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Phenotypic Mixing between Strains of Tobacco Mosaic Virus

By B. KASSANIS AND CONNIE BASTOW
Rothamsted Experimental Station, Harpenden, Hertfordshire

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SUMMARY

Strain Ni 118 of tobacco mosaic virus in tobacco plants kept at 35° exists mainly as free virus RNA and insoluble virus protein but also forms a few intact virus particles. Buffer extracts of infected leaves have, therefore, very little infectivity. Similar extracts from plants inoculated with a mixture of Ni 118 and the type strain tested on plants in which only Ni 118 gives symptoms are very much more infective (40 to 500 times). The increase in the Ni 118 infectivity of leaf extracts is even greater when the leaves are infected with the Nigerian cowpea virus instead of the type strain. The cowpea virus is a strain of tobacco mosaic virus only slightly serologically related to Ni 118. Neutralization of infectivity tests, using homologous and heterologous antisera, showed that the increased infectivity of Ni 118 in dual infections is caused by the nucleic acid of Ni 118 being incorporated in protein of Nigerian cowpea virus.

INTRODUCTION

Ni 118 is a nitrous acid mutant of tobacco mosaic virus (TMV) with a defective protein which does not combine with its nucleic acid to give complete particles as well as the protein and nucleic acid of the type strain do. The defect is most pronounced in plants kept at elevated ambient temperatures, when most of the virus protein is insoluble and its RNA is free, although a few complete particles are formed. For this reason buffer extracts of infected plants kept at 35° have very little infectivity (Jockusch, 1966; Kassanis & Bastow, 1971*b*). The infectivity in buffer extracts was found to be very much greater from plants inoculated with a mixture of type and Ni 118 strains than from plants with Ni 118 alone (Schaskolskaya *et al.* 1968; Sarkar, 1969). The authors suggested that this was because the RNA of Ni 118 became coated with the protein of the type strain. We have confirmed the increase in infectivity when inoculated with type TMV but could not confirm unequivocally the suggestion that there was phenotypic mixing because the two strains are serologically identical and similar in other properties. For this reason we used as an aiding virus, Nigerian cowpea virus (CV), a strain of TMV which is serologically only slightly related to Ni 118. This also increased the infectivity of Ni 118 and specific neutralization tests showed that the increase in infectivity was the result of phenotypic mixing. The same reason led Atabekov *et al.* (1970) to use as a helper virus the Dolichos enation mosaic virus which, like CV, is serologically only slightly related to Ni 118; and the authors were able to demonstrate phenotypic mixing by neutralization tests.

PM 2 is another nitrous acid mutant of TMV but, unlike Ni 118 its protein is non-functional and only free RNA can be isolated from plants. It was for this reason that we used it in dual infection with CV to demonstrate phenotypic mixing. The results with PM 2 have been published (Kassanis & Bastow, 1971*a*), while the results with the mixture of Ni 118 and type strain of CV are presented here.

METHODS

The materials and methods were as before (Kassanis & Bastow, 1971*b*). The Nigerian cowpea virus was the Rothamsted isolate (Bawden, 1958). Sap was extracted in 0·06 M-phosphate buffer pH 7; 1 g. of infected leaf was extracted in 1 ml. of buffer. The antisera to TMV (type strain) and CV had titres of 1024; as the two viruses are only slightly related, the antisera reacted specifically with their homologous antigens at dilutions exceeding 1/64. No serological differences were detected between Ni 118 and the type strain.

RESULTS

Characterization of strains by symptoms

Only inoculated leaves were used and these reacted as follows: the type strain multiplied without causing symptoms in *Nicotiana tabacum* cv. Samsun, White Burley (cv. Judy's Pride) or *N. sylvestris* L. and produced less virus in plants at 35° than at 20°. Mutant Ni 118 produced a considerable amount of virus in Samsun at 20° without causing symptoms. At 35° it produced bright yellow lesions and the virus accumulated mostly as free RNA and insoluble virus protein, although a few complete virus particles were also produced (Jockusch, 1966; Kassanis & Bastow, 1971*b*). White Burley and *N. sylvestris* plants inoculated with Ni 118 and kept at 20° developed necrotic local lesions, and *N. sylvestris* was used to assay the infectivity of Ni 118 in mixture with the type strain. Most of the hosts of CV are legumes, but in some conditions it infects and multiplies in tobacco plants. It multiplied well in White Burley tobacco at 35° without causing symptoms, but at 20° it caused a few necrotic lesions in young plants. Mature White Burley tobacco plants were used at 20° to test the infectivity of Ni 118 mixed with CV. All three strains gave necrotic local lesions on tobacco cv. Xanthi-nc.

Mixed infection with Ni 118 and either type strain or CV at 35°

Samsun tobacco plants were inoculated with Ni 118 alone and Ni 118 mixed with or followed by type strain at different concentrations. At different intervals samples were taken and the extracted sap inoculated to *N. sylvestris*. Sap from leaves inoculated with both strains produced more lesions than from plants with Ni 118 alone. The greatest increase occurred when the two viruses were inoculated as a mixture rather than consecutively (30 times more effective) and sampled 5 days or more after inoculation. The more concentrated the inoculum, the greater the increase, and the concentration of the type strain had to be as great as or greater than that of the Ni 118. We used both viruses at a concentration of 3 mg./ml. In six experiments made under the best conditions, the increase in infectivity was by factors of from 40 to 500 (Table 1). As there is no proportionality between virus content and lesion numbers with very dilute inocula, lesion numbers were converted into infection units by comparing them with the number of lesions produced by inocula containing known concentrations of Ni 118. Although converting lesion number into virus units when lesions are so few is not very accurate, the conversion evens out some of the apparent differences between experiments when based on lesion number (Table 1).

The infectivity of Ni 118 at 35° increased even more than with type strain when inoculated together with CV in White Burley tobacco (Table 2). The infectivity tests were made in White Burley at 20°, but otherwise the conditions of the experiments were as when Ni 118 was mixed with the type strain.

In one experiment with the Ni 118-type strain mixture, we estimated the proportion of the two viruses in the extracts by comparing the infectivity of the mixture, on two different

hosts with that of a series of dilutions of virus of known concentrations. One comparison was made using Xanthi tobacco and the type strain as a control to estimate the total amount of virus in the mixture. The other comparison was made in *N. sylvestris* using Ni 118 as control to estimate the amount of Ni 118. The difference between the two values showed that about 25 % of the mixture was Ni 118.

Table 1. *Inoculation with mixture of Ni 118 and type strain (TS)*

	Average number of lesions/ half leaf of *N. sylvestris*		Virus units†		
Expt	Ni 118	Ni 118+TS	Ni 118	Ni 118+TS	Ni 118+TS / Ni 118
1	5	45	12	500	40
2	1	13	1·5	155	100
3	1	35	2·9	300	100
4	1	135	0·2	100	500
5	1	27	0·6	140	230
6	3*	43	3	1050	350

* The extracted sap was tested diluted 1/10 except where indicated by the asterisk, when it was undiluted.
† Relative virus concentration estimated by comparing the infectivity with that of a series of dilutions of the virus.

Table 2. *Inoculation with mixture of Ni 118 and cowpea virus (CV) strains*

	Average number of lesions/ half leaf of White Burley tobacco		Virus units†		
Expt.	Ni 118	Ni 118+CV	Ni 118	Ni 118+CV	Ni 118+CV / Ni 118
1	1 (1/3)	103 (1/9)	0·03	150	5,000
2	0·5 (1/1)	106 (1/50)	0·005	100	20,000

Figures in parenthesis show the dilution factor of the inoculum.
† As in Table 1.

Table 3. *Neutralization tests with sap from plants infected with Ni 118 + CV using TMV and CV antisera*

Treatment*	Average number of lesions/half leaf of White Burley tobacco
[Ni 118+CV] sap+antiserum to CV	0·5
[Ni 118+CV] sap+antiserum to TMV	15
[Ni 118 sap+20 μg./ml. of Ni 118]+antiserum to CV	85
[Ni 118 sap+20 μg./ml. of Ni 118]+antiserum to TMV	0·5

* Antisera diluted 1/200 were mixed with equal volumes of sap as described in the text.

Neutralization tests

Sap from White Burley tobacco, inoculated with Ni 118 alone or in mixture with CV and kept at 35°, was extracted 5 days after inoculation. The sap was diluted 1/5 and clarified by centrifuging at 10,000 rev./min. As Ni 118 does not produce much virus when inoculated alone at 35°, we added 20 μg./ml. of purified Ni 118 to the sap from such plants after dilution and clarification. The clarified saps were mixed with equal volumes of antisera to TMV or CV, diluted 1/200 and left at 20° overnight, when they were again centrifuged and tested

for the infectivity of Ni 118 by inoculating plants of White Burley tobacco. Table 3 shows that the infectivity of Ni 118 from the mixed infection was eliminated by precipitation with antiserum to CV but not to TMV, indicating that the infectious RNA of Ni 118 in extracts from plants with both viruses was combined with CV protein.

Tests for interference between strains

In interpreting the results of tests of phenotypic mixing, it is important to know whether the strains being used inhibit each other in plants. There can be inhibition during the initiation of infection, which diminishes the number of lesions, or during multiplication when less virus accumulates.

Type strain slightly decreased the number of lesions produced by Ni 118 in *N. sylvestris* at 20°. Ni 118 at 8 μg./ml. was mixed with an equal volume of type strain at 0·5 mg./ml. or buffer. On average, seven lesions/half leaf were obtained with the mixed inoculum and 20 with Ni 118 inoculated alone. This suggests that the factors of increase in the Ni 118 infectivity in mixed infections shown in Table 1 are, if anything, underestimates. Similar results were obtained when comparing the infectivity of Ni 118 alone and in mixture with CV. Using the same concentrations as in the experiments with type strain, an average of 118 lesions were produced by the mixture and 181 by Ni 118 alone/half leaf of White Burley tobacco.

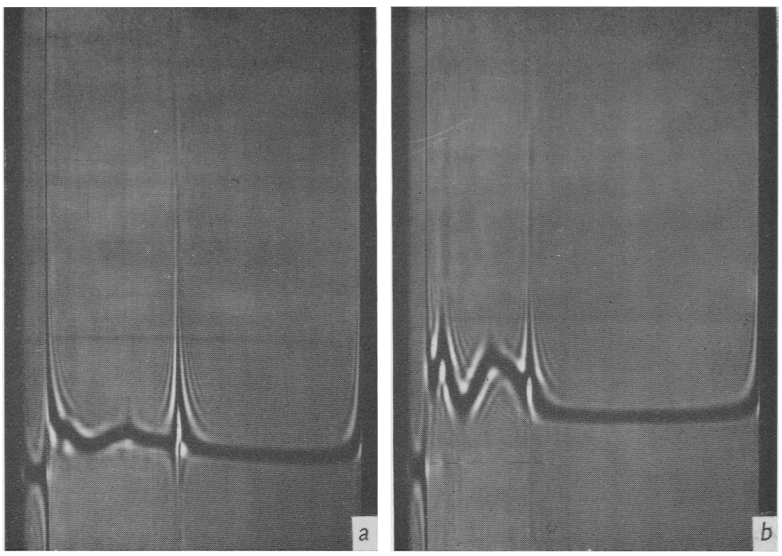

Fig. 1. Sedimentation patterns in the analytical ultracentrifuge of purified virus from plants kept at 35° (*a*) inoculated with type strain (31, 114 and 180s) and (*b*) with the mixture of type strain and Ni 118 (25, 114 and 178s).

To test for interference during multiplication, Ni 118 and type strain were inoculated as in the experiments shown in Table 1 and, for control, plants were inoculated with the type strain alone. Five days later the saps were extracted, diluted and their infectivity compared by inoculating to Xanthi tobacco. There were, on average, 245 lesions/half leaf with the sap from plants infected with type strain alone and 156 from those infected with the mixture. Even if we accept that about a quarter of the lesions produced by the mixed inoculum were caused by Ni 118, the multiplication of the type strain was not excessively inhibited by Ni 118.

However, when purified virus preparations from plants kept at 35° and infected with the type strain alone and in mixture with Ni 118, were compared in the analytical centrifuge, there were considerably more broken virus particles in the mixture (Fig. 1). The significance of this is not obvious.

We also found no evidence that the multiplication of Ni 118 RNA at 35° was affected by the presence of the type strain. Phenol extracts made from Samsun tobacco inoculated 6 days before with Ni 118 alone or mixed with the type strain (plants of Expt. 5 in Table 1) gave an average of 97 and 100 lesions/half leaf of *N. sylvestris*, respectively.

A more accurate estimate of inhibition was possible with the Ni 118-CV combination in plants of 35° by finding the concentration of CV serologically. In three tests, the titre of CV from the mixed infection was the same as the titre from plants infected with CV alone, but in one experiment it decreased from 256 to 64. In contrast, when CV was mixed with the type strain under the same conditions the CV concentration was so diminished as to be undetectable serologically.

DISCUSSION

Our results with the Ni 118-type strain combination agree with those reported by Atabekov *et al.* (1970) but we feel that some of their conclusions were drawn on insufficient evidence. They recovered Ni 118 from necrotic lesions produced in *Nicotiana sylvestris* inoculated with sap from a plant infected with Ni 118 and the type strain at 35° and considered this proof that Ni 118 RNA had been stabilized by the protein of type strain. However, although most of the infective material in plants infected with Ni 118 alone and kept at 35° is free RNA, some intact virus particles are produced (Kassanis & Bastow, 1971*b*). Also, as complete particles can be formed, it could be argued that the increase in infectivity of Ni 118 in mixed infections is because the type strain allows more complete particles of Ni 118 to be formed. There are other examples of one virus increasing the concentration of another for reasons other than phenotypic mixing. For example, the concentration of potato virus X in plants kept at 31° is increased 64 times by simultaneous infection with potato virus Y (Close, 1964). It could be argued that the greater number of imperfect or broken particles in mixed infection with the type strain is because the Ni 118 protein and not the type strain protein coats the Ni 118 RNA.

As Ni 118 is serologically indistinguishable from the type strain, neutralization of infectivity cannot be used to distinguish between the two kinds of protein. Atabekov *et al.* (1970) did neutralization tests using dolichos enation mosaic virus, which is closely serologically related to CV used in our experiments. With both viruses, neutralization of infectivity demonstrated phenotypic mixing, and by analogy this might also be expected to occur with the Ni 118-type strain mixture. In reconstitution experiments *in vitro* with RNA and protein from different strains of TMV, the yield and stability of infective particles increased with increasing closeness of the serological relationship (Fraenkel-Conrat & Singer, 1957; Holoubek, 1962). However, if we accept that the increased infectivity of Ni 118 in mixed infection with type strain is because Ni 118 RNA assembles with the type strain protein, then the results *in vivo* are not the same as those *in vitro*, because more assembles with CV which is less related to Ni 118 than is type strain. This result also is similar to that obtained by Rochow (1970) who demonstrated phenotypic mixing with two serologically unrelated isolates of barley yellow dwarf virus.

The prerequisite for phenotypic mixing is that the two viruses must multiply in close proximity. Therefore, the more distant the relationship, the more likely that mixing will occur, and in our experiments we have used serological tests to measure the relatedness of different strains. Our interference tests indicated very little or no interference between Ni 118

and CV, and unexpectedly little between Ni 118 and type strain which are more closely serologically related. Also, evidence obtained with the light and electron microscope showed that Ni 118 and type strain sometimes occur in the same cell (Kassanis & Milne, 1971). These results make the explanation that phenotypic mixing is the cause of the increased infectivity of Ni 118 in the presence of type strain more acceptable.

Our results also suggest that the ease with which naturally occurring unstable mutants can be isolated from a culture of the type strain is probably because they are protected during transmission by a coat of the protein of the parent strain, and have accumulated in the culture over a long period. Some such mutants are more infective when multiplying in proximity to the parent strain than when alone (Kassanis & Woods, 1969). Another example of an unstable mutant surviving because of phenotypic mixing is that of tobacco necrosis virus. About 5 % of the necrotic local lesions produced by this virus contain only free virus RNA (unstable variant), and these isolates, when recultured, produce no virus protein. As the same proportion of lesions were found to contain the unstable variant, even when a purified preparation was used for inoculum, Kassanis (1968) suggested that the mutant nucleic acid was coated with the protein of the parent virus and could therefore survive during purification.

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