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August 16, 1995

Dr. Harvey Blackburn
Office of Agriculture
Bureau for Research and Development
U.S. AID
Washington, DC 20523-1809

Dear Dr. Blackburn:

Enclosed please find five copies of the final report on the project entitled "Biology and Control of Pigeonpea Witches' Broom in the Tropics" Grant No. DAN 4316-G-00-0072-00 as required by the grant agreement. Two copies of this report were sent to A.I.D. PPC/CDIE/DI Washington, DC 20523-1802 under separate cover.

I'd like to take this opportunity to express my sincere thanks to you and USAID for your financial support. Although this project was officially ended on July 31, 1995, I will continue to work on this important project based on the findings of past research. Thank you again.

Sincerely yours,

James H. Tsai
Professor

JHT/ses
cc: U.S.A.I.D.
PPC/CDIE/DI
Washington, D.C. 20523-1802

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Final Report

Project Title: Biology and Control of Pigeonpea Witches' Broom in the Tropics

Grant No.: DAN-4316-G-00-0072-00

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Reporting Period: October 10, 1990 - July 31, 1995

We want to express our sincere thanks to USAID for the financial support for this project and the extension of this project after Hurricane Andrew. In this Final report we summarized the following accomplishments:

a) Transmission pigeonpea witches' broom agent to periwinkle:

Our earlier attempts to extract pigeonpea (Cajanus cajan) witches' broom (WB) mycoplasmalike organisms (MLO) from the infected pigeonpea plants had been unsuccessful. This was probably due to low titre of MLO in the infected tissue. Our breakthrough came when we were able to transmit WB MLO from infected pigeonpea to periwinkle (Catharanthus roseus). Development of foliar yellowing in periwinkle 6-8 wk after transmission tests were initiated provided the first evidence that MLO infection of those plants had occurred. The appearance of this symptom was closely followed by the production of virescent flowers and a proliferation of small shoots from axillary buds on new growth. When symptoms further intensified, the development of small phylloid flowers was also evident, as was the development of shoots with shortened internodes and stunted leaves from floral organs, which gave a pronounced witches'-broom appearance to affected branches. Grafting of witches-broomed scions to additional healthy periwinkle plants resulted in the appearance of identical symptoms within 21-28 days, as infections progressed systemically in these plants. Transmission electron microscope examination of petiole vascular tissues from symptomatic plants revealed the presence of typical polymorphic MLOs in phloem sieve tube elements; these bodies were absent from similar tissues of noninoculated plants.

b) Isolation and cloning of MLO DNA:

Density gradient centrifugation of DNA from extracts of healthy periwinkle tissues resulted in the separation of one major band with a buoyant density (p) of approximately 1.6 g/cm^3 located between 21.5 and 29 mm from the top of the gradient tube and two minor, less buoyant bands, at 5 and 9 mm below the main DNA band, respectively. Centrifugation of DNA from WB-affected plants produced an identical banding pattern to that observed for healthy extracts, except that an additional faint band (p = 1.59 g/cm^3), located at 2.5 mm above the main host band, was also observed. This unique band was removed from each of three gradients, polled, and recentrifuged as before, revealing two sharply defined, narrowly separated bands. The uppermost of these bands corresponded in approximate location and buoyant density to the unique band that was visualized following the earlier separation. When only the uppermost band was removed and recentrifuged, a single discrete band was resolved. This putative MLO DNA was used for molecular cloning.

A total of 440 recombinant E. coli colonies were obtained from cloning MLO DNA isolated from WB-diseased periwinkle plants. Labeled DNA from healthy plants hybridized with recombinant plasmids from 10 of 80 arbitrarily selected, individual colonies, whereas labeled MLO DNA hybridized with recombinant plasmids from all 80 colonies.

Also, MLO DNA, but no healthy plant DNA, produced discernible hybridization signals with pUC19 plasmid DNA alone when membranes were subjected to moderate stringency (55° C) posthybridization was conditions. By increasing the penultimate wash temperature to 60° C or higher hybridizations between MLO DNA probe and pUC19 was virtually eliminated with little or no reduction in signal intensity from recombinant plasmids. Of the 70 apparently disease-specific

recombinants, relatively strong hybridization signals were produced by 21 recombinants that were clearly evident after only 2 hr of autoradiography. Moderate signals were observed for 46 recombinants, and three gave only weak signals during this time.

c) Characterization of probes:

Fourteen recombinant plasmids were selected to include a range of cloned DNA insert sizes. To determine their specificity for detecting WB MLO DNA, each of the 14 recombinant plasmids or their cloned MLO DNA inserts was used as probes in dot hybridizations, with total undigested DNA extracts from plants affected by several other MLO-associated diseases. A summary of results from these hybridizations is given in Table 1. No hybridizations were observed between any probe and DNA from healthy host plants, which included periwinkle and pigeonpea but not to extracts from any of the additional MLO-infected plants that were examined. Of the remaining probes, WBA13I, pW8A48, and WBB7I also hybridized to extracts containing DNA of Eastern aster yellows or Florida periwinkle witches'-broom MLOs producing discernible, albeit weak, hybridization signals. Only probes WBA12I and WBB2I gave signals with any of the additional MLOs examined that are not known to be indigenous to Florida. Both probes hybridized to DNA extracts from Western-X infected celery; probe WBA12I also hybridized to DNA from periwinkle infected with Western dwarf aster yellows and, to a much lesser degree, with similar extracts containing DNA of the beet leafhopper-transmitted virescence agent.

Each probe was also used in Southern hybridizations against blots of total undigested DNA extracted from healthy periwinkle plants, WB-affected periwinkle and pigeonpeas, as well as extracts from various plant hosts with MLO-associated diseases indigenous to Florida. Distinct differences in the patterns of hybridization between probes WBA12I and WBA28I and MLO DNA were evident. Probe WBA12I hybridized to blots of WB-affected periwinkle and pigeonpea, Florida periwinkle witches'-broom, and Eastern aster yellows DNA extracts at locations corresponding to the position of the visible undigested chromosomal DNA bands in the adjacent gel. By comparison, signals produced by probe WBA28I indicated that this probe hybridized to bands of lower molecular weight extrachromosomal (possibly plasmid) DNA, and that hybridizations were limited only to bands present in DNA from WB-affected periwinkle and pigeonpea.

As a result of the above studies, we have published a paper entitled "Molecular cloning and detection of chromosomal and extrachromosomal DNA of mycoplasmalike organism associated with witches' broom disease of pigeonpea in Florida" in Phytopathology Vol. 4 pp. 300-307. 1991.

Table 1. Results from dot hybridizations of ^{32}P-labeled cloned pigeonpea witches'-broom mycoplasmalike organisms (MLO) DNA to DNA extracts from healthy plants or plants affected by various MLO-associated diseases

Probe DNA[a] (insert size, kbp)	Hybridization with DNA extracts from[b]											
	HP	HPP	WB	WBP	EAY	DAY	FWB	MBSF	MBST	LY	VR	WX
WBA3I(1.1), WBA9I(2.5), WBA16I(1.5), pWBA27(0.6), WBA28I(1.3), WBA36(3.2), pWBA40(0.5), WBB3I(2.0), WBB5I(2.1)	-	-	+	+	-	-	-	-	-	-	-	-
WBA13I(1.6), pWBA48(1.7), WBB7I(2.9)	-	-	+	+	W	-	W	-	-	-	-	-
WBB2I(3.1)	-	-	+	+	+	-	+	-	-	-	-	W
WBA12I(1.4)	-	-	+	+	+	+	+	W	W	-	W	W

[a]Probes consisted of either cloned MLO DNA inserts (I) only, or the recombinant plasmids (p).
[b]+, Positive hybridization; W, weak hybridization; -, no hybridization signal. Each data point represents results of hybridizations of labeled probes with DNA from: HP, healthy periwinkle; HPP, healthy pigeonpea; WB, witches'-broom infected periwinkle; WBP, witches-'broom infected pigeonpea; periwinkle with EAY, Eastern aster yellows; DAY, Western dwarf aster yellows; FWB, Florida periwinkle witches'-broom; MBSF, sweet corn with maize bushy stunt, Florida isolate, or MBST, Texas isolate; LY, Chinese fan palm (Livistona chinensis) with lethal yellowing; VR, beet leafhopper-transmitted virescence agent infected periwinkle; WX, Western-X infected celery. None of the probes hybridized to DNA from healthy corn, palm, or celery.

d) Detection of the WB MLO in pigeonpea:

Dot hybridizations were used to assess the utility of probes for detecting the WB MLO in field-collected pigeonpea samples. Hybridization signals obtained by screening replicate membranes containing DNA samples from nine WB-affected plants by using either WBA28I or WBA12I as probes. Neither probe gave any discernible hybridization signal with DNA from healthy pigeonpea or periwinkle tissues. Both probes gave positive signals with all nine samples that were tested and with DNA from WB-affected periwinkle included as a positive control. Furthermore, the intensity of signals was observed to vary considerably from sample to sample and depended on the particular probe used.

e) Pigeon pea rhabdovirus and mosaic virus:

In the last twenty four months we have observed the occurrence of the rhabdovirus disease and a new mosaic virus disease in the field. The former produces vein yellowing symptom; the latter produces general mosaic symptom. The rhabdovirus was not sap transmissible. The mosaic virus was successfully transmitted to the cucurbits and legume plants. The preliminary results of mechanical inoculation were summarized in Table 2. The punitive vector of the mosaic virus is tentively identified as Aphis nasturtii Kult. We have successfully transmitted the pigeonpea rhabdovirus from pigeonpea to pigeonpea by means of the leafhopper, Empoasca hastosa. The transmission efficiency was at 10-13%. The inoculated pigeonpea plants developed vein clearing symptoms identical to that of the source plants. The electron microscope examination has confirmed the existence of rhabdovirus in the infected tissue. The aggregates of virus particles were mostly found in the cytoplasm of the infected cells (see attached figure).

Table 2. Host reactions and symptoms of pigeonpea mosaic virus

Host	symptoms/transmission
pigeonpea (*Cajanus cajan* L. Millsp.)	veinal clearing & recovered (-)
Zucchini squash (*Cucurbita pepo* L. Dixie)	chlorosis & yellowing (+/-)
watermelon (*Citrullus lanatus* Royal jubilee)	mild mosaic & yellowing (+)
cucumber (*Cucumis sativus* Burpee HybridII)	chlorotic spots (+)
sorghum (*Sorghum bicolor* L. Moench)	red brown necrosis & no symtom (+/-)
corn (*Zea mays* Gardian)	no symtom (-)
pepper (*Capsicum frutescens* Tabasco)	chlorotic spots & recovered (-)
globe amaranth (*Gomphrena globosa*)	chlorosis (+)
soybean (*Glycine max* Century 84 & Evans)	veinal clearing, (+) mild mosaic, & tip marginal necrosis
chenopodium (*Chenopodium amaranticolor*)	chlorotic spot (+/-)
rye (*Secale cereale* L.)	no symptom (-)
bean (*Phaseolus vulgaris*)	(+)
Stringless green pod	chlorosis,
Pencil Pod	mottling, veinal mosaic chlorosis, veinal mosaic
More plants were inoculated, but the results are pending:	Alfalfa (DK125 & Apollo Surpreme) Tobacco (Xanthi and Samsun NN) Watermelon (Crimson Sweet) Bean (Topcrop) Pea (Wando)

f) Pigeon pea mosaic virus.

1) Isolation and purification of pigeonpea mosaic virus.

This virus is saptransmissible to Phaseolus bean. After initial tests on extraction and clarification methods, the following procedure was adopted: fresh tissue was extracted in four volumes (w/v) of cold 0.1 M sodium citrate, pH 6.5, containing 0.01 M disodium EDTA and 0.04 M sodium sulfite, and 4% activated charcoal. After filtration through cheesecloth, the extract is acidified to pH 5.0 by dropwise addition of glacial acetic acid, stored at 4C for 4 hr, and clarified by centrifugation at 17,400 g for 20 min. The virus was concentrated by ultracentrifugation at 100,000 g for 1.5 hr using 20% sucrose cushion in extraction buffer. The pellet was resuspended with 0.05 M sodium citrate (pH 7.8) containing 0.01 M disodium EDTA and 0.04 M sodium sulfite. After centrifugation at 17,400 g for 10 min, repeat high speed centrifugation with 20% sucrose cushion in 0.05 M sodium citrate. The resuspended pellet was centrifuged in 10-40% (w/v) rate-zonal sucrose density gradients using the Beckman SW28 rotor (26,000 rpm for 4 hr). The virus-containing zones were concentrated by centrifugation at 100,000 g for 3 hr.

2) Electrophoresis of intact virus particles.

The viral proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Aliquots of the virus preparations were disrupted in Laemmli sample buffer. Molecular-weight standards were used from Bio-Rad Laboratories. The proteins were electrophoresed in 12.5% SDS-PAGE gels and stained with Coomassie Brilliant Blue.

The analysis of the viral proteins showed two distinctive polypeptide bands, one with the molecular weight of about 17 kD and another of 56 kD. These two components could be observed and separated by repeated rate-zonal gradient centrifugation. These protein profiles were very similar to those of pea enation mosaic virus Tu strains (Reisman and de Zoeten, 1982; Adam et al., 1979; Hull, 1977). Pea enation mosaic (PEMV) is a virus with two centrifugal components which can be transmitted mechanically and by several aphid species in a persistent manner. PEMV has a bipartite genome structure containing either two or three RNAs encapsidated in the two nucleoproteins (Hull and Lane, 1973).

3) Isolation and analysis of viral double-stranded RNA (ds-RNA).

Extraction and purification of ds-RNA were performed by methods of Morris and Dodds (1979) with minor modifications. Three grams of pigeonpea tissue showing mosaic symptoms were ground to a powder in liquid nitrogen with a mortar and pestle. The frozen powder extracted with TSE buffer, bentonite, SDS, 2-mercaptoethanol, phenol, and chloroform:isoamylalcohol. The ds-RNA was purified by one or two cycles of chromatography on CF-11 cellulose column and fractionated on 5% polyacrylamide gels for 4 hr at constant 100 V. Prior to electrophoresis to confirm the nature of the ds-RNA, samples were incubated with DNase I. After electrophoresis, the gels were treated with RNase and stained with ethidium bromide.

4) Electronmicroscopic study of pigeonpea mosaic virus.

Several attempts were made to visualize the pigeonpea mosaic virus (PMV). The final purified preparations were sent to the Electronmicroscope Lab in Gainesville, FL for examinations. No virus particles were found in the samples. Based on EM micrographs of the thin sections, we realized that PMV particles were localized within the chloroplasts of infected pigeonpea and bean tissues. Therefore, the purification methods were changed to disrupt chloroplast membranes by use of Vitris homogenizer and the method of Palmer [Palmer, J. 1988. Isolation of chloroplast DNA. In "Methods for Plant Molecular Biology". Eds. (Weissbahc, A., and Weissbach, H.) Acad. Press]. After isolation of chloroplasts, the preparation was treated with Triton X-100 to disrupt chloroplast membranes. Again no virus particles were recovered from the rate-zonal sucrose gradients (10-40%) or final resuspended pellets.

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g) Pigeonpea rhabdovirus.

During this period a considerable amount of time was devoted to purify the pigeonpea rhabdovirus. Several attempts to purify the rhabdovirus by the procedures developed for Potato yellow dwarf virus (PYDV) (Falk and Weathers, 1983; Hsu and Black, 1973), Sonchus yellow net virus (SYNV) (Jackson and Christie, 1977), Maize mosaic virus (MMV) (Falk and Tsai, 1983), Cynodon chlorotic streak virus (CCSV) (Lockhart et al., 1985), Strawberry crinkle virus (SCV) (Hunter et al., 1990), and Tomato vein-yellowing virus (TVYV) (Maataoui et al., 1985) have failed. In the initial experiments to purify pigeonpea rhabdovirus, we tested several variables and modifications, we found that every one of these factors was critical for efficient recovery of the virus. These

variables included host plants from which virus was purified and purification strategies. The purification procedures, which have been successfully employed for other rhabdoviruses, did not work for pigeonpea rhabdovirus. We have failed to find the typical virus-containing zones in the sucrose gradient or final suspension. Similarly no results were obtained with the PEG method of Hsu and Black (1973). We have used the conventional buffer systems for rhabdovirus purification such as Tris-HCl, phosphate, glycine, and sodium citrate buffers with addition of $MgCl_2$, sodium sulfite or 10% sucrose. To clarify the host contamination we have used celite analytical filter aid (Manville Corp.) or Celite 503, which was known especially critical for virus recovery. In addition, the successful CCSV purification (Lockhart et al., 1985) using activated charcoal filtration also gave us unsatisfactory results. It appears that this non mechanically transmissible pigeonpea rhabdovirus is more difficult to purify than those of known rhabdoviruses.

The EM micrographs showed that pigeonpea rhabdovirus particles were totally bound by cellular organelle-like membrane in the infected tissue. During this reporting period, we have tried many times to homogenize the infected pigeonpea tissue by means of virtis homogenizer at various speeds with various extraction buffer systems. No light scattering band was detected in 5-30% or 10-40% sucrose density gradients. The ultrasonic homogenizer was also used at various speeds from 5-90 seconds at 5 sec. intervals in combination with several buffer systems containing glycine, $MgCl_2$ and EDTA. Again the results were all negative. Since the optimal pH for most rhabdovirus extractions is near neutrality. We have tried to extract the virus from pH 6.5 to 8.4. Another difficulty encountered during the extraction process is the quick oxidation of pigeonpea tissue extract. The addition of reducing agent (2-mercaptoethanol) or autioxidents (sodium sulfate or DIECA) to the extraction process did not overcome this difficulty.

Although we have failed to purify this rhabdovirus from the infected pigeonpea tissue, we are successful to transmit this virus from pigeonpea to pigeonpea by E. hastosa. We will continue to search for an alternate host for the rhabdovirus by leafhopper transmission. Hopefully a better and easier plant can be found.

Literature cited:

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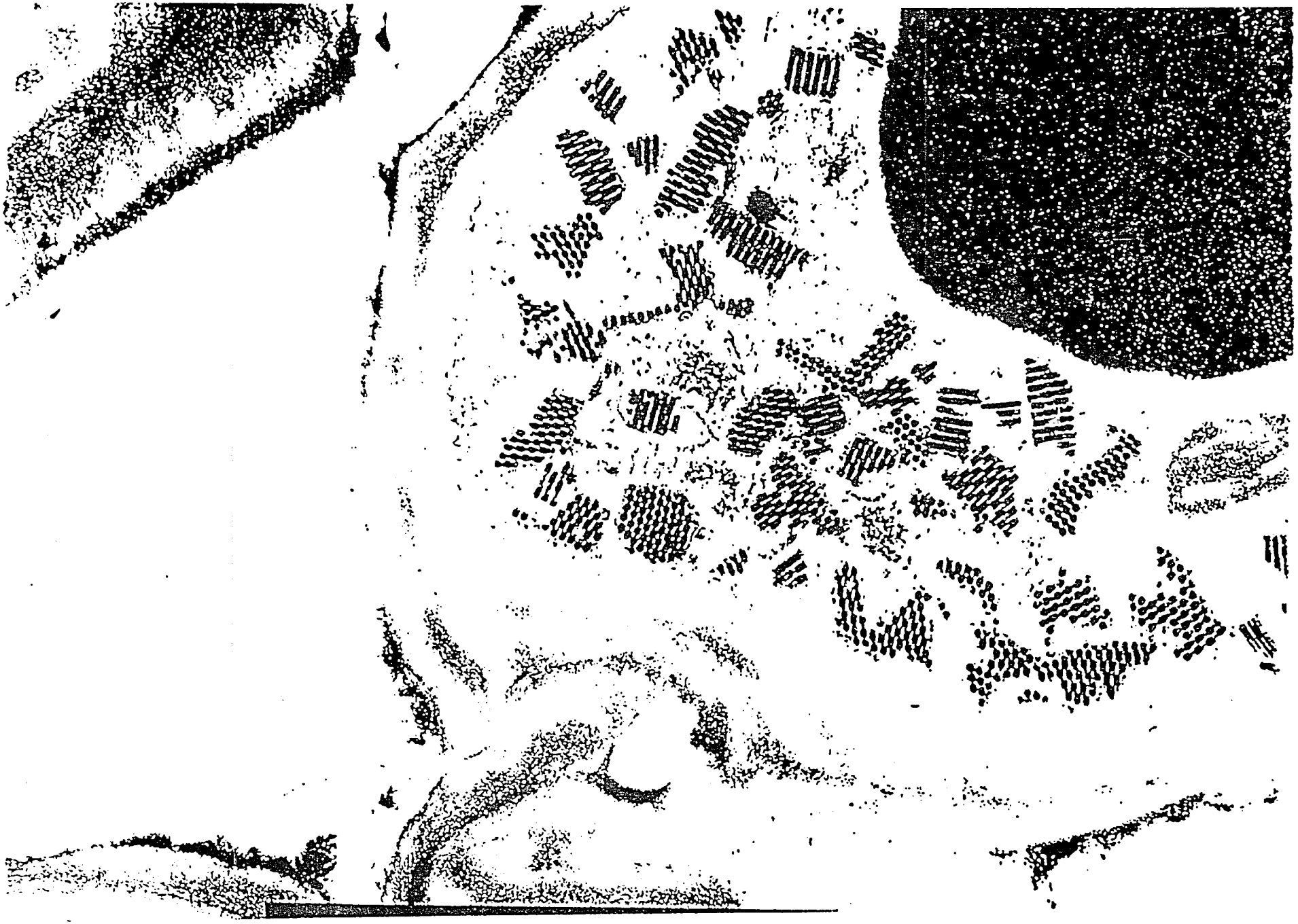
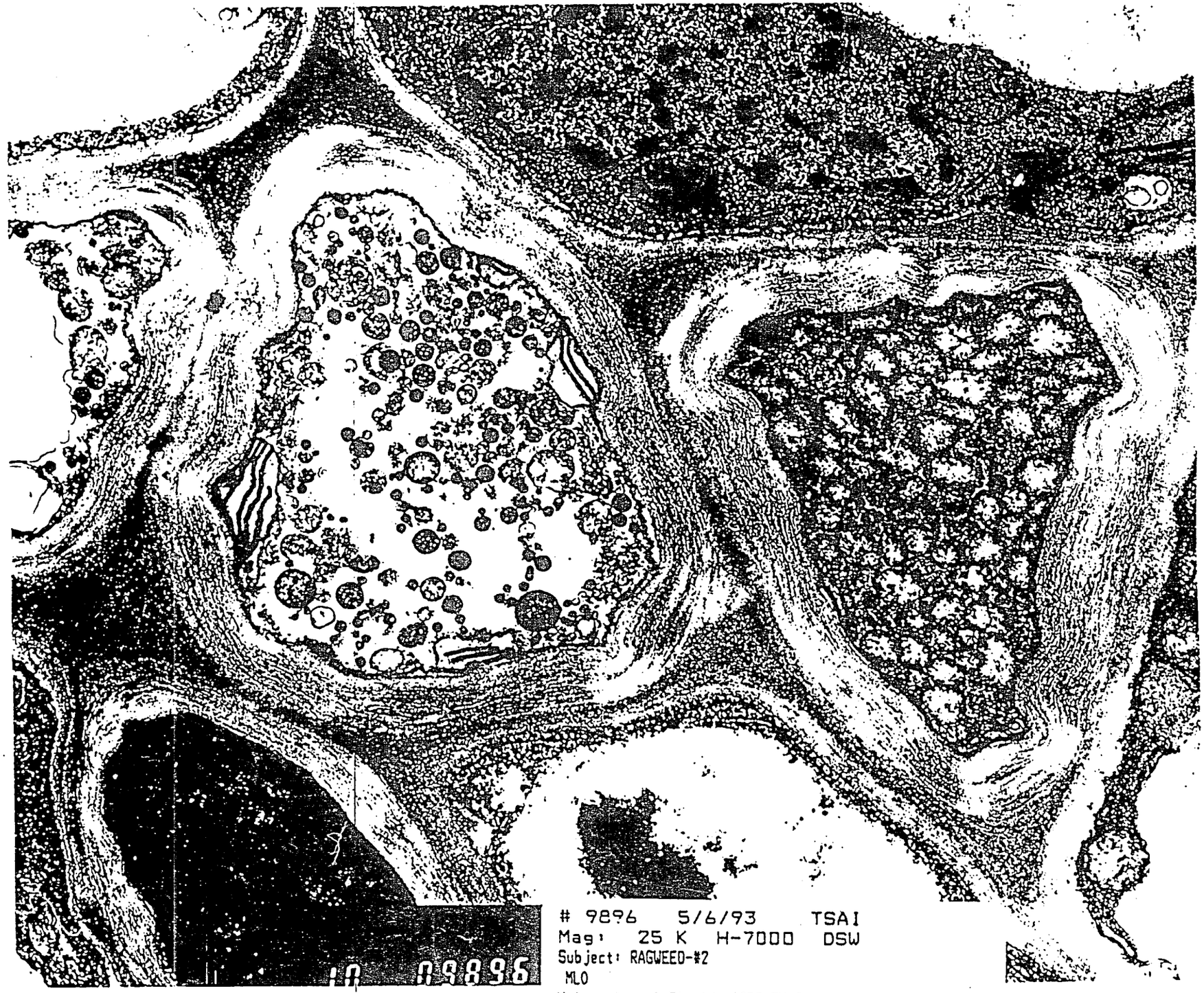
h) Searching for pigeonpea WB MLO alternate hosts:

Using specific DNA probes in search for alternate hosts for pigeonpea witches' broom (WB) mycoplasmalike organism (MLO) in Puerto Rico and Florida we have found that periwinkle (Catharanthus roseus (L.) G. Don) plants in Puerto Rico served as a natural alternate host for the disease agent. In Florida we have found the Crotalaria spectabilis Roth served as a natural alternate host for the pigeonpea WB MLO. In Florida, periwinkle plants have been frequently found to be infected with WB and/or virescence MLOs, however they are not the same MLO as pigeonpea WB based on DNA probe hybridization tests. These findings are significant in studying the epidemiology of this disease.

We have found the ragweed (Ambrosia artemisiifolia L.), a common weed in the pigeonpea fields expressing WB symptoms. The infected tissue was fixed and examined under electron microscope. The MLOs were found abundantly in the phloem cells (see attached figure). This is the first report on ragweed infected with MLOs. Further investigation revealed that ragweed MLOs are more closely related to periwinkle WB MLO than that of pigeonpea WB based on DNA probe hybridization tests. To date we have positively identified two leafhopper vectors of ragweed WB MLO as Gyponana germari (Stal) and Penestragania robusta (Uhler).



Electron micrograph of pigeon pea cell infected with pigeon pea rhabdovirus. Note the aggregates of rhabdovirus particles in the cytoplasm of the infected cell.



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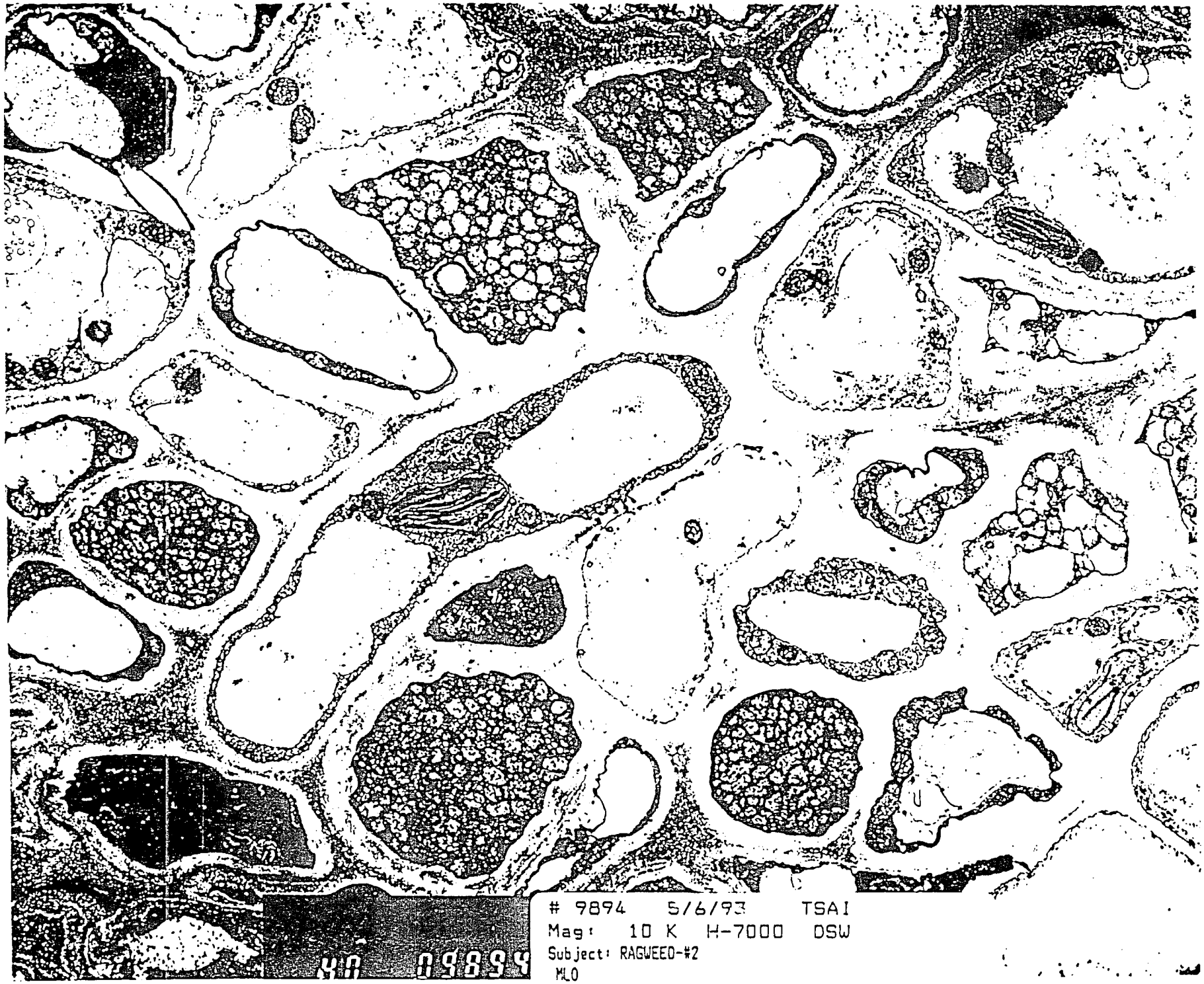
University of Florida ICBR EM Core Laboratory

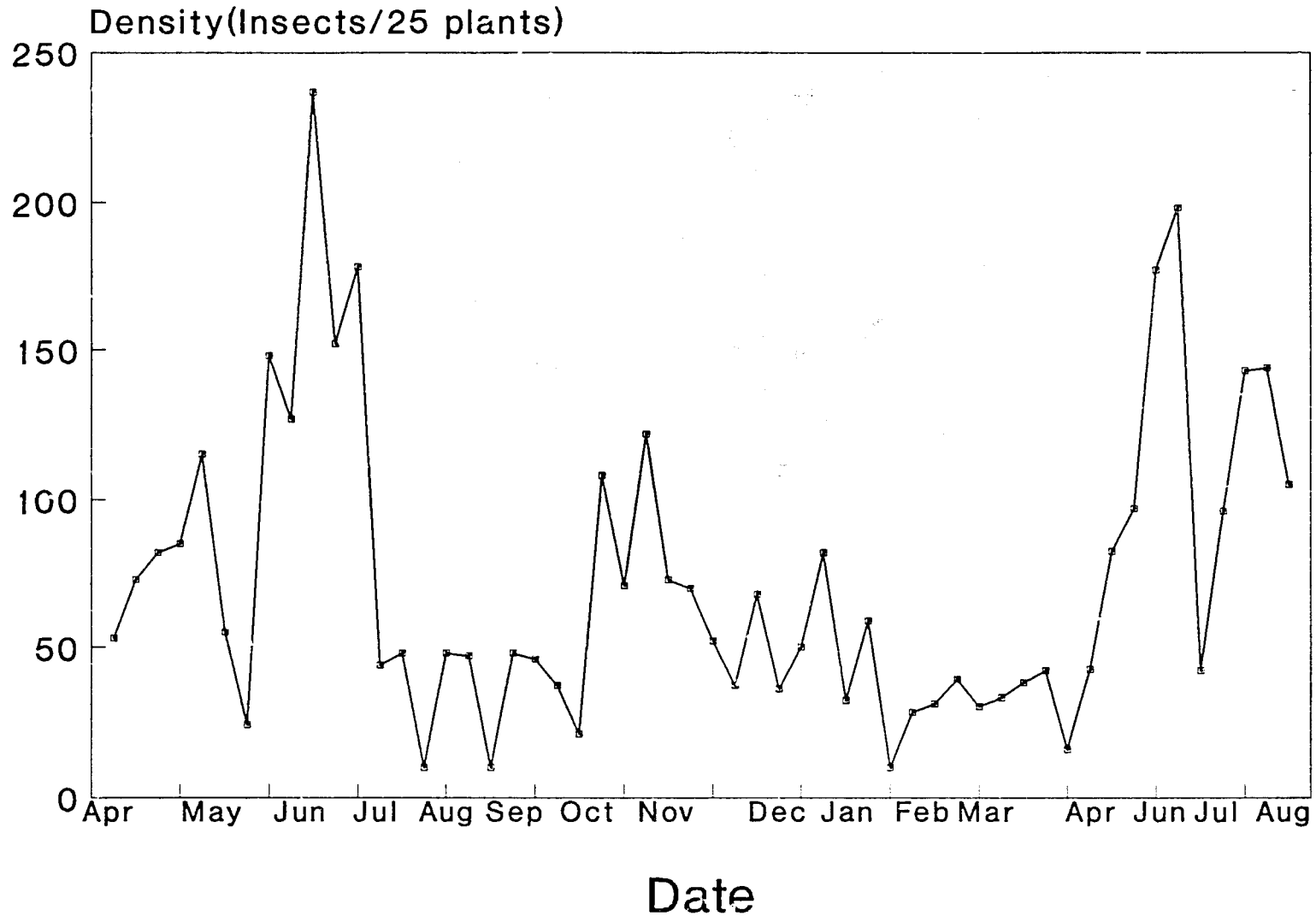


9894 5/6/93 TSAI
Mag: 10 K H-7000 DSW
Subject: RAGWEED-#2
MLO
University of Florida ICBR EM Core Laboratory

Field study of *E. hastosa*.

The population dynamics of *E. hastosa* was studied in a pigeonpea field in Homestead, FL for 16 months. Twenty-five plants were randomly examined and *E. hastosa* counts were recorded weekly. The results were summarized in the following figure. It was evident that the populations fluctuated throughout the year. There were two peaks; a major peak occurred in June and the other occurred in November.

Population Dynamics of Empoasca hastosa
in Pigeonpea Field



Bionomics of Aphid nasturtii.

A detailed study on the biology of A. nasturtii was conducted in the laboratory at seven temperatures. The following is the summary of A. nasturitii biology. It is to be submitted to the Entomological Society of America for publication in "Environmental Entomology".

Introduction.

Pigeonpea (Cajanus cajan (L.) Millsp.) is an important food crop in the Caribbean and other subtropical and tropical countries. About 2.9 million hectares of pigeonpea are grown in the world as a valuable food crop (Kannaiyan et al. 1984). Many diseases and insect pests are known to affect pigeonpea (Barnes 1973, Kannaiyan et al. 1984). The occasional outbreak of pigeonpea aphid (Aphis nasturtii Kult.) and its transmission of unknown pigeonpea mosaic virus have prompted our attention to the study of this insect. Pigeonpea aphid (Buckthorn aphid) is a world wide pest infesting composite, legume and solanaceous crops. It is regarded as a major pest in many potato grown areas in Europe, North America and South Africa (Cook 1984, Miller 1991) and is one of the principal insect vectors of potato virus Y. S. and M. (Damroze 1989, Kostiw 1975a,b. 1984, Kurppa et al. 1986, Piron 1986, Sigvald 1984).

In the last three decades, the emphasis of the research on A. nasturtii was placed on its ability to transmit various isolates of potato virus. A few studies were devoted to monitoring population dynamics in the fields and control of this aphid (Neitzel et al. 1970, Khristova et al. 1981). Little is known of the biology of A. nasturtii. The essential information on development rate, temperature thresholds, age-specific fecundity, and survival of this species is lacking. The development of sound pest management programs must be based on a thorough understanding of the pest's biology and ecology on particular crops. Therefore, we initiated a study on development and reproduction under laboratory conditions to provide an experimental basis for developing predictive models of A. nasturtii on pigeonpea.

Materials and Methods.

Laboratory colonies of A. nasturtii were derived from a single apterous female in the field in Broward County, Florida. The colonies were maintained on potted pigeonpea plants (Cajanus cajan (L.) Millsp.) in a growth chamber at 24 ± 1^{0} C, 70-90% RH and a photoperiod of 12:12. Third leaves from the top of pigeonpea seedlings were detached and used throughout the study. For each experiment, about 200 young apterous adult females of the same age were placed on five pigeonpea seedlings for nymph production at 25^{0} C. Forty to 50 first instar nymphs collected within 4 h of birth were used for each temperature experiment. Each nymph was transferred singly to lower-surface of the detached leaf and caged with a clip cage (2.5 x 1 cm with a nylon cloth top). Fifteen to 20 caged aphids as a group were placed in each of three trays (15 x 15 cm) lined with a moist filter paper. The trays were placed in the growth chambers (Percival Scientific Inc., Boone, IA) at 10, 15, 20, 25, 27, 30 and 35^{0} C, 70-90% RH and a photo period of 14:10 (L:D).

Individual insects were checked twice daily for ecdysis (i.e. for life stage and developmental times) and survivorship. The exuviae were used to determine molting time. After the initiation of nymphal reproduction the adult

mortality and fecundity were then recorded daily and all offsprings were removed from each cage until the death of adult. All test leaves were changed daily throughout the study period.

Differences in developmental times, survivorship and reproduction at different constant temperatures were analyzed by student-Newman-Keul (SNK) multiple range test (GLM procedure, SAS Institute 1988). A linear regression technique was employed using growth rate data as dependent variables (y-axis) and constant temperature treatments of 10-25^{0} C as independent variables (x-axis). Development above 25^{0} C was outside the linear segment of the growth curve and therefore not included in the linear regression. The lower developmental threshold was determined as the x-intercept of the linear equation. The degree-day requirements were determined as the value of the inverse of the linear equation slope.

The nonlinear biophysical model developed by Sharpe and DeMichele (1977) and modified by Schoolfield et al. (1981) was used to describe the temperature-dependent development of immatures:

where r(T) = mean development rate at temperature T(^{0}K); R = the universal gas constant (1.987 cal degree^{-1} mole $^{-1}$); TL (or TH) = Kelvin temperature at which the rate-controlling enzyme is half active and half low-temperature (or high-temperature) inactive; RHO25, HA, HL, and HH are parameters to be estimated. A SAS program developed by Wagner et al. (1984) using Marquardt's techniques (Marquardt 1963) was used to fit the model to various instar data sets.

Life table statistics were calculated for the populations at different temperatures as described by Hulting et al. (1990). The differences in r_m values among populations were also tested after Newman-Keul sequential test (Sokal and Rohlf 1969) based on Jackknife estimate of variance for r_m (Meyer et al. 1986). For any difference between two r_ms from the sequence, in which r_m were arrayed in order of magnitude, to be significant at level, it must be equal to or greater than:

Where k = number of r_m in the set whose range is tested. The degree of freedom equal v. ni and nj were sample sizes of the two r_ms, and Q [K.V] is a value from the table of the studentized range. S^2av is the weighted average variance of r_m and it is calculated as:

Where a equals the number of r_m to be tested, the sample size of ith r_m is ni, S^2i is Jackknife estimate of the variance for ith r_m.

Results.

Development of Immature. Development times for the immature stages at seven temperatures are presented in Table 1. The rates of development of four nymph instars accelerated significantly as constant temperatures increased until they reached the maximum developmental rates (Table 1, Fig. 1). The maximum developmental rates for first through fourth instars occurred at 30° C; the respective developmental periods were 1.4, 1.4, 1.2 and 1.2 d. Extremely high temperature (35° C) caused a decline in development rate compared with the lower temperatures.

A linear regression analysis was applied to the developmental points within the 10-25° C range, within which the development rates of the insect increase linearly with the increase of temperature, and the theoretical developmental thresholds (i.e. the point where the development rate presumably equals 0) were estimated at 4.5, 6.0, 4.6, and 7.5^{o} C, respectively for first instar through fourth instar (Fig. 1, and Table 2). Developmental durations on a physiological time scale were showed in Table 2. Degree-Day (DD) requirements for the first instar through the fourth instar were 39.8, 39.0, 42.3, and 26.0 DD. It required 150.38 DD for the first instar to develop into adult based on 5.4^{o} C developmental threshold for overall immature stages.

The modified nonlinear four-parameter model with high temperature inhibition (Wagner et al. 1984) gave a good fit to the data sets within the range of 10-35° C (Table 2, Fig.1). Therefore, the thresholds of high-temperature inhibition ranged from 29.5 to 35.4^{o} C were derived from the nonlinear model for development of first through fourth instars.

Extreme temperature had a detrimental effect on the survivorship of immature stage (Table 3). The aphids reared at 10 and 35° C had significantly lower maturation of 15.2 and 37.5%, respectively compared with the average survival of 60.0-87.5% at 15-30° C ($P<0.05$). The highest nymphal mortality occurred in fourth instar at extreme temperatures of 10 and 35° C (Table 3).

Adult Longevity and Reproduction. Temperature also affected adult longevity and fecundity significantly (Table 4, Fig. 2). The mean longevity of adults increased with decreasing temperature and reached a maximum of 30.2 d at 15° C, then reduced to 13.1 d at 10° C. No mortality occurred until 22 and 17 d after emergence of adults at 15 and 20° C, respectively. A decline on the survival curve appeared at 5-7 d after emergence when the populations were exposed to temperature above 25° C. The longest longevities for individual females were 20, 42, 38, 25, 21, 16 and 10 d at 10 through 35° C.

Fecundity was also adversely affected by extreme temperatures (10 and 25° C). The females reared at 15 - 27° C range produced approximately ten times as many offsprings (average progenies from 45.8 to 55.0 per female) as those did at 10 and 35° C (2.8 and 5.5 offsprings per female, respectively).

Age-specific nymphal production at various temperatures is shown in Fig. 2. Most females started producing nymphs within 24 h following emergence. The daily rate of nymphal production, the number of nymphs per female per day, varied with ages. The production peaks appeared at 4 through 10 d after emergence with values of 3.0, 2.8, 4.3, 5.3, 3.2, and 1.7, respectively at 15, 20, 25, 27, 30 and 35° C. The duration of reproductive periods lasted as long as adult longevity at all temperatures. The lower temperature limit for reproduction was near 10° C, and the upper limit was near 35° C (Table 4).

The intrinsic rate of increase (r_m), net reproductive rate (Ro), mean generation time (T), and population doubling time (t) were calculated for the populations at different temperatures and summarized in Table 5. The largest r_m occurred at 27^0 C. In contrast, the populations reared at both extreme temperatures of 35 and 10^0 C had a negative r_m value (i.e. - 0.014 and -0.022). The r_m values for populations at 25 and 30^0 C were essentially the same. The intrinsic rate of increase for the population at 15^0 C was only 46.4% of the population at 27^0 C.

Discussion.

Although insects are not always subject to constant temperatures in nature, such controlled study could provide a valuable insight into the population dynamics of a particular species (Summers et al. 1984). Our results reported here clearly showed the effects of temperature on the development time, survival, longevity, fecundity, and fecundity rate of A. nasturtii.

Extrapolation of the temperature-rate curve to the zero- rate axis indicated that the development threshold of the fourth instar was higher than those of other instars (Table 2). It was evident that fourth instar had much higher mortality when exposed to extreme temperature at 10^0 C (Table 3).

It is well known that insects reared at temperatures above the upper thresholds have slower rates of development than insects reared under more favorable conditions (Messenger et al. 1959, Stinner et al. 1974, Sharpe and DeMichele 1977, Curry et al. 1978). Our data showed a similar effect. The extremely high temperature (35^0 C) caused a decrease in development rate from a linear trend (Fig. 1). The nonlinear biophysical model which reflects the adverse effect of extreme temperature on development rate was applied to describe the relationship between development rate and temperature, and the upper-threshold temperatures, at which the rate-controlling enzyme is half active and half high-temperature inactive (Wagner et al. 1984), were calculated for all the immature stages. The smaller upper-threshold 29.5^0 C for fourth instar indicated that high temperature had a more adverse effect on development of fourth instar than it did on other stages. It showed that the fourth instar had significantly higher mortality (31.8%) when exposed to extreme temperature at 35^0 C (Table 3).

The optimal temperature for shortest development time was between 27 and 30^0 C. The highest nymphal production (55.0 nymphs per female) occurred at 15^0 C; the highest mean rate, 5.31 nymphs per female per day, occurred at 27^0 C. The maximal longevity and reproductive period were at 15^0 C. The highest percentage of nymphal survivorship was at 15^0 C. At 20^0 through 30^0 C, the percentages of nymphal survivorship were slightly lower than it was at 15^0 C. The lowest percentages of nymphal survivorship were at both extreme temperatures (10 and 35^0 C) (Table 3).

The intrinsic rate of natural increase (r_m) is a good indicator of temperature at which the growth of a population is most favorable. This population parameter reflects comprehensively the effects of temperature on the development, reproduction and survival characteristics of a population. Table 5 showed that the population reared at 27^0 C had the highest r_m value (0.332)

among all temperatures, this is due to its faster development (Table 1), higher survivorship of immature stages (Table 3) and higher total progeny production (Table 4) as well as considerably higher daily rate of progeny production (Fig. 2). In contrast, the populations exposed to extreme temperatures (15^0 and 35^0 C) had a prolonged development and reduced survivorship of immature stages as well as reduced fecundity. As a result, both populations had negative growth rates. The populations reared at 15^0 and 20^0 C showed much smaller intrinsic rates of increase than the population at 27^0 C despite that they had higher or similar total progeny production (Table 4) and survivorship of immature stages (Table 3). The reduction in r_m could attribute to longer immature development time (Table 1) and longer reproductive period (Table 4), both have resulted in a considerably longer mean generation time (25.2 and 17.8 d). On the other hand, the population reared at 30^0 C had a much lower progeny production than those reared at 20 and 25^0 C (Table 4), but it had a higher r_m (Table 5). The increase in r_m could be due to significantly faster development of immature stages and shorter reproductive period which have resulted in a considerably shorter mean generation time (9.2 D). The similar effects were reported by Messenger (1964) and Graham (1959) on spotted alfalfa aphid, Therioaphis maculata. The maximal r_m of 0.41 at a fluctuating temperature averaging 29.4^0 C or maximal r_m of 0.35 at a constant temperature of 25^0 C were found for the laboratory population of I. maculata. When the populations exposed to the extreme temperatures near 6 and 33^0 C, the populations ceased to grow.

The response to the extreme temperatures varied considerably with aphid species. The developmental thresholds from 4.5 to 7.5^0 C for various nymphal stages in this study are higher than the threshold of 3.45^0 C reported by summers et al. (1984) for Acyrthosiphon kondoi at 11 constant temperatures (4.4 to 32.2^0 C) and lower than the threshold of 9.7^0 C found by Nowierski et al. (1983) for Chromaphis juglandicola at varying temperatures in the field.

Other researches showed that A. kondoi produced an average progeny of 24.81 per female at 7.2^0 C (Summers et al. 1984), and I. maculata produced 40 nymphs per female at 8^0 C (Messenger 1964). However, on an average A. nasturtii produced only 2.8 nymphs per female at 10^0 C.

The upper temperature thresholds for development of A. nasturtii were 29.5^0 C for fourth instar and 35^0 C for other instars. When A. nasturtii exposed to 35^0 C it had a low developmental rate and high mortality (Table 3). Similarly, the upper thresholds for I. maculata were found at 32.2^0 C (Messenger 1964) and 35^0 C (Graham 1959). Other aphids such as C. juglandicola showed an upper threshold at 36^0 C (Nowierski, 1983) and A. kondoi had an upper threshold of 27.1^0 C (Summers 1984).

Liu et al. (1995) have demonstrated that in most instances the differences in development time between constant and varying temperature regimes with the same mean temperature can be ignored in rate functions for phenological models. Therefore, the development-rate model derived from such constant temperature data in this study can be used to estimate the developmental time of insects under natural conditions of varying temperatures within an appropriate range by rate-summation method (Kaufmann 1932).

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Table 1. Developmental periods (d±SE) of immature stages of *Aphis nasturtii* on pigeon pea at seven constant temperatures

Temperture (°C)	n	Stages				
		First Instar	Second Instar	Third Instar	Fourth Instar	Total of Immature
10	17	7.4±0.56a	8.8±0.32a	8.2±0.39a	9.5±0.27a	33.9±0.95a
15	36	3.9±0.17b	4.5±0.09b	4.0±0.12b	3.8±0.10b	16.1±0.21b
20	25	2.4±0.13c	2.9±0.19c	2.7±0.14c	2.0±0.14c	10.0±0.20c
25	28	2.0±0.15c	2.0±0.13d	2.2±0.11d	1.5±0.16d	7.8±0.28d
27	35	1.6±0.08d	1.6±0.09e	1.4±0.09e	1.3±0.09d	6.0±0.08e
30	31	1.4±0.09d	1.4±0.10e	1.2±0.08e	1.2±0.09d	5.7±0.14e
35	12	1.8±0.15cd	1.5±0.14e	1.3±0.13e	2.0±0.17c	6.6±0.19e

Within columns, means with the same letters are not significantly different ($P<0.05$).

Table 2.Parameters of biophysic model and linear model for temperature-dependent developmental rate of immature stages of *Aphis nasturtii*

Life stage	Biophysical Model[a]						Linear Model[b]				
	RHO25	HA	TH[c]	HH	R^{2d}	θ_u[c]	Intercept	Slop	r^2	θ[e]	DD[f]
1st instar	0.5672	14088	307.45	65087	0.98	34.3	-0.1121	0.02510	0.99	4.5	39.8
2nd instar	0.5697	16715	307.50	55023	0.98	34.4	-0.1534	0.02563	0.99	6.0	39.0
3rd instar	0.5471	14905	308.59	173366	0.95	35.4	-0.1095	0.02366	0.99	4.6	42.3
4th instar	0.9019	21633	302.69	54232	0.98	29.5	-0.2886	0.03842	0.99	7.5	26.0

a. The model was modified by removing the first exponential term in the denominator to the form of a four-parameter model with high-temperature inhibition because the article did not have a sufficient number of data points required by the six-parameter model (Wagner et al 1984).

b. The linear model is for the range of 10-25 °C.

c. TH is upper-threshold temperature in Kelvin. θ_u is upper-threshold temperature in Celsius. T(°C)=T(°K)-273.15 .

d. For the non-linear model, R^2 is defined as 1-SSE/CSS, where SSE is the variance of the full model, SSE is the variance of the mean model.

e. The developmental threshold (θ)=-Intercept/Slop.

f. Dregree-Day requirments for development(DD)=1/Slop.

Table 3. Survivorship(%) of immature stages of *Aphis nasturtii* on pigeon pea at seven constant temperatures*

Temperature (°C)	Stages				
	First Instar	Second Instar	Third Instar	Fourth Instar	Total of Immatures
10	72.7d	79.2d	84.2c	31.3d	15.2f
15	95.0a	94.7a	100.0a	97.2a	87.5a
20	77.5c	90.4b	96.4ab	100.0a	67.5c
25	81.2c	89.7b	94.3b	97.0a	60.0d
27	82.5c	93.9a	96.8ab	96.7a	72.5b
30	80.0c	96.5a	93.5b	86.2b	67.5c
35	87.5b	85.7c	73.4d	68.2c	37.5e

*There were three replicates for each temperature,and number of first instars at the begining within each replicate ranged from 15 to 20.

Means within a column followed by different letters are significantly different($P<0.05$).Data were transformed arcsin square root before SNK multiple comparison; untransformed data are presented.

Table 4.Mean fecundity per female and longevity(in days) of female *Aphis nasturtii* on pigeon pea at seven temperatures

Temperature (°C)	n	Mean longevity of female d±SE	Mean no. of progeny / ♀ Mean±SE
10	15	13.1±1.92c	2.8±0.49d
15	32	30.2±2.61a	55.0±2.61a
20	24	26.3±1.37b	47.6±2.65b
25	39	16.6±0.83c	46.8±1.85b
27	35	14.6±0.68c	45.8±2.16b
30	32	8.4±0.62d	17.9±1.27c
35	16	7.0±1.10d	5.5±1.18d

Within columns,means with the same letter are not significantly different at P>0.05(Student-Newman-Keuls multiple range test).

Table 5.Comparison of life table parameters of *Aphis nasturtii* at six constant temperatures on pigeon pea.

Temp (°C)	Parameters					
	n	r	(95%C.I)	R	T	t
10	15	-0.022e	[-0.035,-0.010]	0.42±0.05	40.3	----
15	32	0.154d	[0.150,0.157]	48.10±2.29	25.2	4.51
20	24	0.195c	[0.190,0.201]	32.12±1.79	17.8	3.55
25	39	0.263b	[0.257,0.269]	28.09±1.11	12.7	2.64
27	35	0.332a	[0.322,0.341]	33.23±1.57	10.6	2.09
30	32	0.270b	[0.257,0.283]	12.07±0.86	9.2	2.57
35	16	-0.014e	[-0.048,0.021]	1.03±0.22	8.3	----

n-Number of females in analysis.

r-Jackknife estimate of the intrinsic rate of increase.Within this column,the values with different letters are significantly different ($P<0.05$).

C.I.-Interval estimate for r.

R-Net reproductive Rate(female offspring per adult female).

T-Mean generation Time(in day).

t-Doubling time(in day) for population.

Figure Captions:

Fig. 1. Development rate (r) of first instar (A), second instar (B), third instar (C), and fourth instar as a function of temperature (T) in ^{o} C. Line a is linear regression for the temperature range of 10-25° C; Curve b is nonlinear model for the range of 10-35° C. Circles are observed rates. The parameter estimates are given in Table 2.

Fig. 2. Age-specific survivorship (Lx) and progeny production (Mx) for *Aphis nasturtii* female at 15° C (A), 20° C (B), 25° C (C), 27° C (D), 30° C (E) and 35° C (F).

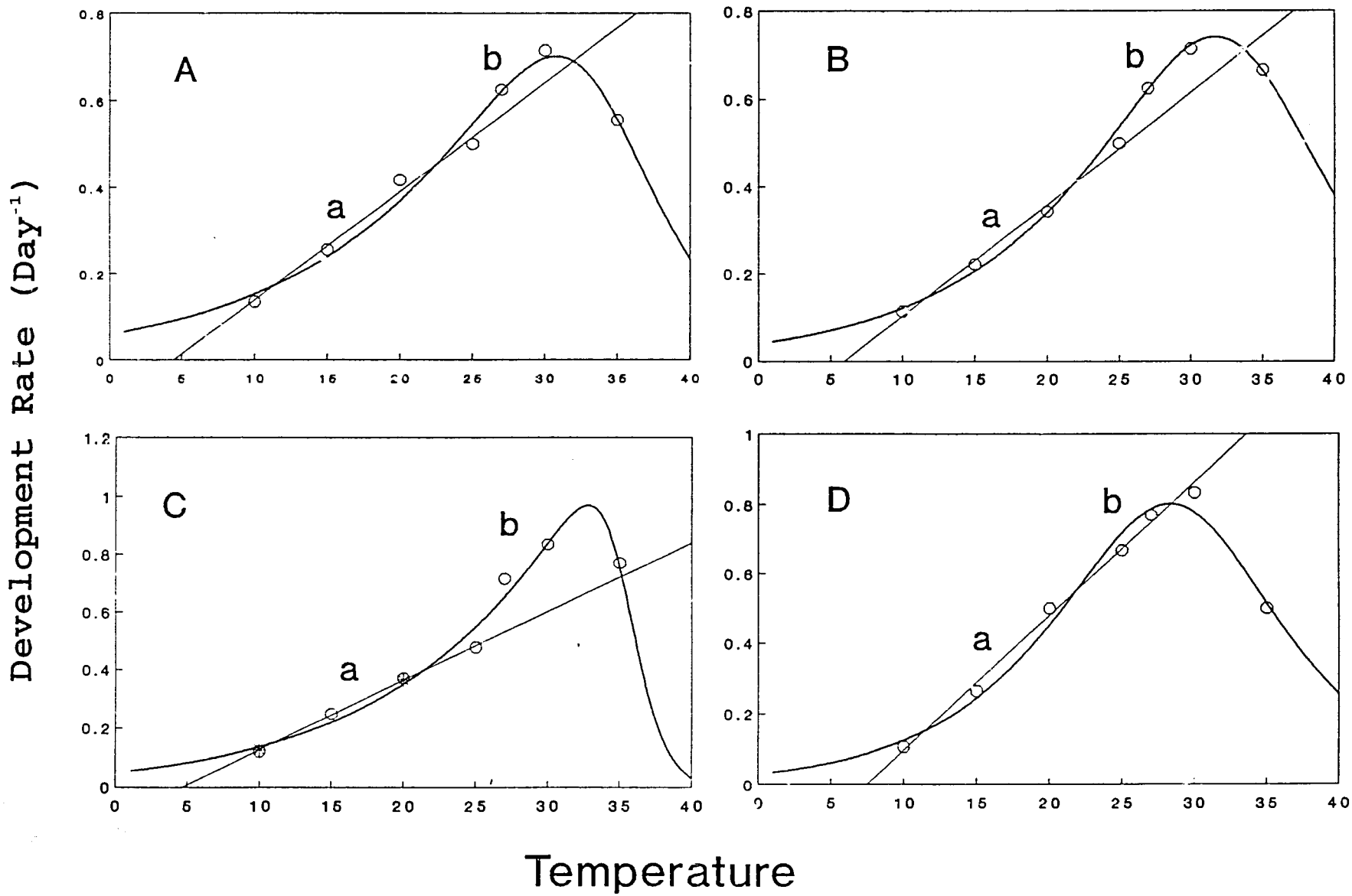

Fig. 1

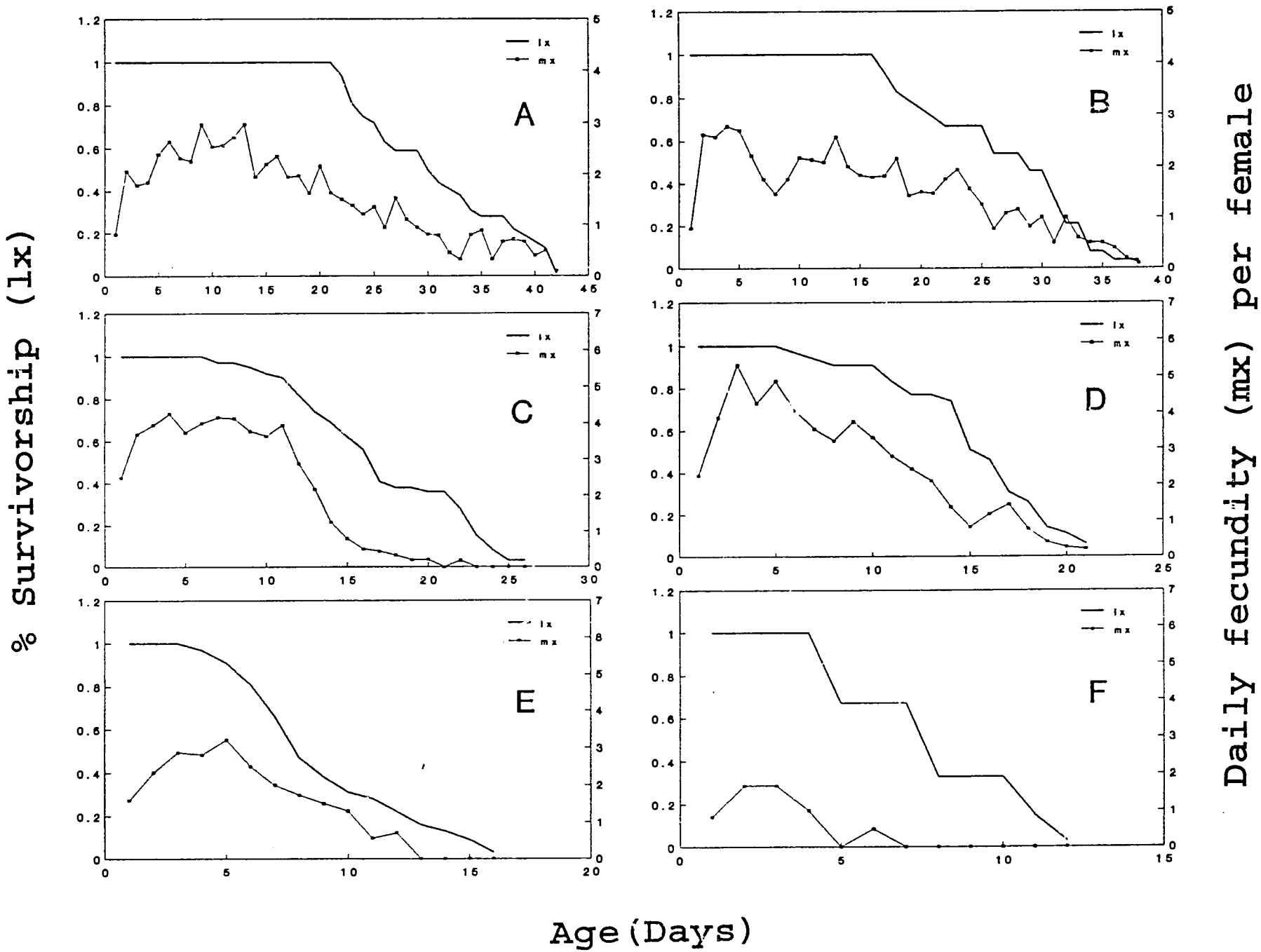
% Survivorship (lx)
Daily fecundity (mx) per female
Age (Days)
A
B
C
D
E
F
lx
mx

Fig. 2.