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SOME CHARACTERISTICS OF TOBACCO STREAK VIRUS ISOLATES FROM QUEENSLAND

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SUMMARY

Tobacco streak virus (TSV) has not previously been reported from Australia. It was isolated from *Dahlia pinnata, Ageratum houstonianum, Solanum auriculatum, Nicandra physalodes, Solanum nigrum* and *Asclepias physocarpa,* growing in scattered locations in south-eastern Queensland. Serological relationship to English and American strains of TSV was demonstrated. Typical TSV virus particles were demonstrated by electron microscopy and a three-component system was visible in sucrose density gradient separations. The host range and symptoms were generally similar to those of the type strain of TSV, but red node in bean (*Phaseolus vulgaris*) resulted from inoculation of this species with some highly infective preparations. Antisera were produced using two different virus purification methods.

I. INTRODUCTION

Tobacco streak virus (TSV) has been reported from Europe and North and South America (Brunt 1968; Costa and Carvalho 1961; Devergne 1961; Fulton 1948; Patino and Zaumeyer 1959) but not previously from Australia. Diseases of crop and pasture plants (Costa and Carvalho 1961; Devergne 1961; Patino and Zaumeyer 1959; Thomas and Zaumeyer 1950) and ornamentals (Fulton 1967; Brunt 1968) can result from TSV infection.

Because the virus isolates used by previous workers have apparently shown different stability characteristics in relation to pH and solvents, the purification methods employed by them have also varied. Mink, Saksena and Silbernagel (1966), working with a strain (TSV-RN) that caused a "red node" disease of bean (*Phaseolus vulgaris* L.), found that methods using chloroform-butanol, butanol 8·5%, heat or pH 4·3 for clarification caused great depletion of the infectivity of resulting preparations. Brunt (1968), however, was able to purify his dahlia isolate using butanol 8·5% and Lister and Bancroft (1970) used a low pH step (5·0) successfully. Fulton (1967, 1970) successfully used hydrated calcium phosphate as a clarification medium together with buffering systems at pH 8·0. Mink, Saksena and Silbernagel (1966) did not buffer their charcoal clarification step. Lister and Bancroft (1970) showed that extraction at pH 7 produced better virus yields than at pH 5. Mink, Saksena and Silbernagel (1966) found that charcoal clarification did not reduce infectivity of TSV-RN preparations.

Bean, tobacco (*Nicotiana tabacum* L.) and *Chenopodium quinoa* Willd. have previously been used as source plants for TSV purification. Mink, Saksena and Silbernagel (1966) found beam stem tissue more satisfactory for charcoal clarification of TSV-RN, while Lister and Bancroft (1970) found *C. quinoa* a poor source of TSV. *Vigna cylindrica* (L.) Skeels, *Dolichos biflorus* L. (*D. uniflorus* Lam. in this paper), *D. lablab* L., *Cyamopsis tetragonoloba* (L.) Taub. (Fulton 1948, 1967), *Chenopodium quinoa* and *Phaseolus vulgaris* (Lister and Bancroft 1970) have been used as local lesion assay plants.

Multicomponent systems have been described for several isometric viruses (Kassanis 1968; Fulton 1970) and considerable work has been done relating to infectivity and relative proportion of the various components of TSV (Fulton 1970; Lister and Bancroft 1970).

While some workers did not report any difficulty in electron microscope observations of negatively strained TSV particles (Brunt 1968; Lister and Bancroft 1970), others (Fulton 1967; Mink, Saksena and Silbernagel 1966) reported particle disruption difficulties with some preparations.

II. MATERIALS AND METHODS

Glasshouse methods.—Plants were grown in steam-sterilized peat-sand mixture with added nutrients. Glasshouses were insect screened and provided with forced ventilation and cooling.

Mechanical inoculations from plant material were made by grinding inoculum in a mortar with O·1M phosphate buffer adjusted to pH 7·7 after addition of 0·1% thioglycollic acid. In inoculations from purification procedures 0·1M, pH 7 phosphate buffer was used for dilutions. Leaves of test plants were dusted with carborundum powder before being inoculated by finger rubbing and were washed with tap water afterwards.

Host range and thermal and dilution end-points were determined using infective sap from *Nicotiana clevelandii* Gray at the speckled necrotic systemic stage or *N. tabacum* L. at the watersoaked systemic lesion stage. Host range plants were indexed back to tobacco and *Chenopodium quinoa.* Virus assay was normally on *C. quinoa,* but tobacco and the young primary leaves of French bean cv. Brown Beauty were sometimes used in addition.

Sources and maintenance of isolates.—Virus isolates used in purifications and preparation of antisera were (A) from *Ageratum houstonianum* Mill. and (B) from *Nicandra physalodes* (L.) Gaertn. Isolates from other species, obtained from field material from south-eastern Queensland, were clarified or partly purified to be used as antigens for serological tests. Isolates were maintained free of variation (Fulton 1970) by three methods:—desiccated leaf cultures; frozen leaf tissue; frozen purified virus. The first of these was found to be most effective in maintaining infectivity.

Purification and serology.—Virus purification runs were made from bean primary leaves as well as tobacco, cucumber cotyledons and petunia (*Petunia hybrida* Vilm.) but *Nicotiana clevelandii* was used in most experiments.

1. Using isolate (A), partially purified virus was obtained by an ether (1/3 vol.)-carbon tetrachloride (1/4 vol.) method resembling that described by Wetter (1960). Following the differential centrifugation steps, this virus was

used for intramuscular injections (each 1 ml + 1 ml Freund's adjuvant) into a rabbit, for preparation of antiserum (A). Three injections at 10-day intervals were given and ear bleedings begun 1 week after the last injection.

2. A second type of purification, resulting in a preparation with less host material, was used with isolate (B). The infected material was macerated with 10–15% activated charcoal after a preliminary maceration with 0·1M, pH 7 phosphate buffer to which 0·1% thioglycollic acid had been added. The homogenate was strained through doubled cheese cloth. After low-speed centrifugation (6,500 *g* for 10 min), a high-speed pellet (Beckman Spinco 30 rotor 120 min at 30,000 r.p.m.) was then obtained from the supernatant. This pellet, containing some charcoal and green material, was resuspended in 0·02M, pH 7 phosphate buffer and adjusted to pH 5·2 with 2% acetic acid. After standing in the refrigerator for 30 min, a low-speed pellet was centrifuged off and the pH of the supernatant raised by addition of 1 vol. 0·02M, pH 7 phosphate buffer. Another cycle of differential centrifugation followed. The virus was then frozen, thawed and given a low-speed centrifugation before being used for intramuscular injections into a rabbit to produce antiserum (B).

Antisera were stored either frozen or 1:1 with glycerol at —20°C and sodium azide at 0·01% was added. All purification procedures were conducted at reduced temperatures.

Gel diffusion tests (Crowle 1961) were carried out in buffered 0·85% saline + 0·75% "Ionogar No. 2" poured in 9 cm plastic petri dishes. Wells were cut with a 5 mm cork borer. Microprecipitin serological tests (van Slogteren 1954) were also performed in 9 cm plastic petri dishes and examined with a stereo microscope.

An antiserum to a bean "red node" strain of TSV was obtained from Dr. G. I. Mink (Washington State University) and antiserum to a dahlia isolate was supplied by Dr. A. A. Brunt (Glasshouse Corps Research Institute, Littlehampton).

Density gradient centrifugation.—One millilitre of purified virus was layered on to Beckman Spinco 25·1 rotor tubes previously prepared with 4,7,7,8 ml of 10%, 20%, 30% and 40% w/v respectively of buffered sucrose solutions. Tubes were then spun for 2 hr at 25,000 r.p.m. and the light-scattering zones removed by hypodermic syringe. UV absorption data on these zones was obtained using a Beckman DB spectrophotometer.

Electron microscopy.—Unfrozen purified virus was fixed for 10 min in 1% glutaraldehyde (pH 6·0) before being mixed with an equal volume of 2% potassium phosphotungstate and applied as a "blotted drop" to electron microscope grids coated with cellulose nitrate and backed with carbon.

III. RESULTS

Field isolations.—Several isolates of TSV were obtained from scattered locations in south-eastern Queensland. Three were obtained from weeds (*Solanum auriculatum* Ait., *A. houstonianum, Asclepias physocarpa* Schlecht.) growing in and around a plantation where a necrotic leaf-spot disease of pineapples (*Ananas comosus* (L.) Merr.) consistently occurred. However, no relationship of TSV to the pineapple disease was proven. Other isolates were obtained from other

weed species (*Solanum nigrum* L. and *Nicandra physalodes*) and from dahlia. In general, it was found that *Nicotiana clevelandii* and *Chenopodium quinoa* were the most useful hosts for inoculations from field plants.

Symptomatology and host range.—Species shown to be susceptible to Queensland TSV isolates were as follows, typical symptoms of the type strain of TSV (Fulton 1948) being produced on most of the hosts tested:

Ageratum houstonianum Mill.
Asclepias physocarpa Schlect.
Bidens pilosa L.
Chenopodium amaranticolor Coste & Reyn.
Chenopodium quinoa Willd.
Cucumis sativus L.
**Cyamopsis tetragonoloba* (L.) Taub.
Dahlia pinnata Cav.
Datura stramonium L.
Dolichos uniflorus Lam.
Nicandra physalodes (L.) Gaertn.
Nicotiana clevelandii Gray
Nicotiana glutinosa L.
Nicotiana tabacum L.
Petunia hybrida Vilm.
Phaseolus lathyroides L.
Phaseolus mungo L.
Phaseolus vulgaris L.
Portulaca oleracea L.
Solanum auriculatum Ait.
Solanum nigrum L.
Vicia faba L.
Vicia sativa L.
Vigna sinensis (L.) Endl. ex Hassk.

There were some anomalies, the most conspicuous of which was the production of a "red node" disease of bean closely resembling that described by Thomas and Zaumeyer (1950), and including almost identical pod symptoms. This severe systemic disease did not result from inoculation with any isolate unless very concentrated inoculum was used. When highly infective purified preparations were inoculated to Brown Beauty bean, a high density of lesions was produced on the primary leaves. These lesions varied from necrotic to chlorotic small rings usually surrounding a greener area with a small necrotic spot in the centre. The primary leaves often became extensively necrotic and abscissed, with the severe systemic "red node" disease developing later.

On Blackeye cowpea, brown rings formed on inoculated primary leaves. Sometimes these developed another concentric ring. Systemically infected trifoliate leaves showed a mottle and some flecking. The stems of some plants became necrotic just below the primary leaves and these plants died. Vein necrosis was a characteristic symptom on inoculated leaves of several legumes.

The lesions on inoculated leaves of guar (*Cyamopsis tetragonoloba* (L.) Taub.) appeared to be identical with those obtained by Fulton (1948). The virus did not become systemic in this host. In some other legumes such as broad bean (*Vicia faba L.*) the virus was systemic but symptomless. *Dolichos uniflorus*

*Local lesions

Lam. and *D. lablab* gave characteristic symptoms (Fulton 1967; Brunt 1968). The lesions on inoculated leaves of *Chenopodium quinoa* were usually less necrotic but otherwise the disease development resembled that described by Brunt (1968). The bleached rings and herringbone patterns induced on Turkish tobacco, in addition to the notched leaf margins of some of the later leaves, are characteristic of TSV (Figure 1). Systemic infection followed inoculation of *Nicotiana glutinosa.*

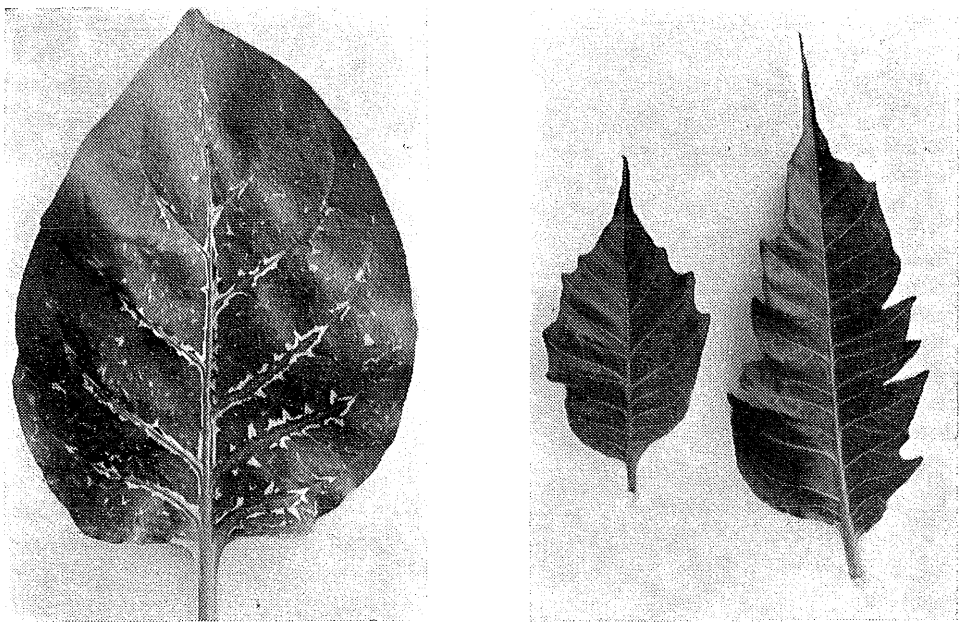

Fig. 1.—Xanthi tobacco leaves showing white herringbone necrosis pattern (left) and notched margins (right), following infection by tobacco streak virus, isolate (A).

An unusual symptom in *Asclepias physocarpa* made diseased plants easy to detect in the field. Infection in this species resulted in somewhat narrowed laminas with a conspicuous protrusion of the midrib beyond the lamina blade (Figure 2).

Dahlia seedlings inoculated with TSV showed no consistent symptoms (Brunt 1968). Field dahlia plants from which TSV was isolated showed some mild symptoms which could have been due to other causes. In one instance these mild symptoms were shown to be due to a virus serologically and morphologically related to potato virus X, which was associated with TSV as a complex.

Purification source hosts.—Assays to local lesion hosts showed that virus infectivity from tobacco fell rapidly after the first systemic necrotic symptoms of infection. Mechanical transmission of the virus from plants which had been infected for some time was often difficult, although it could always be re-established by leaf grafts to other tobacco plants.

Fig. 2.—*Asclepias physocarpa* leaves naturally infected by tobacco streak virus, showing midrib extensions and narrow laminas.

Tests on other hosts such as petunia and *Nicotiana clevelandii* showed less rapid fall of infectivity and the latter produced a very high relative infectivity at the necrotic speckle systemic stage. Preparations from this species were also found to be relatively easy to clarify compared to those from tobacco or bean leaves, in which the pigments were more persistent.

Purification.—Before the Queensland TSV isolates had been identified, several purification methods were tried. Butanol or chloroform destroyed infectivity completely, but it was found that the ether-carbon tetrachloride clarification method (Wetter 1960) with two cycles of differential centrifugation produced a highly infective preparation with little or no residual pigmentation. Resuspension in borate buffer, 0·02M, pH 8·0 appeared to maintain infectivity at least as well as phosphate of the same molarity at pH 7·5. When this purification method was used to prepare an antiserum (A), it was evident from gel diffusion reactions that a considerable amount of host material had been included in the antigen. Freezing and thawing, followed by low-speed centrifugation, did not remove much of this host material. However, acid precipitation using citric acid to pH 5·2 (Fulton 1967) and repelleting the supernatant by

high-speed centrifugation did produce a virus preparation that was highly infective and with good serological activity. Gel diffusion tests with antiserum (A) showed this latter preparation to contain little host material.

After the virus had been identified by reacting it in gel diffusion and micro-precipitin serology tests with Mink's TSV-RN antiserum, some further methods of purification were tested. The charcoal-freezing method (Mink, Saksena and Silbernagel 1966) appeared to leave some host material during these tests and a modification incorporating an acid precipitation step (Lister and Bancroft 1970) was included. This was preferred to the ether-carbon tetrachloride + acid precipitation procedure because the charcoal clarification step is less laborious. Antigen produced in this way gave no host lines with undiluted and unabsorbed antiserum (A). However, assays indicated that all acid precipitation steps caused some loss in infectivity.

Serology.—Antiserum (A) and a second antiserum (B) were prepared by the methods already detailed. The use of antiserum (A) in combination with Mink's antiserum was sufficient to confirm identification of the virus. Because of the host reaction of antiserum (A) some improvement was attempted by absorption with host material prepared from healthy plants using an ether-carbon tetrachloride procedure. Following absorption, only a minimal host reaction remained, but an imbalance of host antigen components resulted in an excess of some components. In consequence of this a reaction occurred between absorbed and unabsorbed antisera.

Comparisons were made between the two imported antisera and antisera to locally obtained isolates (Figure 3). Confluence of the precipitation lines in gel diffusion was evident with antisera to a dahlia isolate (Brunt 1968), the

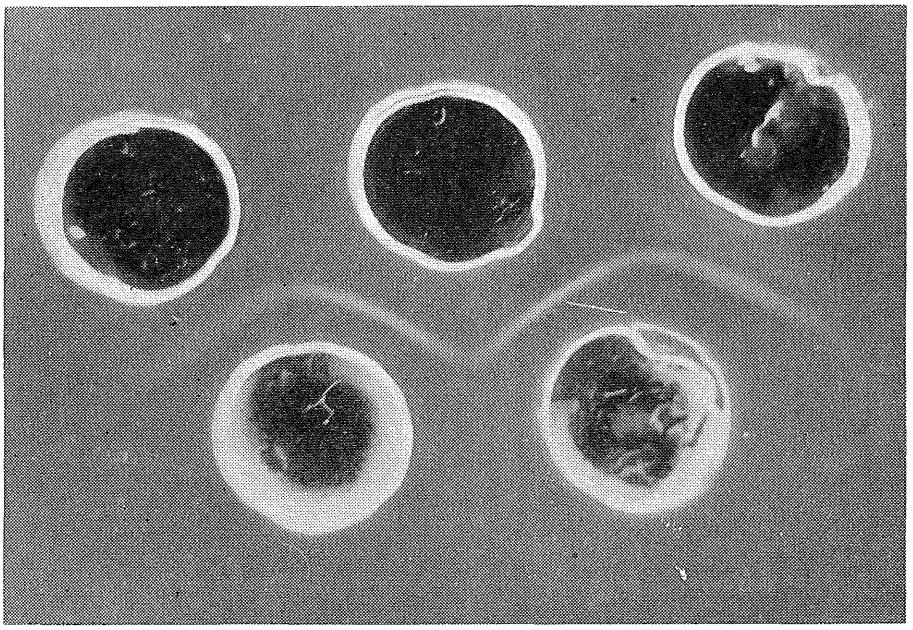

Fig. 3.—Reaction of tobacco streak virus isolate from *Nicandra physalodes* (lower two wells), with 3 antisera:—left, English isolate from dahlia; centre, American isolate from bean; right, Queensland isolate from *Ageratum houstonianum*.

Queensland isolates and TSV-RN (Mink, Saksena and Silbernagel 1966). The reaction of Brunt's antiserum was relatively weak with Queensland isolates and no precipitate was visible above a 1 in 4 dilution. However, this antiserum had a strong homologous reaction (A. A. Brunt, personal communication).

Sucrose density gradient centrifugation.—Centrifuge tubes prepared as described and layered with 1 ml of virus prepared by ether-carbon tetrachloride clarification plus differential centrifugation showed two light-scattering bands of host material and a diffuse band containing infectious material below these. Considerable light scatter made accurate observation of the virus band rather difficult and no separation of components was observed.

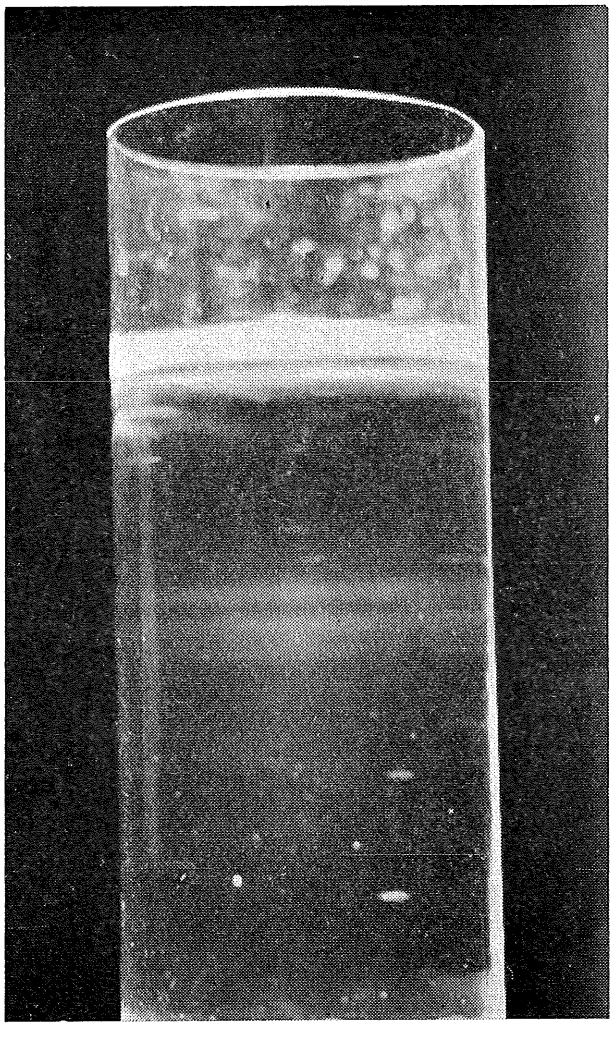

Fig. 4.—Rate zonal centrifugation of tobacco streak virus showing three bands, with lower band less dense and closer to the middle band.

However, when charcoal-acid precipitated preparations were centrifuged in the same manner, no upper host bands could be seen and three distinct bands were present in the virus zone (Figure 4). The lower band was less dense and was somewhat closer to the middle band than was the top component. The method of removal of these bands by hypodermic syringe possibly allowed some contamination of adjacent bands. When assayed, all were infective at dilutions of 1:4. The UV absorption spectra of the bands had similar maxima and minima (A260 and A243) with less relative absorption from the lower band. The A260/280 ratio of the middle band was 1·62.

Electron microscopy.—Purified preparations presented a highly disrupted appearance when negatively stained with potassium phosphotungstate and viewed in an electron microscope. Fixation with 1% formaldehyde produced no noticeable improvement. However, when preparations were fixed for 10 min in 1% glutaraldehyde at pH 6·0 before adding the negative stain, undisrupted isometric particles typical of TSV were observed.

IV. DISCUSSION

It appears that TSV is established in a range of weed species in south-eastern Queensland. The search for the virus was not extended beyond this area. Some of the field infections were remote from any vegetatively propagated exotic ornamentals, such as dahlias, which could have introduced the virus, and there would presumably have to be an active vector and/or efficient dispersal mechanism unless the virus was long-established in the area.

The various Queensland isolates appeared to be symptomologically similar and, in general, more closely resembled the type strain on a symptom basis. However, the ability of some preparations to establish a typical systemic red node-pod lesion disease syndrome in French bean possibly distinguishes our isolates from the type strain. With the heavy inoculum density required to produce this disease, it is conceivable that mutants are involved in the systemic penetration of the bean plants. Inoculations direct from the stock desiccated leaf cultures did not produce "red node" in beans.

A multicomponent system similar to previously described TSV strains was demonstrated. Some further work using better tube fractionating apparatus is planned for more precise component analysis.

In Queensland experiments, homogenization with activated charcoal tended to lower the pH of unbuffered sap considerably, sometimes as far as pH 4. preliminary homogenization and later addition of charcoal could thus represent an approximately neutral extraction with subsequent lowering of the pH.

The sensitivity to butanol, which destroyed both detectable infectivity and serological activity, contrasts with the dahlia isolate described by Brunt (1968). The serological relationship with this isolate also appeared to be more distant than with the TSV-RN isolate described by Mink, Saksena and Silbernagel 1966). The sensitivity to negative stain disruption corresponds to Fulton's observations (1967), where glutaraldehyde fixation was also necessary for satisfactory electron microscopy.

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