were as severe as in comparable healthy plants inoculated with potato bouquet virus. One possible explanation of these results was that the culture of potato bouquet virus contained a mixture of two viruses, one of which was related to beet ringspot virus. Seven local-lesion isolates of potato bouquet virus were therefore made, and each was passed through a single local lesion twice. All the local-lesion isolates protected plants completely from infection by the stock culture of potato bouquet virus, showing that the latter was not a mixture of two viruses. Further tests showed that potato bouquet virus produced the same kind of symptom in plants already infected with each of seven different isolates of beet ringspot virus.

The two isolates of tomato black ring virus protected completely against one another. The isolate from lettuce usually protected completely against beet ringspot virus: sometimes, however, faint chlorotic lesions developed in the inoculated leaves and a very mild systemic symptom developed in one plant. More clearly defined chlorotic lesions and mild systemic symptoms

were produced when beet ringspot virus was inoculated to plants infected with tomato black ring virus (tomato). Beet ringspot virus appeared to protect against the tomato isolate but never against the lettuce isolate of tomato black ring virus. The latter produced symptoms in plants infected with beet ringspot virus very like those caused by potato bouquet virus, but slightly less severe. Potato bouquet virus protected against both isolates of tomato black ring virus; it caused faint chlorotic lesions and mild systemic symptoms in plants infected with the tomato isolate but only did this rarely in plants infected with the lettuce isolate.

Table 1. *Symptoms produced in the plant-protection tests*

Second virus	Symptoms in inoculated leaves already infected with					
	Beet ringspot virus	Tomato black ring virus (tomato)	Tomato black ring virus (lettuce)	Potato bouquet virus	Tobacco ring spot virus	Peach† yellow bud mosaic virus
Beet ringspot virus	0	+	t*	0	++*	++*
Tomato black ring virus (tomato)	0	0	0	0	0*	—
Tomato black ring virus (lettuce)	++*	0	0	0	++*	++*
Potato bouquet virus	++*	+	t*	0	++*	++*
Tobacco ring spot virus	++++*	++++*	++++*	++++*	0	+
Peach yellow bud mosaic virus	+++	—	—	—	+++	0

Degree of local lesion formation in inoculated leaves: 0, none; t, trace; +, weak; ++, moderate; +++, strong; —, not tested.

* Systemic symptoms observed.

† Recovered *Petunia hybrida* plants used. Tests with the other viruses were made in recovered tobacco plants.

Tobacco ring spot virus produced many local lesions and systemic symptoms in plants already infected with beet ringspot, potato bouquet or tomato black ring viruses: also, plants infected with tobacco ring spot virus were susceptible to these three viruses and to peach yellow bud mosaic virus. The latter readily infected plants containing beet ringspot virus. Because peach yellow bud mosaic virus does not infect tobacco systemically, reciprocal tests were made in infected *Petunia hybrida* plants which had 'recovered'. These plants were readily infected with beet ringspot, potato bouquet and tomato black ring viruses, and less readily by tobacco ring spot virus, which was slow to produce systemic symptoms. The results of these plant-protection tests are summarized in Table 1.

Other tests in tobacco showed that beet ringspot virus did not protect against raspberry ring spot, potato stem mottle or lucerne mosaic viruses, potato bouquet virus did not protect against raspberry ring spot or potato stem mottle viruses, tobacco ring spot virus did not protect against raspberry ring spot virus, and lucerne mosaic virus did not protect against beet ringspot, potato bouquet or raspberry ring spot viruses.

An experiment of the type described by Holmes (1956) was made using beet ringspot, potato bouquet and tobacco ring spot viruses. Groups of plants were inoculated with each virus and with preparations containing each possible pair of viruses. The effects of the viruses on the growth of the plants after recovery were recorded. Plants infected with the mixture of tobacco ring spot + beet ringspot viruses were most stunted and those with potato bouquet virus least stunted. The degree of stunting increased in the order:

potato bouquet virus = potato bouquet virus + beet ringspot virus
 < beet ringspot virus < tobacco ring spot virus
 < tobacco ring spot virus + potato bouquet virus
 < tobacco ring spot virus + beet ringspot virus.

The ability of potato bouquet and beet ringspot viruses to enhance the stunting produced by tobacco ring spot virus and the failure of potato bouquet virus to enhance the stunting caused by beet ringspot virus suggest that the two latter are related virus strains, but that neither is related to tobacco ring spot virus. Twelve weeks after infection with the mixture of potato bouquet + beet ringspot viruses, sap from the 'recovered' leaves of the plants was inoculated to *Chenopodium amaranticolor*. Nearly all the lesions were of the potato bouquet virus type but a few were characteristic of the lesions produced by beet ringspot virus. Potato bouquet virus therefore seems to have suppressed almost completely the multiplication of beet ringspot virus in the systemically infected leaves of plants which received a mixed inoculum.

Serological tests

In the first series of experiments, virus antisera diluted 1/35 were mixed with graded dilutions of infective sap. Antiserum to beet ringspot virus only precipitated beet ringspot virus but antiserum to potato bouquet virus precipitated potato bouquet virus and both isolates of tomato black ring virus. Precipitation end-points of sap were usually 1/16. When used diluted 1/16 or less, however, both antisera precipitated all four virus isolates. These results suggested that the viruses were serologically related but that different pairs had different numbers of antigens in common. To estimate the extent of the serological relationship a second series of experiments was done in which the infective saps diluted 1/5 were mixed with graded dilutions of the antisera. The results are summarized in Table 2.

Potato bouquet virus and the two isolates of tomato black ring virus appear to share at least half their antigens, for antiserum to potato bouquet virus had the same precipitation end-point against all three. In an attempt to detect antigenic differences between potato bouquet and tomato black ring viruses, antiserum to potato bouquet virus was absorbed with tomato black ring virus (lettuce) before testing. One ml. antiserum was incubated for 3 hr. at 37° with 7 ml. sap containing tomato black ring virus (lettuce), the mixture kept overnight at 2°, centrifuged and the clear supernatant fluid used. The precipitation end-point of this preparation was much lower than that of the untreated antiserum, but the same whether measured using potato bouquet or tomato

black ring (lettuce) viruses. There is, therefore, little or no antigenic difference between these two viruses.

Beet ringspot virus, by contrast, seems to share less than 20 % of its antigens with potato bouquet and tomato black ring viruses. The precipitation end-points of beet ringspot virus antiserum against potato bouquet and tomato black ring viruses were the same, but scarcely greater than that against normal tobacco sap. Despite the small end-point, antibodies to the virus were present, for after the serum was absorbed with normal tobacco sap, it

Table 2. *Precipitation end-points against different ringspot viruses of virus antisera*

Antiserum tested against	Reciprocal of precipitation end-point				
	Beet ringspot virus antiserum		Potato bouquet virus antiserum		
	Untreated	Absorbed with 3 vol. normal tobacco sap	Untreated	Absorbed with 3 vol. normal tobacco sap	Absorbed with 7 vol. undiluted tomato black ring virus (lettuce) preparation
Beet ringspot virus	128	128	16	16	—
Potato bouquet virus	16	8	128	128	16
Tomato black ring virus (lettuce)	16	—	128	—	16
Tomato black ring virus (tomato)	16	—	128	—	—
Tobacco ring spot virus	—	0 (4)	—	0 (4)	—
Normal tobacco sap	8-16	0 (4)	4-8	0 (4)	0 (8)

Precipitation end-points of the antisera were recorded after 5 hr. incubation at 37°. The figures in parentheses are the smallest dilutions of antiserum tested. The virus preparations consisted of infective tobacco sap diluted 1/5. Absorption was done by incubating the mixture for 3 hr. at 37°, keeping overnight at 2° and clarifying by centrifugation before use.

still precipitated potato bouquet virus. In the same way it was shown that antiserum to potato bouquet virus had a low but real precipitation end-point against beet ringspot virus. Also, when potato bouquet virus was incubated with antiserum to beet ringspot virus for 3 hr. at 37° and centrifuged, no infectivity remained in the supernatant fluid: by contrast, a preparation of potato bouquet virus similarly treated with normal serum was highly infective. Neither the antiserum to beet ringspot virus nor the antiserum to potato bouquet virus precipitated tobacco ring spot virus, although highly infective virus preparations were used.

DISCUSSION

The results of the serological and plant-protection experiments show that beet ringspot, potato bouquet and tomato black ring viruses should be regarded as strains of one virus. As tomato black ring virus (tomato) seems to have been

the first of the strains to be named, beet ringspot and potato bouquet viruses are probably best considered as strains of tomato black ring virus. The isolates of tomato black ring virus from tomato and lettuce, and potato bouquet virus are closely related strains but beet ringspot virus is more distantly related to these three. The degree of relationship between the different isolates is represented by the series:

potato bouquet virus—tomato black ring virus (lettuce)—tomato black ring virus (tomato)———beet ringspot virus,

and is reflected in the slight difference between the symptoms caused by beet ringspot virus and the other strains. There was no evidence of strain relationship between any of this group and tobacco ringspot virus or peach yellow bud mosaic virus.

Ringspot viruses with the most antigens in common were usually also the ones that appeared most closely related in the plant-protection experiments, a conclusion similar to that reached by Matthews (1949) from studies on potato virus *X*. Detailed examination of the results from the two kinds of tests, however, shows that the correlation between them is not complete. This is seen in the following examples. Reciprocally protecting virus strains can be antigenically distinguishable by cross-absorption tests (potato virus *X*; Matthews, 1949) or indistinguishable (isolates of beet ringspot virus) from one another. Similarly, incompletely protecting virus strains can be antigenically distinguishable (potato bouquet and beet ringspot viruses) or indistinguishable (potato bouquet and tomato black ring viruses) from one another. That protection should not be complete between two viruses which are antigenically indistinguishable is especially interesting but it should be remembered that very small differences in antigenic constitution might not be detected in the experiments described above. Whether or not very slight antigenic differences exist between potato bouquet and tomato black ring viruses, the comparisons made above seem to provide new evidence for the view that serological and plant-protection tests measure different properties of the virus particles. The relative value of the two kinds of tests for establishing virus relationships has been fully discussed by Bawden & Kassanis (1951) and need not be further considered here.

Non-reciprocal protection is not a new phenomenon, but it happened unusually often in my tests, perhaps partly because the virus-host system favoured observing it. When testing possible relationships between viruses by plant-protection tests it is obviously important to do reciprocal tests whenever possible. When protection is incomplete, as between beet ringspot and potato bouquet viruses, the lesions which develop after inoculating leaves containing beet ringspot virus with potato bouquet virus are much less severe than those caused by potato bouquet virus in previously healthy leaves. From the size of the potato bouquet virus lesions in leaves containing beet ringspot virus, it can be deduced that changes, probably the multiplication of potato bouquet virus, occurred in a hundred or more cells in each lesion, in all of which beet ringspot virus had presumably multiplied before the leaves

were inoculated with potato bouquet virus. Possible explanations for the poor development of the potato bouquet virus lesions are that the virus invaded fewer cells in each lesion than usual, that it multiplied less than usual in such cells or that the cells were partially protected from the usual deleterious effects of multiplication of potato bouquet virus. The ability to multiply and cause symptoms in cells in which a serologically related virus has already multiplied seems to be possessed to different degrees by different virus strains, and the strains of tomato black ring virus can be arranged in order of decreasing ability to do this:

potato bouquet—tomato black ring (lettuce)—beet ringspot—tomato black ring (tomato).

Because infectivity seems to be a property of the nucleic acid part of tobacco mosaic virus (Fraenkel-Conrat, 1956; Gierer & Schramm, 1956) and possibly of other viruses, it is tempting to speculate that some property of the virus nucleic acid determines the result of plant-protection tests. Whether or not it is possible for the properties of virus nucleic acid to vary in different virus strains independently of variations in virus antigenicity is unknown, but if such variations do occur they may cause the discrepancies in estimates of the degree of relationship between virus strains as determined by the results of plant-protection and serological experiments.

Presumably viruses evolve from forms with all their antigens in common to those with but few in common, and finally into types in which no common antigens can be detected and which would be described as separate viruses. A parallel series of reactions in the plant-protection tests can be divided into four stages: (1) Complete and reciprocal protection; for example, lettuce and tomato isolates of tomato black ring virus. (2) Non-reciprocal protection; tomato black ring (lettuce) and beet ringspot viruses. (3) Partial protection, with infection being reciprocal but the symptoms caused by the second virus being milder than usual; potato bouquet virus and the pseudo-*aucuba* strain (Bercks & Gehring, 1956). (4) No protection; tobacco ring spot and potato bouquet viruses. Divergence between strains will probably be more gradual than suggested by these four categories: for instance, a relationship might appear to be of stage 3 type when tests are done in one set of conditions or in one host, and of stage 2 type in another. Also, all gradations between stages 3 and 4 are possible.

There seems good evidence that tomato black ring virus has evolved rather differently in different countries. Isolates of the beet ringspot virus type from different localities in Scotland were indistinguishable and the differences between the two English tomato black ring virus isolates were very slight. The German isolate (potato bouquet virus) was just distinguishable from the English isolates and readily distinguishable from the Scottish ones: the English isolates were rather less distinct from the Scottish isolates than was potato bouquet virus. Thus the greatest differences were between isolates from the most widely separated sources, with differences becoming smaller with decreasing distance between sources until isolates found in localities only a few miles apart were

indistinguishable. When still greater distances are involved, such as those between the sources of the European and North American ringspot viruses used, the differences were greater than those between related virus strains: this last point, however, is at the most only suggestive, for it is by no means certain that strains of tomato black ring virus do not occur in North America. There is some suggestion that the same kind of geographical variation occurs in other soil-borne viruses. For instance, there are considerable antigenic differences between potato stem mottle virus found in Holland and a form found in Scotland (Cadman & Harrison, unpublished), and although this virus has not been found in North America, the soil-borne wheat mosaic virus occurring there has particles of a similar shape and size (Gold, Scott & McKinney, 1957) and may be a representative of the same major virus group. Here too, the differences between the European types are almost certainly much smaller than those between European and North American types. Although it is possible that soil-borne viruses are particularly prone to geographical variation, perhaps because they have less chance than other viruses of becoming widely disseminated, adequate comparisons must be made with variation in viruses which have flying vectors before such a view can be accepted or refuted. If more geographical variation occurs with soil-borne than with other viruses, and if viruses of the ringspot type are commonly soil-borne as has been suggested (Harrison, 1956), then the existence of many apparently distinct viruses that produce similar symptoms of the ringspot type (see Smith, 1957) is only to be expected.

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