Some Effects of Temperature on the Early Stages of Tobacco Necrosis Virus Multiplication

By D. McCARTHY, SUSAN P. HAWKES AND JOANNA THORNE
Department of Plant Biology and Microbiology, Queen Mary College, London E1 4NS

(Accepted 16 June 1976)

SUMMARY

The number of infective centres which were established successfully following the manual inoculation of French bean leaves with tobacco necrosis virus strain D (TNVD) or with TNVD RNA, decreased with increasing temperature between 13 and 30 °C. At 30 °C or above, primary and probably also secondary infections could not be established, though it is likely that a limited amount of virus RNA and nucleoprotein was produced at 30 °C in cells in which infection had been established previously at 23 °C. During the first day after inoculation, 23 °C was optimal for virus accumulation. Between 23 and 30 °C the rate at which lesions increased in diameter decreased with increasing temperature. The inhibitory effect of supra-optimal temperatures on the establishment of infection may be due to degradation of the infective entity by ribonuclease(s).

INTRODUCTION

Kassanis (1952) found that lesions formed in French bean leaves which had been inoculated with the Rothamsted culture of tobacco necrosis virus (TNV) if they were maintained at 20 °C after inoculation but not if they were maintained at 36 °C. Later, Harrison (1956) noticed that between 10 and 30 °C the number of lesions which developed seemed to be related to the ambient temperature, but he did not test this directly. He found also that (i) the virus could both multiply and be inactivated at 30 °C; (ii) increasing the virus content of the inoculum above that sufficient to produce 1 lesion/cm² did not affect the amount of virus which accumulated at different temperatures and (iii) the optimum temperature for accumulation was 22 °C. Thus, he provided some direct evidence for the hypothesis (Kassanis, 1952) that the virus content of plant cells at any time might reflect an equilibrium between synthesis and degradation. Babos & Kassanis (1963a) subsequently demonstrated that strains A, B, D and E of TNV (Babos & Kassanis, 1963b) all infected less readily at 30 °C than at 20 °C and that the relative amounts of virus produced by the different strains at 30 °C could be attributed to the relative numbers of lesions which were formed by the different strains at that temperature. It therefore seemed worthwhile to examine in more detail how variation in the ambient temperature affects the accumulation of infective RNA and nucleoprotein, and the establishment of infection by both TNV-RNA and nucleoprotein. For this purpose, we chose to use TNV strain D because it produces discrete lesions and does not support the multiplication of a satellite virus (Babos & Kassanis, 1963b; Kassanis & Phillips, 1970). Because the accumulation of infective RNA and infective nucleoprotein at 23 °C can be detected from about 6 and 8 h after inoculation respectively (Kassanis, 1960; Kassanis &
Welkie, 1963), we decided to concentrate our studies on the period 0 to 24 h, for this seemed to be the time when differential effects of temperature on the rates of accumulation might be distinguished most readily.

METHODS

Virus. Strain D of TNV (Babos & Kassanis, 1963 b) was propagated in the leaves of French bean (Phaseolus vulgaris L. cv. The Prince), from which preparations of infective RNA, infective sap and purified virus were made as described previously (McCarthy et al. 1972; Babos & Kassanis, 1963 b).

Experimental procedure. French bean plants were grown at 20 to 25 °C in a glasshouse. Ten days after sowing, plants of uniform size were selected for use and repotted. Plants were routinely kept at 25 to 28 °C in the dark for 24 h before inoculation on the eleventh or twelfth day. Excess inocula were rinsed off within 1 min of application and the plants were maintained thereafter in temperature controlled cabinets (±0.5 °C). Illumination was by sunlight during the day, supplemented with mercury/tungsten lamps (Philips MBTL) during the winter months to provide a 16 h light:8 h dark diurnal cycle. Mung beans were germinated, inoculated and the excized hypocotyls maintained as described previously (McCarthy et al. 1972). For studies on the rate of virus multiplication (Fig. 1, 2) infections were initiated in French bean leaves with inocula containing concentrations of infective RNA and Celite sufficient to produce local lesions which were spaced so closely that they coalesced within 40 h at 23 °C. The same preparations when inoculated to mung bean hypocotyls (Fig. 9) produced about 200 lesions per hypocotyl. Infective RNA was prepared from 2 g, and infective sap from 1 g, of French bean leaves or mung bean hypocotyls (McCarthy et al. 1972) and assayed on the leaves of French beans using Celite to enhance apparent infectivity. Each sample was inoculated, without further dilution, to at least eight half-leaves; but because many samples often had to be assayed from one experiment, relative infectivity was not determined by interpolation with a dilution series as is customary. Instead, if all samples were assayed on one group of plants, infectivities were expressed as average numbers of lesions per half leaf; alternatively if assays were made on more than one group of plants, infectivities were expressed as a percentage of the number of lesions produced by a standard inoculum which was included in each assay. This procedure should not greatly affect the interpretation of the results as all samples in any one experiment were treated in the same manner and in most instances produced between 10 and 250 lesions per half leaf. For experiments concerned with the establishment of infection (Fig. 3 to 8 and 10) in which countable numbers of lesions were required, inocula contained phosphate-buffered (0.067 M or 0.1 M, pH 7) infective RNA (Fig. 3), or purified virus (Fig. 4 to 8) at concentrations sufficient to produce between 200 and 400 lesions per leaf at 23 °C; Celite was not used.

When assayed on French bean leaves the specific infectivity of TNV-RNA prepared either from sap or from purified virus were about 1 to 4 % of the same amount of RNA contained in nucleoprotein. However, the relative values obtained varied both between preparations and when the same preparations were assayed on different occasions.

RESULTS

Virus multiplication

Infectivity assays were made on extracts prepared from the leaves of French bean plants which had been inoculated with TNV-RNA and maintained at 23 °C, or at 23 °C for 2 h, before transfer to either 14 or 30 °C. At 12 h after inoculation more infective RNA than
infective nucleoprotein was detected in extracts made from plants kept at 14 or 23 °C, but no infective RNA or nucleoprotein was detected in extracts made from plants kept at 30 °C. Between 12 and 18 h after inoculation, the accumulation of infective RNA was greater at 23 °C than at 14 °C, whereas the accumulation of infective nucleoprotein was similar at the two temperatures. During the same period, the rates of accumulation of infective nucleoprotein and RNA in plants kept at 30 °C were severalfold lower and accumulation ceased after 18 h (Fig. 1). Lesions subsequently appeared on plants maintained at 23 or 14 °C, but not on those maintained at 30 °C. In an experiment on mung bean hypocotyls in which a largely similar protocol was used, more infective RNA and nucleoprotein was detected at 12 h and the accumulation of both continued more rapidly than in French bean leaves (Fig. 2) but, except at 30 °C, the effects of temperature were largely similar in the two experiments. These results are in accord with earlier observations on the multiplication of the Rothamsted culture of TNV at different temperatures (Kassanis, 1952; Harrison, 1956) and TNV strain D (Babos & Kassanis, 1963a). In addition they demonstrate the failure of infective RNA, as well as infective nucleoprotein, to accumulate when plants are kept for prolonged periods at 30 °C. In our experiments (Fig. 1, 2), we had hoped that infective centres would be established during the periods when plants were maintained at 23 °C, 2 h and ½ h respectively, so that the effects of temperature would have been on subsequent virus multiplication.
Fig. 2. The infectivity of TNV-RNA (----) and nucleoprotein (-----) extracted from mung bean hypocotyls which had been inoculated with TNV RNA. After inoculation plants were kept at 23 °C for ½ h, the hypocotyls were excized and maintained thereafter at 30 °C (□ and ■), 23 °C (△ and ▲) or 16 °C (○ and ●).

In equal numbers of infective centres. As it seemed necessary to test this supposition, yet difficult to do so directly, attempts were made instead to ascertain the extent by which exposure to different temperatures immediately after inoculation might affect the establishment of infective centres.

Establishment of infection

In a preliminary experiment, French bean leaves were inoculated with virus or infective RNA, the plants placed at 14, 18, 23, 28 or 30 °C for 18 h and then transferred to 23 °C to allow lesions to develop (Fig. 3). Irrespective of whether the inoculum contained TNV-RNA or nucleoprotein, low temperatures were more favourable than high for the establishment of infective centres. Exposure to 30 °C beginning immediately after inoculation and extending for various times (0.25 to 20 h) until transfer to 23 °C was more effective in preventing the establishment of infections initiated by inocula of RNA than of nucleoprotein (Fig. 4). Within the temperature range 30 to 44 °C, the higher the temperature to which plants were exposed immediately after inoculation with infective RNA, the more rapidly were potential infective centres inactivated (Fig. 5). However, in a similar experiment in which infection was initiated by inoculation with TNV nucleoprotein, increases in temperature failed to increase the rate at which potential infective centres were inactivated (Fig. 6). If plants which had been inoculated with infective RNA were maintained at 23 °C for 0, 1 or 2 h before exposure to 30 °C for various periods (0.25 to 2 h), the longer that infective centres
Tobacco necrosis virus multiplication

Fig. 3. The primary leaves of French bean plants were inoculated with either TNV$_D$ RNA (unshaded areas) or TNV$_D$ containing sap (shaded areas) and the plants maintained at 28, 23, 18 or 13 °C for 18 h before transfer to 23 °C. Lesions were counted 3 days after the plants were transferred to 23 °C. Values are the mean of three experiments in which the total lesion numbers ranged from ~294 (28 °C) to 4700 (13 °C). The values given for 30 °C (both zero) were obtained from experiments performed on other occasions using the same protocol and inocula, but in which plants were maintained at 30, 23 and 13 °C.

were allowed to become established at 23 °C, the more resistant they became to inactivation by subsequent exposure to 30 °C (Fig. 7). Similarly, within the period examined (0 to 21 h), the longer plants were kept in excess of 6 h after inoculation at 12 °C before transfer to 23 °C, the greater the number of infective centres which were established (Fig. 8), though the increases caused by exposure to 12 °C after inoculation were small when compared with the decreases caused by exposure to 30 °C or above for similar periods (e.g. compare Fig. 4 and 8).

**Temperature shift experiments**

It is apparent from these results (Fig. 3 to 8) that although the temperature at which plants are kept immediately after inoculation can markedly affect the number of infective centres which develop into lesions, it is likely that a period of 2 h at 23 °C after inoculation with infective RNA is sufficient time for the majority of the potential infective centres to become established. Furthermore, although it seems that infections cannot be initiated successfully in plants kept at 30 °C, it may be possible for a limited amount of accumulation to occur at 30 °C in centres which have been established previously at 23 °C (Fig. 1). To investigate in more detail the effects of temperature on the accumulation of infective RNA and nucleoprotein in infective centres already established, we chose to use excised mung bean hypocotyls, because under our conditions, the times at which infective RNA and
infective nucleoprotein begin to accumulate rapidly could be more closely reproduced than when French bean leaves were used. Hypocotyls were inoculated with infective RNA, excised and maintained at 23 °C for 10 or 13 h, when batches were transferred to 30 or 13 °C. The minute pale brown localized necrotic lesions, which first became visible at 23 °C between 9 and 10 h after inoculation, continued to increase in size and darken in colour as is usual at this temperature. By comparison, in hypocotyls which were transferred to 13 °C at either 10 or 13 h the lesions did not increase in size so quickly and they remained pale brown in colour. Transfer to 30 °C at either time prevented any further increase in lesions size, as had been observed previously in leaves (Kassanis, 1952), but the lesions continued to darken progressively. Infectivity assays of extracts prepared from the hypocotyls (Fig. 9a, b) showed that transfer to 13 °C at 10 h but not 13 h decreased the rate of accumulation of infective RNA and that transfer at either time decreased the rate of accumulation of infective nucleoprotein, though at the end of the experiment the apparent concentrations of infective RNA and nucleoprotein were higher than in hypocotyls maintained continuously at 23 °C. After transfer to 30 °C at either time, the accumulation of infective RNA and infective nucleoprotein continued for a short while (3 to 6 h), but ceased thereafter.


to decrease as the temperature is increased above 22 °C (Harrison, 1956) and since our studies have demonstrated that both the establishment and the enlargement of lesions is
Fig. 5. The effect of exposing French bean plants to 30 (●), 37 (▲) or 44 °C (■) for various periods of time beginning immediately after inoculation with TNV$_\beta$ RNA. The average numbers of lesions per leaf which developed when the plants were transferred subsequently to 23 °C have been expressed as fractions of those which developed on leaves maintained continuously at 23 °C. Results given are the means of four experiments. The average number of lesions produced per leaf on 56 leaves at 23 °C was 208.

Prevented at 30 °C, it seemed desirable to examine how the rate of increase in lesion diameter is affected by temperature, within the range 23 to 30 °C. Supraoptimal temperatures clearly inhibited the rate at which true necrotic lesions increased in size between 48 and 120 h after inoculation (Fig. 10). On the leaves of plants which had been maintained at 29 °C many small (0.2 to 0.5 mm diam.) non-necrotic lesions, which subsequently did not increase much in size, were visible at 48 h. A few similar lesions also occurred on plants maintained at 27 °C, but not on those at 25 or 23 °C. These lesions have been observed frequently before on leaves manually inoculated with TNV or TNV-RNA (B. Kassanis, personal communication, 1967; our unpublished observations) and although they are often more numerous and occur more frequently on plants which have been exposed to higher than normal (20 to 23 °C) temperatures their nature is not known, and their occurrence is unpredictable.

**DISCUSSION**

Our experiments have demonstrated that the temperature sensitive stage which prevents the accumulation of TNV$_\beta$ in plants at 30 °C occurs early in the replication cycle before the production of infective RNA. It seems unlikely that it is the uncoating process which cannot be completed successfully at 30 °C, because infection could be established readily.
Fig. 6. The effect of exposing French bean plants to 30 (•), 37 (▲) or 44 °C (■) for various periods of time beginning immediately after inoculation with TNV<sub>D</sub>. The average numbers of lesions per leaf which developed when the plants were transferred subsequently to 23 °C have been expressed as fractions of those which developed on leaves maintained continuously at 23 °C. Results given are the means of four experiments. The average number of lesions produced per leaf on 87 leaves at 23 °C was 126.

With TNV<sub>D</sub> RNA at 23 °C but not at 30 °C. If plants were inoculated with infective RNA and kept at 30 °C for various periods before transfer to 23 °C, the numbers of infective centres which were successfully established decreased in a pseudo first-order manner, with respect to the length of time for which the plants had been kept at 30 °C. Also, for a given period of exposure, increases in temperature, within the range 30 to 44 °C, promoted the rate at which potential infective centres initiated by RNA were inactivated (Fig. 4, 5). Thus, it would appear that temperatures above 30 °C not only prevented the establishment of infective centres by blocking some early step in multiplication, but also enhanced the destruction of the infective entity in vivo. However, when plants were kept at 23 °C for 1 to 2 h after inoculation with infective RNA the infective entity underwent some changes which made potential infective centres more resistant to inactivation by subsequent exposure to 30 °C (Fig. 7). Possibly these changes are the same as those by which resistance of infective centres to inactivation by ultraviolet radiation is increased (Kassanis, 1960).

To explain the results in Fig. 4, 5, 6 and 8 we would propose the following hypothesis. Firstly, that virus particles may be divided into two categories: in the first, uncoating occurs almost immediately after inoculation and the rate of uncoating is not the rate limiting step in the destruction of potential infective centres at 30 °C. The initial steep fall in the curve (Fig. 4), which resembles that obtained after inoculation with TNV<sub>D</sub> RNA, could then be
correlated with the numbers of infective centres successfully established by the RNA released from this category of virus particles. Particles in the second category would uncoat at various times over a much longer period, and for these particles the rate of uncoating becomes the rate limiting step in the destruction of potential infective centres at 30 °C, though this is not enhanced by increases in temperature within the range 30 to 44 °C. Secondly, that a period at 30 °C, albeit after inoculation, increased the susceptibility of some potential infective centres occupied by intact virus particles from the latter category so that if uncoating occurred after the plants were returned to 23 °C, a number of infective centres were successfully established which would not have been established in plants maintained continuously at 23 °C. The remainder of the curve (Fig. 4) could then be correlated with the number of infective centres successfully established by virus particles of the latter category. A brief rise in the curve occurring when the dominant influence of exposure to 30 °C, for those periods of time, was the increase in susceptibility, but followed by a prolonged fall, when it was the destruction of potential infective centres. If both categories of particles occurred within a single cell, it is possible that RNA from particles of the latter category might successfully establish infection if RNA from those of the former category had failed.

Two electrophoretic forms have been observed in preparations of particles of some other isometric viruses, e.g. bean pod mottle virus (Bancroft, 1962) and cowpea mosaic virus (Agrawal, 1964; Niblett & Semancik, 1969, 1970) but we have been unable to detect any differences by electrophoresis of TNV₉ preparations through phosphate-buffered 2·5 %
Fig. 8. The effect of exposing French bean plants to 12 °C for various periods of time beginning immediately after inoculation of the opposite halves of each leaf with TNV₀ RNA (■—■) or TNV₀ (○—○). The average numbers of lesions per half-leaf which developed subsequently on transfer to 23 °C have been expressed as percentages of those which developed on plants maintained continuously at 23 °C. Results given are the means of five experiments in which the average numbers of lesions per half-leaf produced on 29 plants at 23 °C were 197 (virus) and 279 (RNA). The average of the standard errors for all observations was 24.2% (19.7% minimum to 27.6% maximum).

(w/v) polyacrylamide gels at pHs between 5 and 8 (our unpublished observations). The presence of two forms in equilibrium has been suggested by in vitro heat inactivation studies (Babos & Kassanis, 1963) but, because TNV-RNA also exhibited the same behaviour, the relevance of those observations to our present hypothesis is uncertain. The possibility that the postulated categories are a consequence of differences amongst the cells in which infective centres may develop must also be considered.

In contrast, the effects of changes in temperature on multiplication in infective centres already established seem less complex. Transfer from 23 °C to lower temperatures (< 16 °C) slowed the rates of accumulation of both nucleoprotein and infective RNA but only slightly affected their relative proportions. It also allowed the accumulation of greater amounts of both than occurred at 23 °C, probably because it delayed the onset and decreased the extent of necrotic damage. It is not clear from our results whether transfer to 30 °C immediately halted multiplication or whether, as seems more likely (Fig. 1 and 9), it continued for a while afterwards. Prolonged exposure to 30 °C (Fig. 1, 2 and 9) caused some net loss of extractable progeny infective RNA and nucleoprotein, as had been reported previously (Harrison, 1956).

The rates of accumulation of infective RNA and nucleoprotein which were observed at different temperatures (Fig. 1, 2) would thus appear to have been determined by (i) the number of cells in which primary and secondary infections were established and (ii) the subsequent
Fig. 9. The infectivity of (a) TNVd RNA and (b) nucleoprotein extracted from mung bean hypocotyls following inoculation with TNV-RNA. The plants were kept at 23 °C for ½ h after inoculation, the hypocotyls excised and maintained at 23 °C (○). At 10 and 13 h batches were transferred to 30 °C (口) and 33 °C (△) respectively. The inset shows temperature measurements taken with a copper-constantan thermocouple placed inside a mung bean hypocotyl during the period after transfer from 23 to 30 °C.

Fig. 10. Frequency distributions of the 'diameter' of necrotic lesions present on French bean leaves 48 and 120 h after inoculation with TNVd RNA. The values given are the means of three experiments in which the plants were kept at 23 °C for 2 h before batches were transferred to 29, 27, 25 or 23 °C. The 'diameter' of every lesion on a leaf was measured once in a random plane using a binocular microscope fitted with a calibrated eyepiece.
rates of virus multiplication and inactivation within them. Between 12 and 23 °C, increases in temperature usually increased the rates of accumulation in established infections, but conversely, between 12 and 44 °C, increases in temperature increased the rate at which potential infective centres were inactivated after inoculation with infective RNA, vide infra, and above 30 °C primary infective centres could not be established. If the rates at which lesions enlarge (Fig. 10) is determined mainly by the rate at which secondary infections are established, and if this process is affected by temperature in a manner similar to that determined experimentally for primary infective centres, then it is likely that (i) the limited amount of accumulation observed at 30 °C (Fig. 1, 2 and 9) occurred only within cells in which infection had been established previously at a permissive temperature and (ii) the loss by degradation of infective RNA and nucleoprotein at 30 °C within cells in which multiplication had occurred, or was occurring, was small in our experiments relative to these other factors. Thus, we would consider our results compatible with the suggestion (Kassanis, 1952) that the virus content of infected plant cells represents an equilibrium between the rates of synthesis and degradation, if, in this instance, it is not inferred that increasing the temperature increases the rates of two opposing processes, the temperature coefficients of which are so related that in infected cells net accumulation occurs below and net loss above about 30 °C. Instead, the rates of accumulation at different temperatures seem more likely to be a consequence of the opposing effects of temperature on the number of cells in which primary and secondary infections are established and the rates of virus multiplication within them.

We are grateful to Dr B. D. Harrison and Dr B. Kassanis for valuable discussions, to Mrs E. Palfreyman and Miss A. Trotter for technical assistance and to the Science Research Council for a support grant to D.M. (no. BSR 6961) and a research studentship to S.P.H.

REFERENCES


(Received 15 March 1976)