1992

The Inhibitory Effects of the Extracts of Zingiber Plants on the Adsorption, Growth, and Replication of Phage Lpp-1 in Cyanobacterium

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THE INHIBITORY EFFECTS OF THE EXTRACTS OF ZINGIBER PLANTS ON THE ADSORPTION, GROWTH, AND REPLICATION OF PHAGE LPP-1 IN CYANOBACTERIUM

by

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A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the requirements for the Degree of

Master of Science

January

1992

ACKNOWLEDGEMENTS

I am thankful to Dr. Amrik S. Dhaliwal, Chairman of my thesis committee and my advisor, for his interest, help and continued support throughout this project. I am also thankful to the other members of my committee, Dr. F. Wezeman (Department Chairman), Dr. W. Jones, and Dr. E. Palinscar, for their suggestions and guidance concerning the experimental procedure and the preparation of this manuscript. My thanks also go to Dr. W. Jones and Mr. J. Schluep for teaching me Transmission and Scanning Electron Microscopy and Photographic Procedures. Without their help no Electron Microscopy would have been possible.

I am also grateful to Mr. M. Dean and Mr. J. Walsh for all the help and guidance that they have given me. Thanks also go to my mother, Olga, my sister Denise, and my aunt Victoria for their patience and for their encouragement and financial support.

VITA

The author, Ebby Paul Jido, is the son of Paul Jido and Olga (Yousefi) Jido. He was born November 8, 1965, in Tehran, Iran.

In 1979, he immigrated to the United States and graduated from Saint Gregory High School, Chicago Illinois, in 1982.

He entered Loyola University of Chicago in September, 1982 and received the degree of Bachelor of Science, Cum Laude, with a major in Biology and a minor in Chemistry in May, 1986. While attending Loyola University, He became a national member of Beta Beta Beta Biological Honor Society, Lambda Omega Chapter. He also became a national member of Alpha Epsilon Delta, PreMedical Honor Society.

In September, 1987, he was admitted to the Graduate School of Loyola University leading to a Master of Science degree in Biology under the direction of Dr. Amrik Singh Dhaliwal Ph.D.. He presented an abstract and a seminar at the Illinois State Academy of Science Meeting at the Bradley University in Peoria, Illinois in 1988. He also presented an abstract and a seminar at the Annual American Institute of Biological Science Meeting in the division of Phycological Society of America at the University of Toronto in Toronto,

Canada. As a graduate student, he also became an associate member of Sigma Xi, the Scientific Research Society. The author is currently studying Medicine at the Southern Illinois University School of Medicine in Springfield Illinois.

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INTRODUCTION

Currently, there are only few effective chemotherapeutic agents available for viral infections. Although significant results have been achieved in the chemotherapy of non-viral parasitic diseases, years of research in the development of antiviral drugs has not produced a substantial number of potent chemicals that can be used for the treatment of viral infections with greater success. The problem is in part due to the way the viruses multiply. Unlike other pathogens, viruses are intimately dependent on their hosts for reproduction. Consequently, any compound that can interfere with the multiplication of virus particles also has a tendency to interfere with the metabolism of the host. Should the idea of viral chemotherapy be abandoned?

The opposition toward viral chemotherapy mainly comes from researchers working on the development of vaccines against the viral diseases. Undoubtedly, the success of vaccination against diseases like smallpox and poliomyelitis has been tremendous. However, the development of vaccines is not risk free, particularly when live vaccines are used for the purpose of immunization. Furthermore, the development of vaccines against herpes and influenza viruses has given considerable challenge to the researchers in the field. It is

therefore obvious that if an effective vaccine can not be produced, viral chemotherapy remains the only alternative and the only solution.

There are a variety of natural and synthetic chemotherapeutic compounds available which interfere with the process of viral infection. These compounds, when applied to the host before, at, or after the time of inoculation of the host with virus, reduce the number of infective viral particles. The present thesis deals with the mixture of natural compounds of this type.

The term chemotherapy can be defined in various ways. In this thesis, the word is used to include the effects of compounds which delay or inhibit virus multiplication or disease development.

Most of the work done in viral chemotherapy has been attempted on plant and animal viruses for obvious reasons. Plant viral diseases are of utmost concern in the area of agriculture and animal viral diseases have great importance in the area of clinical medicine. Bacterial viruses (bacteriophages) on the other hand may not provide the best model for usage in viral chemotherapy. An inhibitory compound that is useful against a phage particle will not necessarily be effective against plant or animal viruses. Nevertheless, there are certain advantages in using the bacterial viruses. For example, they offer a system for screening the effects of many viral inhibitory compounds and their assay results of the

infective viral particles are more accurate than assay results of plant or animal viruses. The infectious cycle is extremely rapid in bacterial viruses; in some cases it is completed in few minutes. The host cells in bacterial virus systems are much easier to manipulate than the host cells of plant and animal viruses. Phage chemotherapy is also important because if a chemotherapeutic agent is discovered which operates through its effect on a particular viral enzyme common to all viruses, then its use can be extrapolated against the plant and animal viruses.

The purpose of the present study is to measure the antiviral activity of the rhizome extracts of two plants, Zingiber officinale (fig. 1) and Zingiber zerumbet (fig. 2), on the infectivity and replication of the LPP-1 virus (fig. 3) in its blue-green bacterium host, Plectonema boryanum (fig. 4,5, and 6). This study shows the changes produced by the rhizome extracts in the rate of adsorption of the virus particles on to the host, in the average number of virus particles produced within an infected culture of the host, and in the number of virus particles produced within specific host cells. Furthermore, this study shows the ultrastructural changes in the extract treated cyanobacteria using transmission electron microscope and cationic-probe highlighting techniques.

Fig. 1 60-90 day old plant of Zingiber officinale. Note that the leaves are long and narrow.

Fig. 2 60-90 day old plant of Zingiber zerumbet. Compared to Zingiber officinale, the leaves of Zingiber zerumbet are shorter and wider.

Fig. 3 LPP-1 virus. The viral head capsid is a polyhedron. A short tail is attached to one of the vertices of the head. This virus structurally is very similar to bacteriophage T7. X750,000

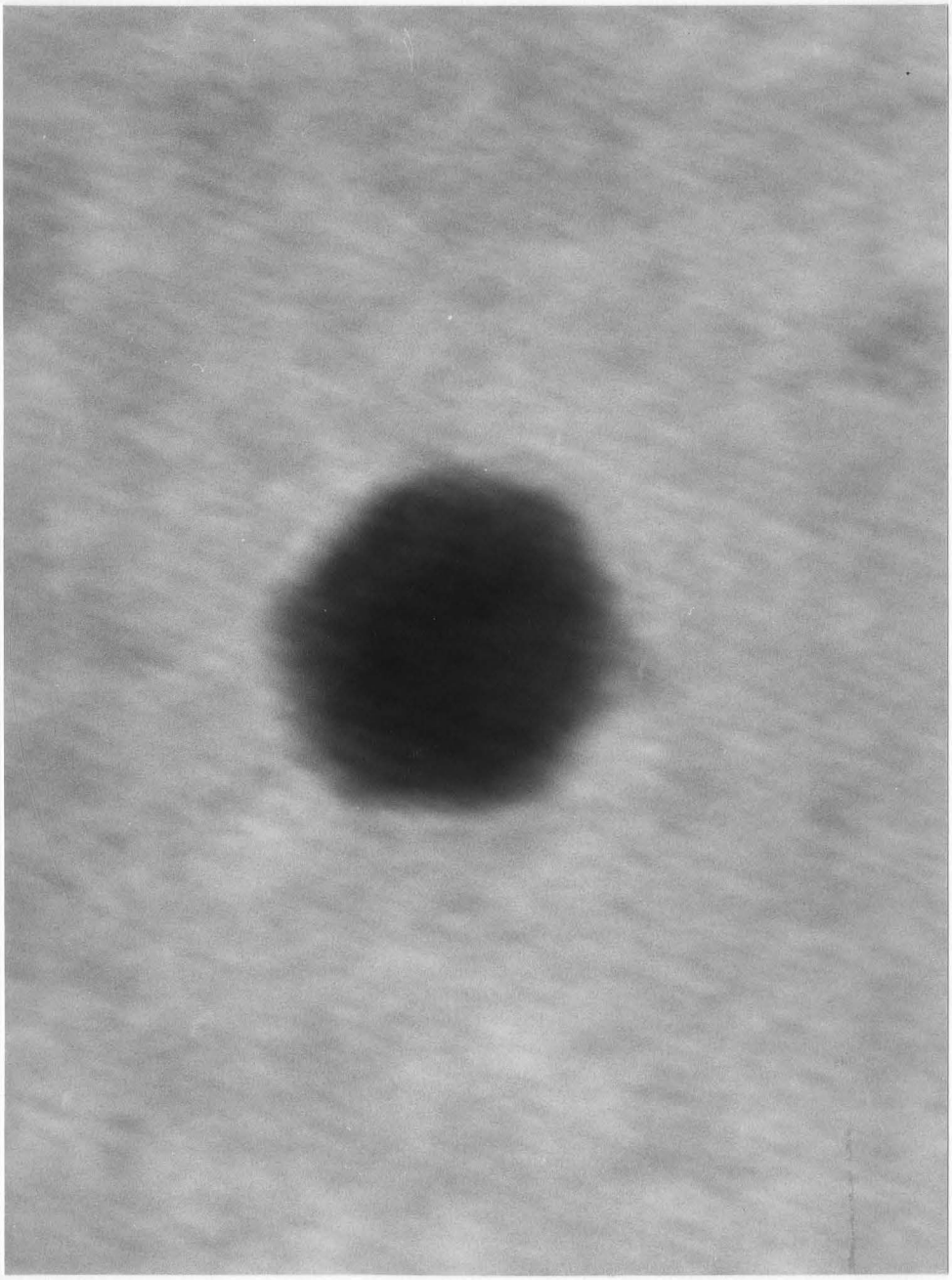
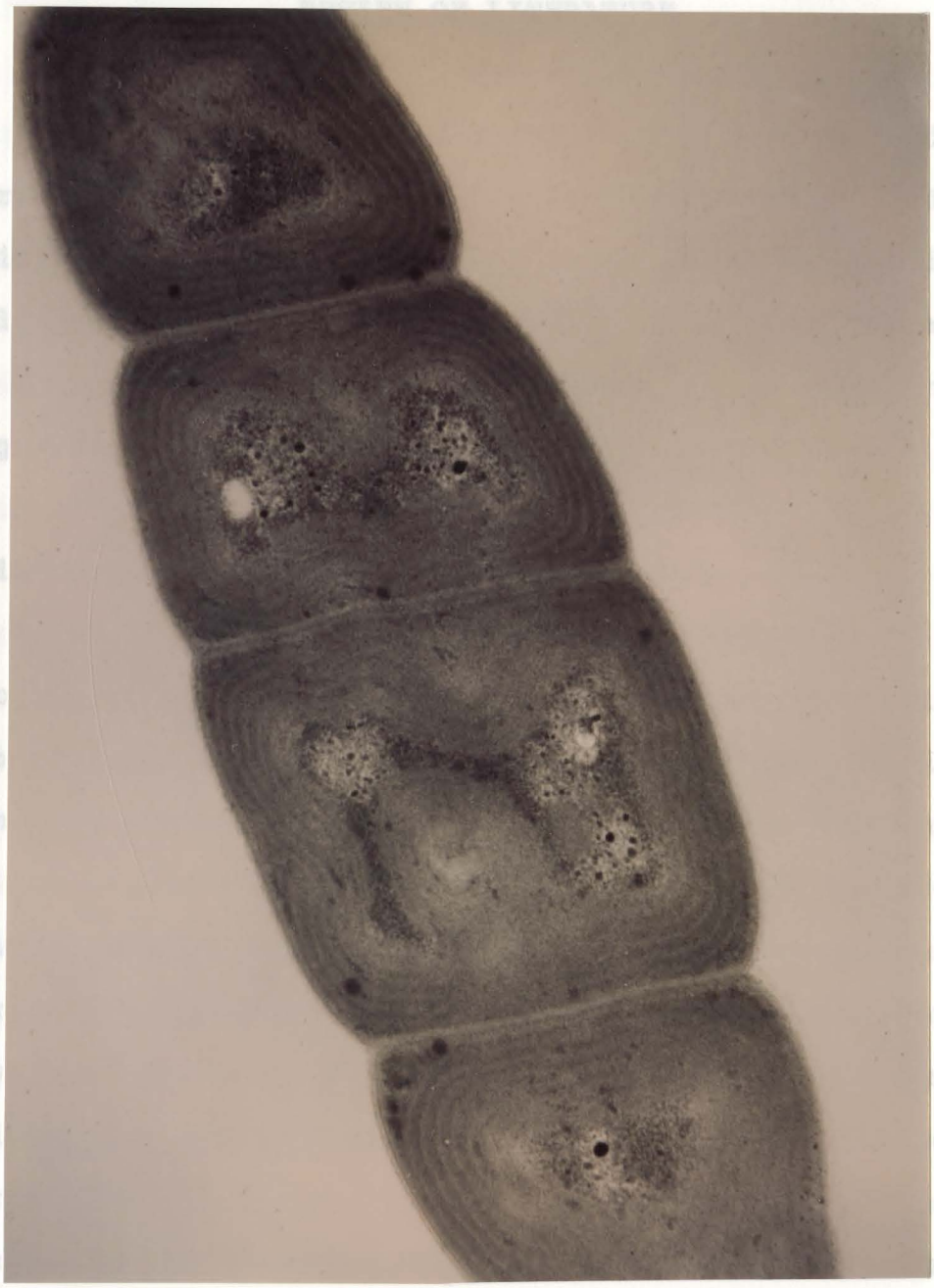


Fig. 4 Light micrograph of Plectonema boryanum, the host of LPP-1 virus. Note the filamentous nature of this cyanobacteria. There is a close resemblance between the cyanobacteria in general and the bacteria. X1500

Fig. 5 Scanning electron micrograph of Plectonema boryanum. Cyanobacteria contain a mucilaginous sheath, composed of polysaccharides, which covers the entire filament. As a result, no surface detail can be seen. X5100

Fig. 6 Transmission electron micrograph of Plectonema boryanum. The cell in which the cyanophage develops is a highly organized prokaryote. The photosynthetic membranes are organized in sheaths at the periphery and compartmentalize the cell.X20,000



REVIEW OF LITERATURE

The screening for different types of synthetic and natural chemicals which affect the viral infection and viral replication has been done by many researchers in the field of viral chemotherapy. The findings of many of these authors will be discussed in the following section. In addition, some background on phage LPP-1 research will be provided.

I. Viral Chemotherapy _ Synthetic Compounds

A. Plant Viruses- Most of the work done in viral chemotherapy has been on the use of synthetic chemicals that are purine, pyrimidine, and amino acid analogues. The idea is to incorporate the synthetic compound into the viral nucleic acid and protein synthesis in order to stop or interfere in the viral reproduction. One of the substances used in this regard was 8-azaguanine, a guanine analogue. This compound delayed the development of turnip yellow mosaic virus in small Chinese cabbage plants (Matthews, 1954). It readily incorporated itself into the viral nucleic acid and was also effective on many other plant viruses like lucerne mosaic virus, tobacco mosaic virus, and cucumber mosaic virus. This compound had no significant effect on henbane mosaic virus,

tomato spotted wilt virus, pea mosaic virus, tropaeolum virus II in tobacco, potato virus X, and on potato virus Y (Matthews and Smith, 1955).

Experiments were conducted by Lindner, kirkpatrick, and Weeks (1959) involving the screening of 233 chemicals for their ability to inhibit TMV and fruit ring spot disease. The viruses were multiplied in the cotyledons of cucumber. Only 15 chemicals were found to be effective inhibitors. These chemicals were nucleic acid base analogues like thiouracil, 2,6,8,-trichloropurine, six biological stains, four antibiotics (chloramphenicol, actidione, cytovirin, and noformicin), sodium selenate, A-methopterin, and A-denopteran. Fluorouracil was reported to reduce the yield of TMV by 50% when tobacco leaves were treated with fluorouracil after the virus had infected the cells (Gordon and Staehelin, 1958). This uracil analogue was found to be incorporated into the TMV-RNA.

Varma (1968) reported the inhibition of Tobacco necrosis virus (TNV) in Phaseolus vulgaris, var. Saxa. In this case the viral solution was mixed with various concentrations of guanidine carbonate compound and the mixtures were tested on intact primary leaves attached to the bean plants. This investigator found that 0.05M guanidine carbonate completely prevented TNV infection in the leaves after being absorbed into the leaf tissue either with the virus or within four hours after inoculation. When this compound was tested in

vitro, no inhibitory activity was observed. Guanidine carbonate was found to have no toxicity to the leaves of the host if used at pH 10.5. If the pH was reduced to 7.0 the chemical proved to be highly toxic to the leaves. Several 6-substituted purines and two oxypurines were studied by kurtzman et al. (1967) in order to determine whether there was any inhibitory activity in these compounds against TMV multiplication in infected Nicotiana tabacum. Hypoxanthine, 6-mercaptopurine, and 2-oxypurine showed no antiviral activity. On the other hand 6-methyl purine along with 6-chloropurine proved to be inhibitory. Recently, Dawson and Boyd (1987) tested the relationship between chemical modifications of normal nucleic acid base or nucleoside precursors and the ability to inhibit multiplication of TMV or cowpea chlorotic mottle virus in disks from mechanically inoculated leaves with 131 analogues. The authors selected chemicals from 10 general classes of modifications to determine the types of modifications of normal nucleic acid precursors that have greater probabilities of inhibiting virus multiplication. No inhibitory chemicals were found in several classes. Eight new inhibitors of virus multiplication were identified: 6-aminocytosine; 6-ethylmercaptopurine; isopentenyladenosine; 2-thiopyrimidine; 2,4-dithiopyrimidine; melamine; 5'-iodo-5'-deoxyadenosine; and 5'-methyl-5'-deoxythioadenosine.

B. Bacterial Viruses- Bacteriophages also have been used for the screening process of viral chemotherapy. Many chemicals have been found which interfere with the process of adsorption, penetration, or the intracellular growth of bacteriophages. The first recognition that chemical agents which inhibit the action of phage can do so by preventing adsorption was discovered by d'Herelle (1926). He explained the inhibition of mass lysis by viscous colloids such as gelatin, agar, and egg albumin as a physical barrier between the phage and its host. Nonviscous colloids such as colloidal silver and colloidal sulfur had no effect. Kellenberger and Arber (1955) manipulated the tail of phage T2 and T4 by hydrogen peroxide and ethanol in order to observe the inactivation process. These phages adsorbed reversibly but they were unable to proceed to the irreversible step of attachment and hence could not kill the host nor could they establish infection.

Some compounds also exist which prevent the injection of the phage DNA into the bacterial host. Kozloff and Henderson (1955) reported that when T2 phage is adsorbed on the bacteria in the presence of a chelator, versene, the bacteria are killed but no production of phage ensues. That this is indeed interference with injection is shown by the fact that 90% of the adsorbed phage DNA can be removed from the bacteria by the Blendor technique as compared to 30% in the absence of versene.

Inhibitory action against the phage nucleic acid metabolism was attempted with a number of analogues of the purine and pyrimidine bases. For example triazine, triazole (Mathews and Smith, 1955; Wooley, Murphy, Bond, and Perrine, 1952), and 2,6,-diaminopurine (Asheshov, Hall, and Flon, 1955) were used effectively, but these substances also proved to be equally inhibitory to the host's nucleic acid machinery.

C. Animal Viruses- Several compounds have been developed which by inhibiting viral DNA synthesis inhibit replication of the animal viruses such as herpes simplex type 1 (HSV-1) and type 2 (HSV-2), cytomegalovirus (CMV), varicella zoster virus (VZV) and Epstein Barr virus (EBV). The properties of these compounds (analogues of naturally occurring nucleosides) have been explained (De Clercq, 1983 and 1984). The most effective of the antiviral nucleoside analogues is 9-(2hydroxyethoxy-methyl)guanine (acyclovir) which is now in clinical use.

Metal chelating agents are one class of compounds which might inhibit the replication of a range of viruses (Hutchinson, 1985). Phosphonoacetic acid (Boezi, 1979) and the related phosphonoformic acid (Oberg, 1983) can inhibit a wide variety of enzymes involved in nucleic acid synthesis, for example, DNA and RNA polymerases and reverse transcriptase.

Influenza viruses have been the main class of viruses which have been studied for the development of drugs that are inhibitors of RNA synthesis. 1-B-D-ribofuranosyl-1,2,4-

triazole-3-carboxamide (ribavirin) inhibits the replication of influenza viruses by inhibiting the capping of mRNA of influenza viruses (Goswami,Borek, and sharma, 1979). In addition, 1-aminoadamantane hydrochloride (amantidine) and the related a-methyl-1-adamantane methylammonium hydrochloride (rimantidine) are agents for the prophylaxis and treatment of influenza virus A infections (Oxford and Galbraith, 1980). Rimantidine has fewer effects on the central nervous system than amantidine and is widely used in the USSR (Zlyndikov,Kubar,Kovaleva,and Kamforin, 1981). Genetic analysis and other studies indicate that the primary site of action of these drugs is a protein which is expressed in the plasma membrane of the infected cells (Hay,Wolstenholme,Skehel,and Smith, 1985). When the drug interacts with this membrane protein, the virus no longer can penetrate the host cells.

Many synthetic compounds also exist which inhibit the RNA transcriptases. One of the most important synthetic nucleoside analogue used currently is 3'-azido-3'-deoxy-thymidine, AZT (Mitsuta,Weinhold,and Furman, 1985). This agent inhibits the reverse transcriptase of HTLV-III, the AIDS virus.

II. Viral Chemotherapy _ Natural Compounds

Plants are known to have many different compounds in them, most of which are the products of the plant's

metabolism. But plants also synthesize some interesting proteins which have been known to have some antimicrobial function. One of the best examples of such proteins are lectins (Goldstein and Hayes, 1978). These proteins are capable of killing microbial or eukaryotic cells by binding to the cell surface of the invading organism, entering within the cytoplasm, and inactivating the ribosomes (Olsnes and Pihl, 1982a). The first protein of this kind was discovered due to its antiviral action. This protein was isolated from Phytolacca americana (pokeweed). It was reported that this antiviral agent was capable of reducing the infectivity of tobacco mosaic virus (Duggar and Armstrong, 1925). The authors at first had tried to infect the pokeweed plant with TMV. Failing to accomplish this task, they then tested the activity of the sap from the pokeweed plant directly on TMV and found that it reduced the infectivity of the virus in tobacco plants even after the sap was diluted several fold. In 1973, Obrig et al., reported that this antiviral agent had also a tendency to inhibit protein synthesis in cell-free extracts derived from eukaryotic organisms. It was also found that the purified protein was equally effective against a number of other plant viruses. The experiments were done in a similar way; the antiviral protein was mixed with the virus which was then rubbed on plant leaves in the presence of an abrasive substance such as carborundum to damage the tissue in order to allow the entry of the virus and the antiviral agent.

Using this method, Wyatt and Shepherd (1969) found that highly diluted solutions of this agent were still capable of inhibiting local lesion formation caused by southern bean mosaic virus and cucumber mosaic virus (Tomlinson et al., 1974). Using this type of assay system, it was shown that the antiviral substance was extremely effective at very low concentrations. Complete inhibition of local lesions produced by tobacco mosaic virus was obtained with 50nM solution (Irvin et al., 1980). Pokeweed plants themselves did not receive any protection from this antiviral protein because it was shown that at least one virus, pokeweed mosaic virus, was transmitted to the pokeweed plant (Shepherd et al., 1969) and that the purified antiviral protein did not protect the plant from local lesion formation caused by tobacco mosaic virus (Grasso and Shepherd, 1978).

Some work was also conducted on the effect of this antiviral protein against the animal viruses. It was shown that the antiviral protein was effective in the inhibition of both the influenza virus (Tomlinson et al., 1974) and the poliovirus (Ussery et al., 1977) multiplication. This antiviral agent affected the attachment of poliovirus to Hela cells and that treatment of virus with this protein, following its removal by sedimentation of the virus, did not affect viral infectivity.

The antiviral activity of the protein compound was tested also against herpes simplex virus (HSV) infection of both Vero

and Hela cells and it was found to inhibit viral multiplication at μM concentrations. This action of the protein was independent of the cell type being infected by the virus and maximum inhibition of virus required the continued presence of the inhibitory compound in the culture medium (Aron and Irvin, 1980).

The most recent work on the action of this antiviral compound on HSV infection of Vero cells produced some interesting observations. It was found that greater than 90% inhibition of virus yield was obtained by pretreating Vero cells with μM concentrations of antiviral compound for 12 hours and washing the cells before viral inoculation. Virus production inhibited to a slightly greater extent if the antiviral protein was added at the time of inoculation and kept in the culture medium during viral replication (Teltow et al., 1983). These results suggested that the antiviral activity of this compound depends upon an interaction of the compound with the cell rather than with the virus.

Other antiviral proteins have been reported to be present in a number of plants besides pokeweed but only a few of these proteins have been purified and characterized. Two proteins from the leaves of Dianthus caryophyllus (carnation) were shown to be inhibitors of tobacco mosaic virus lesion formation (Ragetli and Weintraub, 1962a,b). These proteins were reported to be basic in nature with molecular weights of 32,000 and 30,000 (Stirpe et al., 1981). The two proteins

proved to be more inhibitory to the tobacco mosaic virus local lesion formation and cross-reacted weakly with the antibodies against the pokeweed inhibitory substance described above (Grasso and Shepherd, 1978). Dhaliwal and Dhaliwal (1971) also found inhibitory activities in the extracts of Allium cepa and Allium sativum (garlic plants). These extracts inhibited the multiplication of the virion in TMV local lesion host Phaseolus vulgaris.

Proteins from a number of other plant species such as Chenopodium album, Atriplex nitens, and Amaranthus caudatus were partially purified along with the Chenopodium amaranticolor protein and all were found to have similar properties (Smookler, 1971). More recently, 14 different plant species reported to contain virus inhibitors were subjected to the same purification procedure used to isolate the pokeweed antiviral protein. In all cases proteins of similar properties and molecular weight were isolated though many were found to be present in the plant extracts in very small amounts (Grasso and Shepherd, 1978). Many of these proteins had strong antiviral activity and some were found to cross-react weakly with the antibodies against the pokeweed antiviral protein.

It has been shown that a large number of seed extracts contain proteins which inhibit protein synthesis and may be very similar to pokeweed antiviral protein (Gasperi-Campani et al., 1977,1980). In fact a number of these purified proteins

were all shown to inhibit the formation of local lesions by tobacco mosaic virus (Stevens et al., 1981).

A number of viral inhibitors have been purified and characterized from diverse sources such as wheat germ (Roberts and stewart, 1979), Gelonium multiflorum (Stirpe et al., 1980), Momordica cherantia (Barbieri et al., 1979), and Zingiber officinale (Roy, Sinha and Gupta, 1979). Two recent studies, however, are of particular interest since they report broad surveys of a large number of plant extracts as well as the effects of these extracts upon a number of different viruses. In one of these (May and Willuhn, 1978), 178 different plant extracts were tested against herpes virus, influenza virus, vaccinia virus, and poliovirus. The authors showed that 75 of the plant extracts provided antiviral activity against at least one of the viruses and many of the extracts showed activity against all four of the viruses. In a similar study using extracts from 100 different plants tested against six different mammalian viruses it was found that eight of these extracts showed antiviral activity against one or more of the viruses (Van Den Berghe et al., 1978).

III. Cyanophage LPP-1 and Cyanobacteria

In 1963, Safferman and Morris reported the discovery of a virus which attacks and lyses several species of cyanobacteria. These viruses are now known to attack a wide range of cyanobacteria, and have been designated phycoviruses

(Schneider, Diener, and Safferman, 1964), algophages (Goriushyn, and chaplinskyaya, 1968), and cyanophages (Padan and Shilo, 1969). The last designation seems to be the most suitable because of their close resemblance to bacteriophages and the close resemblance of the cyanobacteria to the bacteria (Stanier and Van-Niel, 1962).

The discovery of cyanophages, produced a great interest in the scientific community, particularly those involved in the study of plants, plant pathology, and photosynthesis. Until the discovery of the cyanophages, photoautotrophic metabolism under viral infection could only be studied in tissues of higher plants. However, plant tissue systems are difficult to isolate in tissue cultures, thereby creating a problem of not being able to infect the cells all at once. Cyanophages offered a system that resembled that of bacteriophages. Since photosynthesis of cyanobacteria is like that of higher plants, cyanophage-cyanobacteria system would offer a model for studying plant photosynthesis under viral infection.

LPP-1 cyanophage attacks three genera of cyanobacteria that are classified as being non-heterocystous filamentous organisms. These are Lyngbya, Phormidium, and Plectonema (Safferman and Morris, 1964). Its morphology has been studied extensively (Luftig and Haselkorn, 1968). The viral head capsid is a polyhedron, which appears hexagonal in projection. A short tail (20nm long, 15nm wide) is attached to one of the

vertices of the head (fig. 3). LPP-1 cyanophage was included in group C of bacteriophages defined by Bradley (1967) as "short noncontractile tail and head with six-sided outline." Adolph and Haselkorn (1972) stressed the similarity of LPP-1 phage to bacteriophage T7.

Analysis of the nucleic acid of LPP-1 virus indicated a linear double stranded DNA (Luftig and Haselkorn, 1967). This agrees with the prediction that head-tail bacteriophages contain mostly this type of nucleic acid (Bradley, 1967). The major proteins of LPP-1 virus were characterized by sodium dodecyl sulfate-gel electrophoresis of bulk viral proteins and separate head-tail preparations (Sherman and Haselkorn, 1970). Head proteins were 39,000 and 13,000 molecular weight species. The major tail protein has a molecular weight of 80,000. These molecular weights account for about 35% of the coding capacity of LPP-1 DNA.

LPP-1 virus requires Mg^{+2} for its stability (Goldstein et al., 1967). In bacteriophages like T3 and T7, the Mg^{+2} requirement was related to the diffusion of polyamines out of the head during purification and their replacement by the cations, which neutralize phosphate groups of the viral DNA to maintain structural integrity (Ames and Dubin, 1960). Compared with bacterial viruses, which are stable from pH 5 to 8 , the LPP-1 virus was stable from pH 7 to 11 (Safferman and Morris, 1964).

Goldstein et al. (1967) found that the adsorption of the

LPP-1 virus to its host was slow and nonlinear. This was related to the variability in the host filament length. The one-step growth curve of LPP-1 virus indicated that the general pattern of the growth cycle of cyanophages resembles that of the bacteriophages; however, it is much slower. The latent period lasts for 6 to 6.5 hours, the first 3 hours of which are the eclipse period. Six hours after the latent period, the rise period gets completed. The average burst size was 350 plaque forming units per cell (Sherman and Haselkorn, 1970).

By studying sections of Plectonema boryanum cells infected with LPP-1, Smith and his co-workers (1966) observed that the first sign of infection is invagination of the photosynthetic lamellae. The space between the folded lamellae and the plasma membrane in which viral particles appear was called the virogenic stroma. The invagination is first seen 3 hours after infection and is clearly visible after the 4th hour, indicating the end of the eclipse period (Sherman and Haselkorn, 1970). This was rather interesting because the bacteriophage development is usually not accompanied by any gross cytological changes in the host cell until just before lysis (Bradley, 1967). Smith et al. (1967) suggested that after infection the cyanophage DNA reaches the nucleoplasm, replicates there and then migrates into the spaces between the photosynthetic lamellae where long helices are formed. These helices then migrate into the virogenic

stroma where assembly occurs (see fig. 7,8, and 9).

All of the cyanophages show characteristics of virulent phages. But LPP-1D, and LPP-1SPI are shown to be temperate phages (Padan, Shilo and Oppenheim, 1972). Induction of the prophage with ultraviolet light, x-rays, or antibiotic treatments proved to be unsuccessful. However induction of a mutant of LPP-1SPI prophage by raising the temperature of the culture from $25°^{c}$ to $35°^{c}$ indicated that temperature changes might have tremendous significant effects on the population control of cyanobacteria in nature.

Fig. 7 TEM micrograph of a Plectonema cell 5 hours after the infection with LPP-1 virus. Note the appearance of viral capsids at the peripheral region of the cell. Cells were fixed in Karnovsky fixative in 0.1M cacodylate buffer at pH 7.4 and postfixed in 2% osmium tetroxide in the same buffer. X30,000

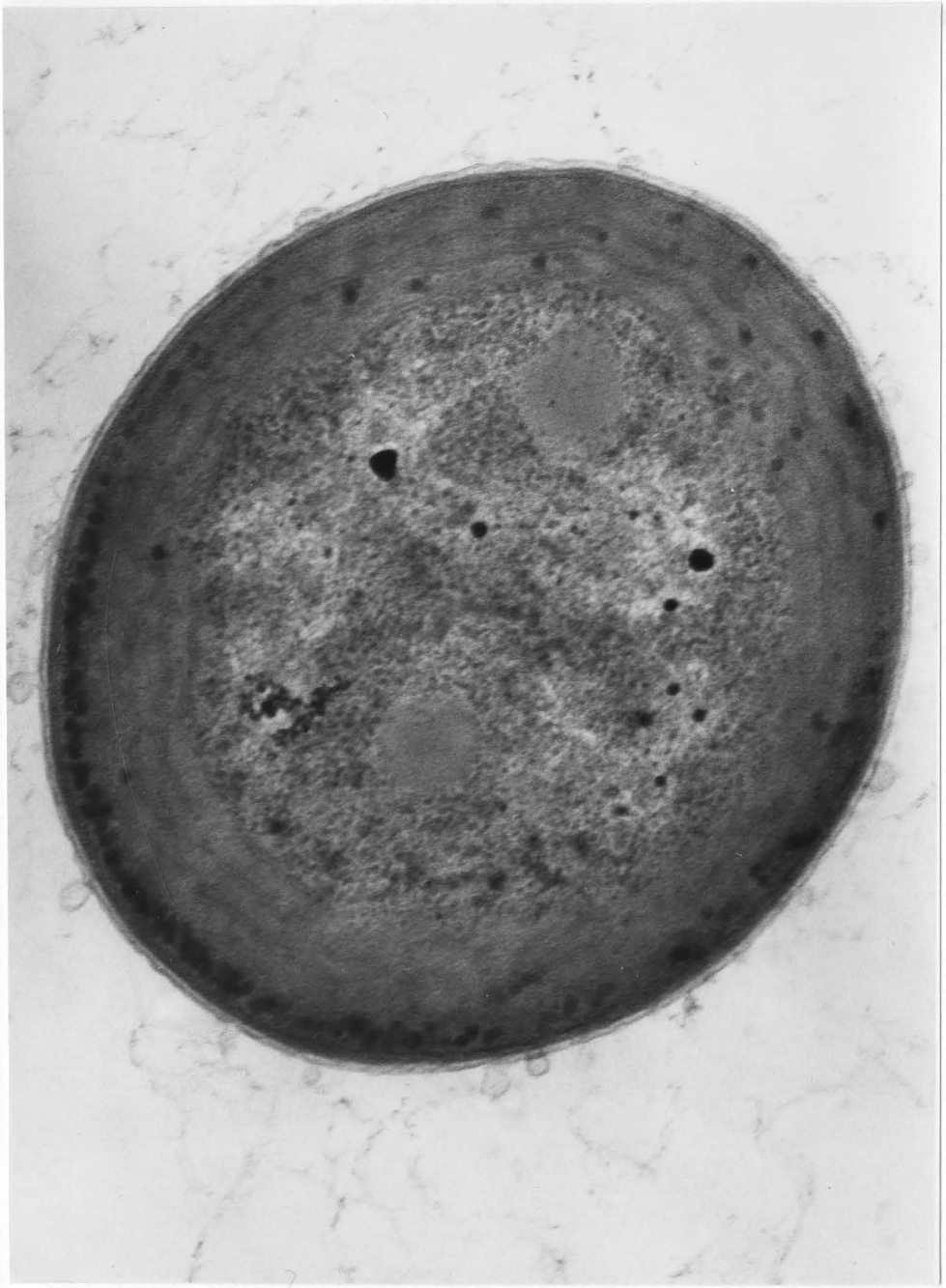
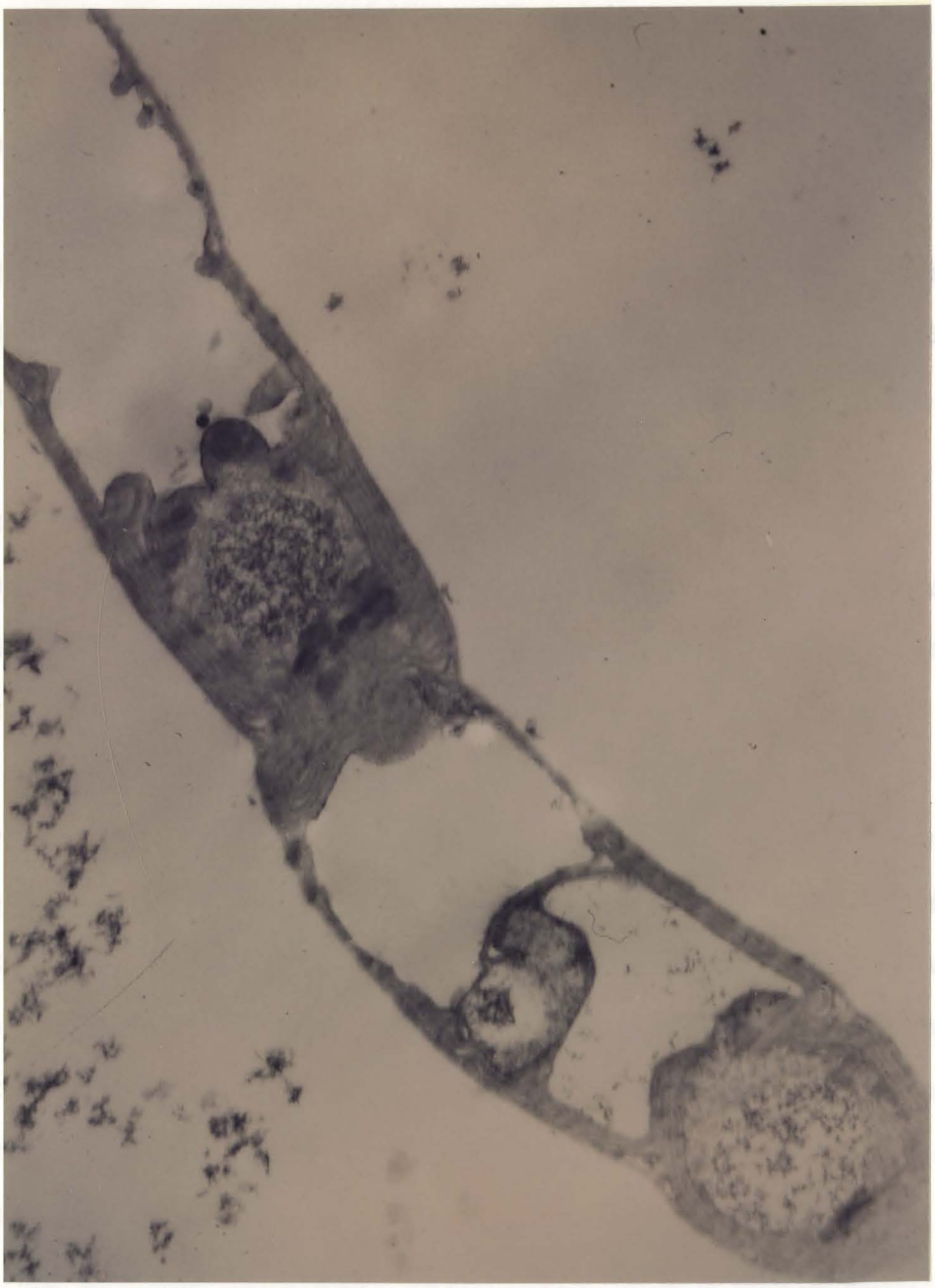


Fig. 4 Plectonema cell 7 hours after the infection with LPP-1 virus. Note the invaginated photosynthetic [illegible] the

Fig. 8 Plectonema cell 7 hours after the infection with LPP-1 virus. Note the invaginated photosynthetic membranes and the virogenic stroma containing the viral heads. X30,000

Fig. 9 An LPP-1 infected filament of Plectonema boryanum after occurrence of lysis. Note the absence of cytoplasm due to lysis in some of the cells within the trichome. X14,000



MATERIAL AND METHODS

I. Medium For Cyanobacterium:

Throughout all the work contained in this study, Plectonema boryanum, strain 594 was used. This organism was cultivated in 6-liter Erlenmeyer flasks containing 4,500 ml. of Bold's synthetic medium. In a growth chamber, these flasks were stirred, aerated, and incubated at 22-25°C. The Erlenmeyer flasks were illuminated with "cool white" fluorescent light at 150 ft-c. Photo period was 14 hours and all of the cultures for experimentation were kept at their log phase of growth.

The Bold's synthetic medium contained $NaNO_3$, $MgSO_4$, K_2HPO_4, KH_2PO_4, $CaCl_2$, NaCl, $FeSO_4$, H_3BO_3, $ZnSO_4$, MoO_3, $Co(NO_3)_2$, $MnCl_2$, $CuSO_4$, and EDTA (see Appendix D). For most phage work about 30 ml of agar containing Bold's medium/Petri plate was used. The plates were stored at room temperature overnight before they were used. For routine cultivation of the host organism, slants with two per cent agar containing Bold's medium were used.

II. Assay of Host Organism:

Most of the quantitative work with the bacteriophage or

cyanophage system requires an accurate measurement of the concentration of the host. Aliquots (1.0 ml.) of serial dilutions of the host organism were spread over the surface of agar plates and after incubation the number of colonies were counted. From this count the number of viable organisms/ml. of the original culture can be calculated. Since P. boryanum is a filamentous organism, the method of colony count was not suitable. Because the organism grows very slowly, the colony count method was not practical time wise. Furthermore, the colonies of P. boryanum, when visible, would represent a progeny from a single filament, not a single cell. As a result the population count was achieved by the use of hemacytometer, 0.1 mm. deep (Appendix E).

III. Assay of Phage LPP-1:

The concentration of the virus was assayed using agar layer technique, described by Gratia (1936c). The cyanobacteria host and virus particles were mixed in a small volume of warm 0.7 per cent agar and the mixture was poured over the surface of an ordinary agar plate and allowed to harden to form a thin layer. The cyanobacteria grew as tiny colonies in this layer and got their nutrients from the deep layer of 1.7 per cent agar (fig. 10). The plaques appeared as clear holes in the green layer of cyanobacterial growth (fig. 11). The soft 0.7 per cent agar was melted in an oven and then was cooled in a $48^{\circ c}$ water bath. Four ml. of

Fig. 10 Growth of Plectonema boryanum in the solid media. In order to form a lawn in the agar media, a concentration of greater than 100,000 trichomes per ml. is usually used.

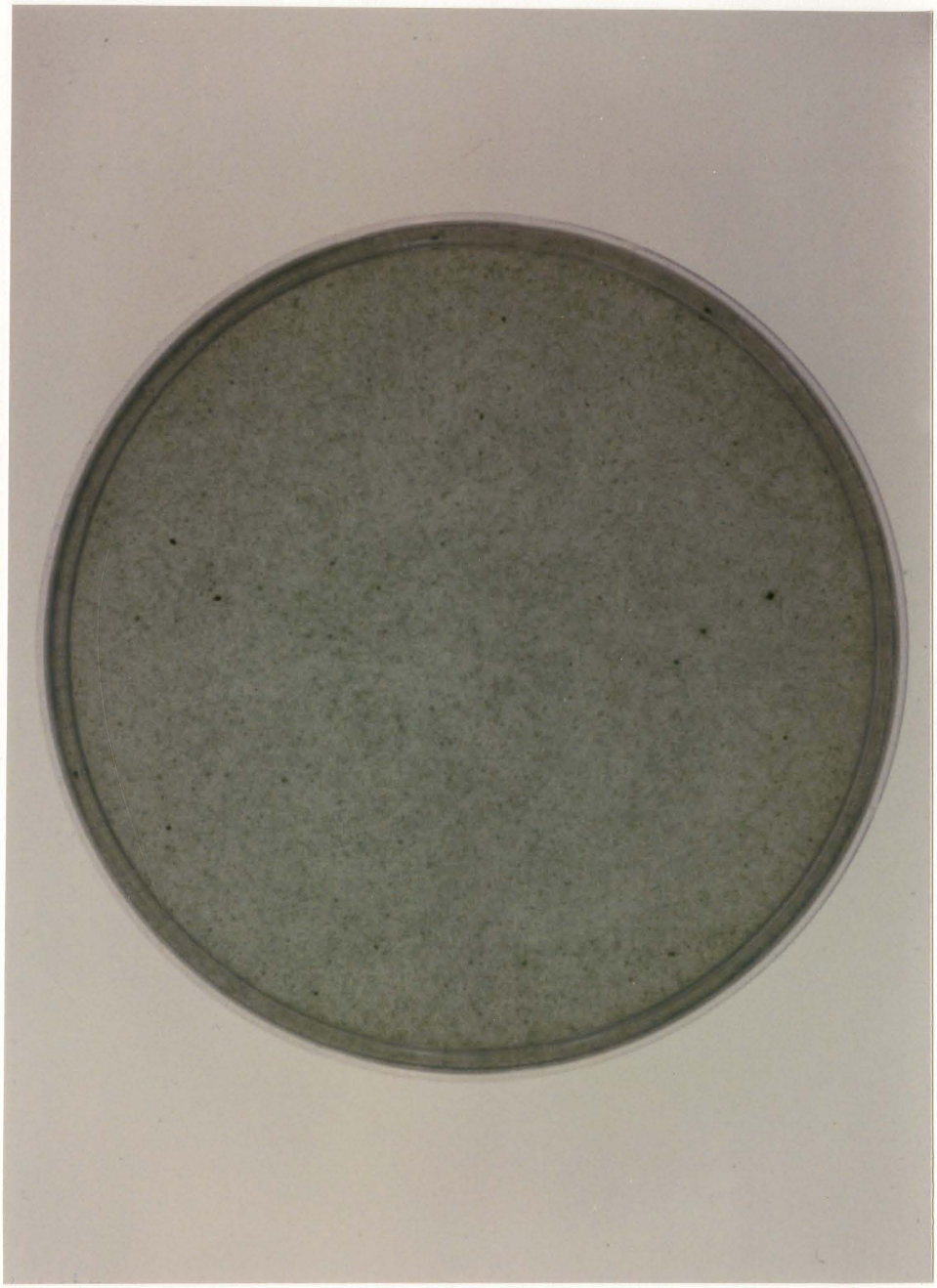
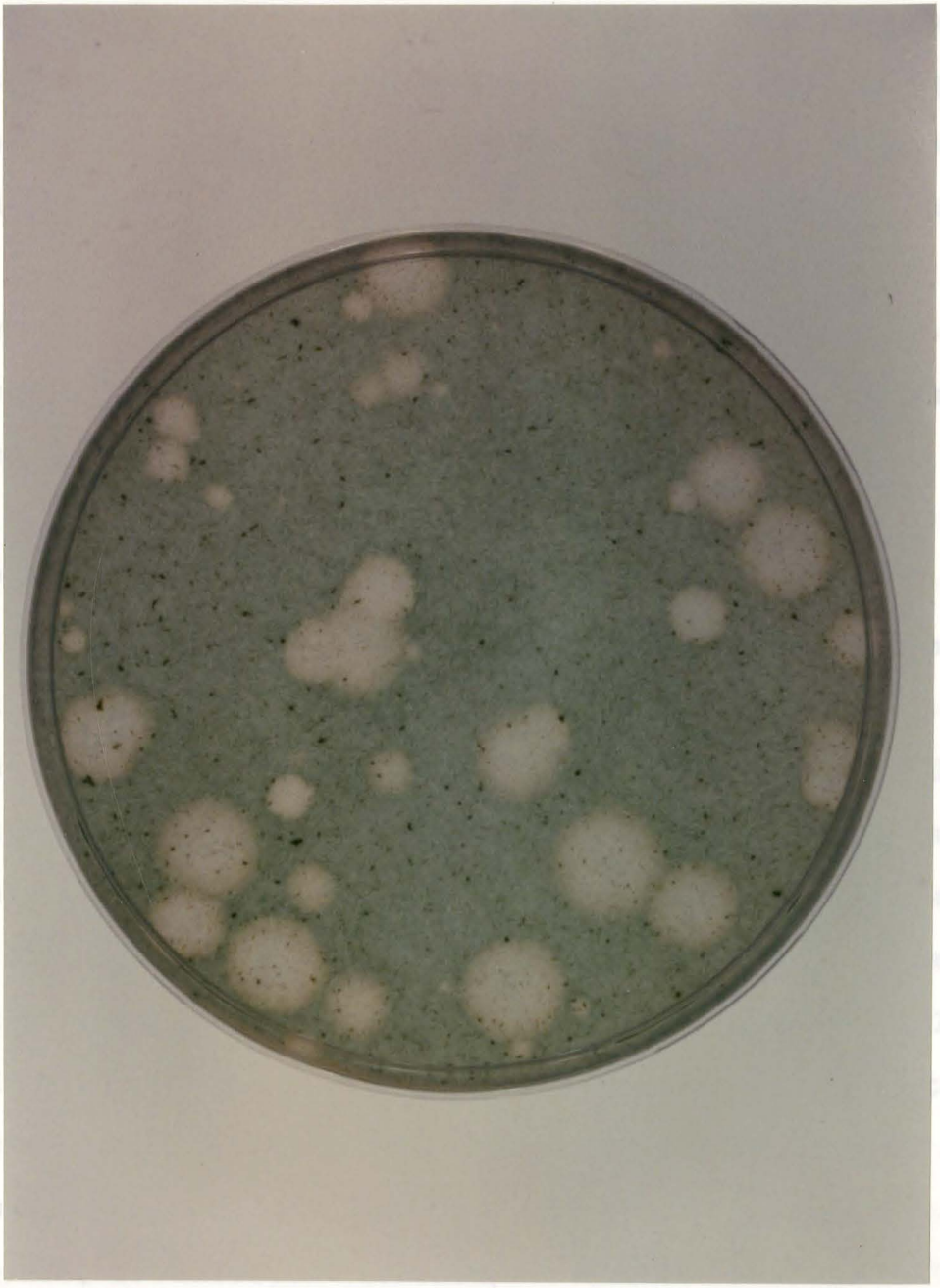


Fig. 11 Plaques of LPP-1 virus appearing after 4 days of incubation in the solid media (agar) containing Plectonema cells. The clear areas represent the areas where complete lysis has occurred.



cyanobacterial inoculum was added to test tubes containing 5 ml. of soft agar. Then, the diluted virus in any volume up to 1 ml. was pipetted into the test tubes of soft agar and the mixture was poured quickly over the surface of an agar plate. The plate was rocked gently to mix the cyanobacteria and virus particles and was set aside to harden. The petri plates were then incubated at room temperature underneath 150 ft-c fluorescent light. Plaques appeared after 3 or 4 days depending on the concentration of the host organism used during inoculation.

IV. Preparation of High Titer Phage Stocks:

Plectonema boryanum was grown in Bold's medium with continuous aeration and light intensity of 150 ft-c. The concentration of cyanobacteria reached about $5x10^9$ cells/ml. within about two weeks after the inoculation. If such a culture is grown to a concentration of $5x10^9$ cells/ml. and is then inoculated with a drop of LPP-1 virus with concentration of $5x10^9$ phage particles/ml., the cyanobacteria will all lyse completely within a week, and a final titer of 10^{10} to 10^{11} infectious particles/ml. can theoretically be achieved. Since the average yield per infected bacterium is 100-300 phage particles, 10^9 infected bacteria/ml. should liberate 10^{11} phage particles/ml.. However, this yield is usually not obtained in practice because large number of the progeny phage is either adsorbed to infected but unlysed host or they are adsorbed to

debris from lysed host cells. Fresh cyanobacterial lysates therefore have to be centrifuged at low speed to remove the debris to prevent the loss of the phage titer. The lysed filtrates were centrifuged at 10,000 r.p.m. for 30 minutes and then they were passed through 0.22μm Millipore filters and stored at $4^{\circ c}$.

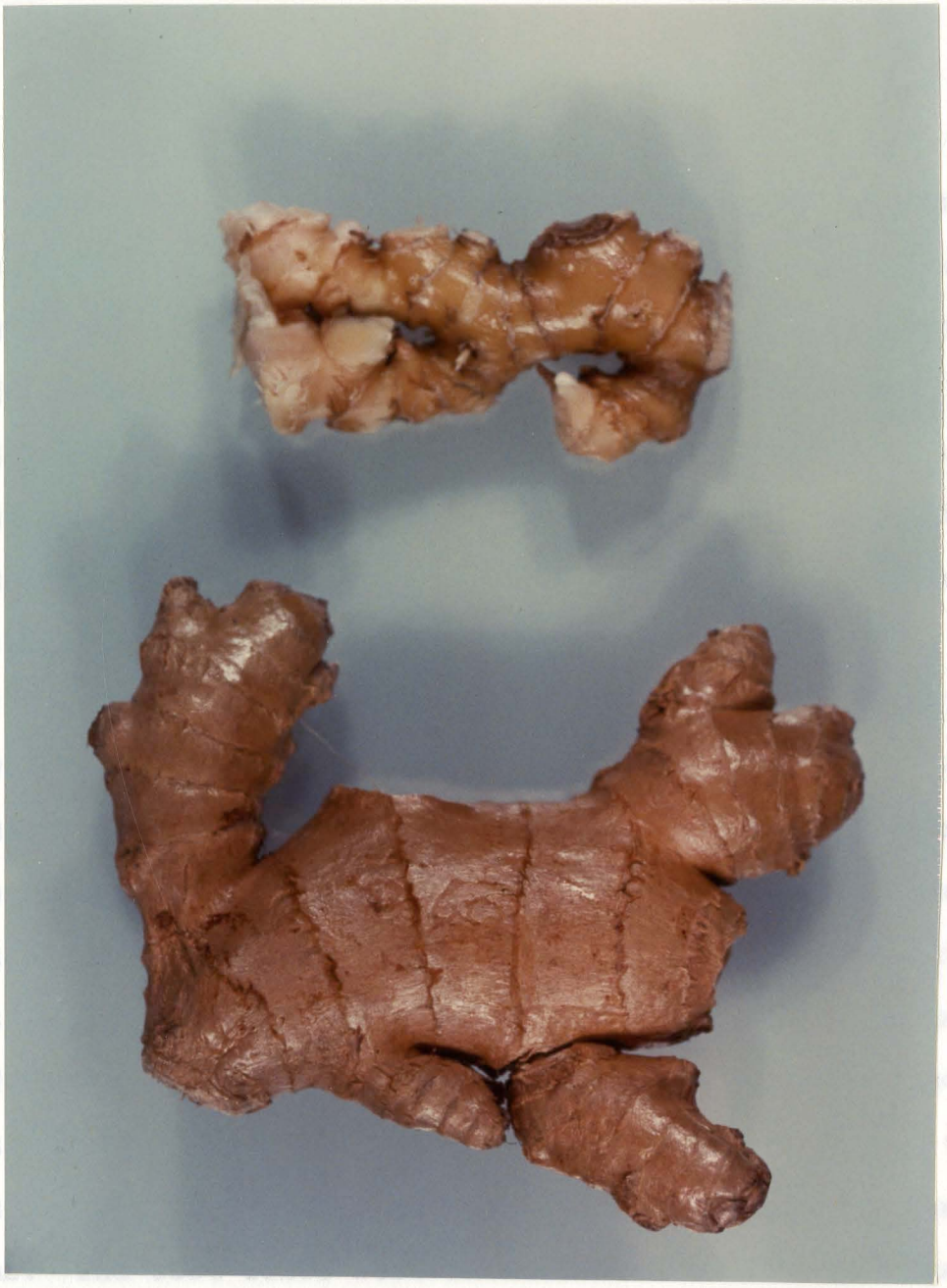
V. Rhizome Extract Preparation:

The rhizomes were weighed after being washed and dried (fig. 12). The extract was prepared by grinding the rhizomes with mortar and pestle. An equal weight of sterile, saline solution was added to the extract and then the extract was passed through several layers of cheesecloth. The extract was centrifuged at 10,000 r.p.m. for 30 minutes to remove the solid materials further. The extract was then used at whatever concentration necessary throughout the study.

VI. Viral Inhibition Test:

To determine whether the two rhizome extracts of Z. officinale and Z. zerumbet have any inhibitory activities against the LPP-1 virus, a test for inhibition was conducted. In this test, concentrated cyanobacteria cells were inoculated with 6% (v/v) officinale, and 4% (v/v) zerumbet extract, separately, and incubated for a period of up to 24 hours and the ability of the virus to produce plaques on the solid media was assayed by inoculating the treated cultures as well as the

Fig. 12 Rhizomes of the Zingiber plants used throughout this study. Note that the rhizome of Zingiber zerumbet is smaller and thinner than the rhizome of Zingiber officinale.



controls with known concentration of the LPP-1 virus at different time intervals.

VII. Transmission Electron Microscopy:

Transmission electron microscopy was used to study the nature of the charges existing on the cell wall, and its immediate environment, of P. boryanum and to see whether the addition of the extract interferes with the charge distribution. The cyanobacteria cells were inoculated with cationized ferritin (Sigma Chemical Co.) to study the distribution of anionic sites on the cell wall by visualizing the charges on the cell surface.

Concentrated cyanobacteria cells were treated with 10% (v/v) of Z. officinale extract and incubated under 150 ft-c light at 23°C with constant shaking for four hours. The cells were then centrifuged (1000 r.p.m.) for 5 minutes and the pallet was inoculated with 0.1 ml. of CF (cationized ferritin) that was diluted to 1.0 ml. by the addition of cacodylate buffer, pH 7.4. The test tubes containing the pallet were agitated so that the cells would be resuspended into the CF solution rather than remaining on the bottom of the test tubes. The test tubes were incubated at room temperature for 30 minutes after which the cells were fixed with Karnovsky fixative (Karnovsky, 1967) for 1 hour. Then the centrifugation was resumed for 5 minutes and the pallet was

washed three times in cacodylate buffer. Secondary fixation was done by osmium tetroxide (2% w/v) for 1 hour. The cells were washed again three times with cacodylate buffer. Then the cells were dehydrated by transferring them into 30% acetone for 15 minutes, after which the cells were transformed through 50%, 70%, 90%, and three changes of 100% acetone.

After dehydration, the cells were embedded in 1:1 ratio of spurr's medium (Spurr, 1969) to acetone solution, followed by 3:1 ratio and finally with 100% of the embedding medium. After the polymerization of the embedding medium, the sample capsules were trimmed by the glass knives and thin sections were obtained by the use of microtome. All sections were mounted on 300 mesh copper grids and were stained with uranyl acetate, lead citrate and bismuth subnitrite.

VIII. Toxicity Test:

Since plant extracts are known to have antibacterial as well as antiviral activity, a toxicity test was conducted. It was expected that if the extract was toxic to the cyanobacteria, the phage progeny per cyanobacterium would decrease considerably since the rate of the production of the virions in an infected cell depends on the rate of the metabolism of the host. This effect was to be avoided at all costs because no meaningful assay of the intracellular inhibition could be done. As a result before testing the antiviral activity of Zingiber officinale and Zingiber

zerumbet, it was necessary to measure the level of toxicity of the extract to the cyanobacterium. This was determined by qualitative assay of the growth of the cyanobacterial colonies in petri plates after inoculation with various concentrations of the extract. Time required to cause toxic effects on bacterium by various concentrations of the extracts was also assessed. This experiment allowed a standard growth pattern to be established. Then the experimental population was analyzed against the control population.

Toxicity test was done like a phage burst experiment described below, however, no virus was added to the host. Instead at the end of the experiment, the treated cyanobacteria was plated to see if it would grow and form colonies like the control population. The host cells were at first inoculated with the extract at various concentrations and incubated at various time intervals. Then the extract treated culture was diluted 1:100 with Bold's medium and the cyanobacteria were plated at various time intervals to determine whether the cells were healthy enough to grow and reproduce. The petri plates were incubated at room temperature under 150 ft-c fluorescent light.

IX. Quantitative Examination of the Antiviral Activity:

The rate of the viral adsorption to its host, under certain conditions, determines the rate of its growth. Therefore, the first step in the test of a chemotherapeutic

compound against viral growth should be the measurement of its effect on the viral adsorption process. If the rate of attachment changes, under certain conditions, rate of the viral growth changes also. The purpose of this current study was to determine the rate of the adsorption so that it could be taken into account in the interpretation of growth experiments that were to be performed on the virus when treated with the extract. These growth experiments were the phage burst, and single burst experiments. Phage burst experiment measured the number of virus particles formed within the host treated with the inhibitory compounds, prior to the occurrence of lysis. This experiment also measured the latency period in which the cells were infected but they were not lysed. The results shown by this experiment were the averages of all the bursts occurring in the medium in which the host cells were suspended. To measure the burst size of a single cyanobacterium cell within a filament, a single burst experiment was performed.

A. Determination of the Rate of Adsorption:

The first step in the growth cycle of a virus is adsorption of virus to host cells. Knowledge of the adsorption rate is an absolute necessity for the design and interpretation of certain kinds of experimentation, described later in this study. In order to measure the rate, the cyanobacterial culture was temperature-equilibrated and a sample taken for the determination of cyanobacterial assay

just before the addition of the virus. Four different populations were set up and the rate of adsorption in each was measured.

1. Officinale Treated Population _ In this case, 72 ml. of an accurately known concentration of cyanobacteria was treated with 20 ml. of officinale extract (20% v/v). The culture then was incubated for four hours under 150 ft-c of fluorescent light and was constantly shaken at 23°^{c}.

2. Zerumbet Treated Population _ Similarly, 72 ml. of an accurately known concentration of cyanobacteria was treated with 20 ml. of zerumbet extract (20% v/v). The culture was incubated for four hours under 150 ft-c of fluorescent light and was constantly shaken at 23°^{c}.

3. Officinale Treated Virus _ Eight ml. of accurately known concentration of LPP-1 virus was treated with 20 ml. of officinale extract. The mixture was then incubated for four hours under 150 ft-c fluorescent light and was shaken constantly at 23°^{c}.

4. Control Group _ The control group consisted of 72 ml. of accurately known concentration of cyanobacteria to which instead of extract, 20 ml. of Bold's medium was added, followed by incubation at 23°^{c} under 150 ft-c light intensity, shaken constantly for four hours.

To the officinale, zerumbet, and control group, mentioned above, 8 ml. of an accurately known quantity of the LPP-1 phage was added so that the initial concentration of phage in

the adsorption mixture could be determined. The virus treated group on the other hand received 72 ml. of cyanobacteria which had the same concentration as in the other groups.

The four different adsorption mixtures were then incubated at 25°^{c} under fluorescent light of 150 ft-c, and were constantly shaken. After thorough mixing, samples of the adsorption mixture were removed at different time intervals and diluted 1:100 with Bold's medium in order to stop the adsorption process. Then 5 ml. amount of the diluted samples were centrifuged at 3000 r.p.m. for 5 minutes to sediment the cyanobacteria and the adsorbed phage. A 1 ml. aliquot of the supernatant fluid was then accurately assayed for the free unadsorbed phage particles. The entire procedure from the start of the adsorption process to the assay of free phage particles in the centrifuge supernatant fluid had to be completed before the end of the latent period of phage growth, so that lysis of infected cyanobacteria would not liberate additional virus into the medium.

Aside from the centrifugation, another method for measuring free (unadsorbed) phage was to treat the infected cyanobacterial culture with chloroform which inactivates the infected cyanobacteria. In this procedure, the adsorption mixture was diluted into Bold's medium containing chloroform (4.5 ml. of Bold's medium with 0.5 ml. chloroform), shaken vigorously and then assayed for free phage. This method has been successful with phage particles (Sechaud and

kellenberger, 1956) and it was essential to treat the adsorption mixture with chloroform early in the latent period of phage since infected host cells containing mature virions would lyse and could contribute viable phage to the medium.

B. Determination of the Average Burst Size:

Ellis and Delbruck (1939) made an important contribution to the phage research by developing the one-step growth experiment. They designed this technique to determine quantitatively 2 important characteristics of the bacteriophages, the latent period of intracellular virus growth and the burst size. The latent period is the minimum length of time from adsorption of the virus to the host cell to lysis of the host cell and release of the progeny of the infecting virus particle. On the other hand Burst Size is the average yield of virus particles per infected host cell. The one-step growth method allows one to determine in one experiment both the latent period and the burst size. The technique is extremely adaptable, permitting the experimenter to change the physical or chemical environment of the host cell to see the effect that these changes will have on the infection process. As an example, in this study extracts from two different plants were added in order to change the chemical environment of the host cells and to see whether this alteration would have any effect on the latency period and the burst size.

The one-step growth experiment was modified in this study

to fit the cyanobacteria/cyanophage system rather than bacteria/bacteriophage system which it was originally developed for. The virus used was cyanophage LPP-1 and the host cell was strain 594 of P. boryanum grown in the Bold's medium which was the medium used throughout. The number of infected cyanobacteria was not determined by neutralization of unadsorbed phage with anti-LPP-1 serum, followed by plating of a suitable dilution for plaque count. Rather the infected cyanobacterial filaments were assayed by measuring the number of total infective centers (free phage plus infected cyanobacteria) and then subtracting the number of unadsorbed phage particles from the number of total infective centers. Single infection method was used, multiplicity of infection was 0.1 p.f.u./filament. All platings for phage assay were done by agar layer technique.

Six different experimental groups were set up for the phage burst experiment and the measurement of burst size in each and every one of them was conducted.

1. Officinale Treated Population _ In this case, 72 ml. of an accurately known concentration of cyanobacteria was treated with 20 ml. of officinale extract (20% v/v). The culture was then incubated for four hours under 150 ft-c of fluorescent light and was constantly shaken at $23^{\circ c}$.

2. Zerumbet Treated Population _ Similarly, 72 ml. of an accurately known concentration of cyanobacteria was treated with 20 ml. of zerumbet extract (20% v/v). The culture was

incubated for four hours under 150 ft-c of fluorescent light and was constantly shaken at 23°c.

3. Officinale Treated Virus _ Eight ml. of accurately known concentration of LPP-1 virus was treated with 20 ml. of officinale extract. The mixture was then incubated for four hours under 150 ft-c fluorescent light and was shaken constantly at 23°c.

4. Control Group _ The control group consisted of 72 ml. of accurately known concentration of cyanobacteria to which instead of the extract, 20 ml. of the Bold's medium was added, followed by incubation at 23°c under 150 ft-c light intensity, shaken constantly for four hours.

5. Officinale treatment after adsorption _ This group was set up by inoculating 72 ml. of the cyanobacterial culture of known concentration with 20 ml. of the Bold's medium. Then the culture was incubated for four hours at 23°c under 150 ft-c fluorescent lighting with constant shaking. After four hours the culture was inoculated with 8 ml. of known concentration of phage LPP-1 and incubated in the same manner for one hour so that the adsorption process would proceed normally. After 1 hour, the culture was diluted 1:100 by taking 0.1 ml aliquot and diluting it in 7 ml. of the Bold's medium plus 2 ml. of officinale extract. The final concentration of extract in this mixture was 20% (v/v). The culture was incubated in this condition for four hours after which it was further diluted 1:100 but this time in Bold's

medium.

6. Zerumbet treatment after adsorption _ This group was treated exactly the same way as step 5, mentioned above, except that zerumbet extract was used instead of officinale extract.

For each of the six different categories, explained above, a culture of P. boryanum was grown in the Bold's medium with active aeration to a concentration of $5x10^7$ cells/ml. A stock of LPP-1 virus was diluted in saline to a concentration of $5x10^7$ p.f.u./ml.. In all of the six categories, a culture of cyanobacteria whether treated or not with the extract was mixed with the virus solution and was incubated for 1 hour with constant agitation under fluorescent light of 150 ft-c. After an hour, the cultures were diluted 1:100 to stop the further adsorption of virus. From the tube containing the hundred fold dilution two growth tubes were prepared. One growth tube had about 100 infected cyanobacteria/ml (first growth tube) and the other was a hundred fold dilution of the first growth tube, named appropriately, the second growth tube. The second growth tube contained only 1 infected cell/ml.. At 1 hour intervals, 1 ml. samples from the first and second growth tubes were plated by the agar layer technique. After incubation at 23^{oc} under fluorescent light of 150 ft-c for 3 days, the plaques were counted.

C. Determination of Single Cell Burst Size:

In this method a suspension of infected cyanobacteria was

diluted to the point that small samples of the dilution contained no more than 1 infected host cell. These samples were then incubated until all infected cyanobacteria lysed, then the samples were plated to determine the phage progeny coming from a single cell.

For single burst experiment, six different groups were set up and the measurement of the burst size in single infected cells in each and every one of these groups was conducted.

1. Officinale Treated Population _ In this case, 72 ml. of an accurately known concentration of cyanobacteria was treated with 20 ml. of officinale extract (20% v/v). The culture then was incubated for four hours under 150 ft-c of fluorescent light and was constantly shaken at 23°^{c}.

2. Zerumbet Treated Population _ Similarly, 72 ml. of an accurately known concentration of cyanobacteria was treated with 20 ml. of zerumbet extract (20% v/v). The culture was incubated for four hours under 150 ft-c of fluorescent light and was constantly shaken at 23°^{c}.

3. Officinale Treated Virus _ Eight ml. of accurately known concentration of LPP-1 virus was treated with 20 ml. of officinale extract. The mixture was then incubated for four hours under 150 ft-c fluorescent light and was shaken constantly at 23°^{c}.

4. Control Group _ The control group consisted of 72 ml. of accurately known concentration of cyanobacteria to which

instead of extract, 20 ml. of Bold's medium was added, followed by incubation at 23°C under 150 ft-c light intensity, shaken constantly for four hours.

5. Officinale treatment after adsorption _ This group was set up, as in the one-step growth experiment, by inoculating 72 ml. of the cyanobacterial culture whose concentration was accurately determined with 20 ml. of the Bold's medium. Then, the culture was incubated for four hours at 23°C under 150 ft-c fluorescent lighting with constant shaking. After four hours, the culture was inoculated with 8 ml. of accurately known concentration of phage LPP-1 and incubated in the same manner for one hour so that the adsorption process would proceed normally. After 1 hour, the culture was diluted 1:100 by taking 0.1 ml. aliquot and diluting it in 7 ml. of the Bold's medium plus 2 ml. of officinale extract. The final concentration of the extract in this mixture was 20% (v/v). The culture was incubated in this condition for four hours after which it was further diluted 1:100 but this time in Bold's medium.

6. Zerumbet treatment after adsorption _ This group was treated exactly the same way as step 5 mentioned above, except that zerumbet extract was used instead of officinale extract.

Cyanophage LPP-1 was used to infect the six groups containing the culture of P. boryanum at an input ratio such that one filament out of ten filaments would be infected with only one virus particle during one hour adsorption period.

The six separate cultures were diluted with the Bold's medium to an extent that only 0.7 singly infected cyanobacterium filament existed in 1 ml. This dilution was then distributed into 50 samples of 1.0 ml. each for each of the six groups. Distribution was completed before the end of the latent period. The samples and the remainder of the suspension in the sample flask were incubated at 23°C under 150 ft-c light intensity. The sample flask was shaken constantly. Incubation continued until well after the end of the rise period (15 hours) to insure that all bursts have taken place. A flask of 0.7 per cent agar was melted in the oven and cooled to 48°C. Then, 4.5 ml. aliquots of plating cyanobacteria were added to each test tube followed by 5 ml. of the 0.7 per cent agar. The contents of the test tubes were then immediately poured over the surface of the agar plates. The plates were rocked gently for a few seconds to mix the sample with the agar and to insure uniform layering of the agar. In addition to this, several 1 ml. samples from the sample flask containing the remainder of the diluted suspension were also plated to determine average burst size in the suspension, and to compare them to the results of the single burst values.

RESULTS AND DISCUSSION

In order to facilitate the reader's understanding of the experimental data, discussion accompanies the results. It must be pointed out that the experiments that will be discussed indicate whether the plant extracts used throughout the experiments have any inhibitory activities; and if so, when and where in the infectious cycle of the virus the inhibitory activity exists.

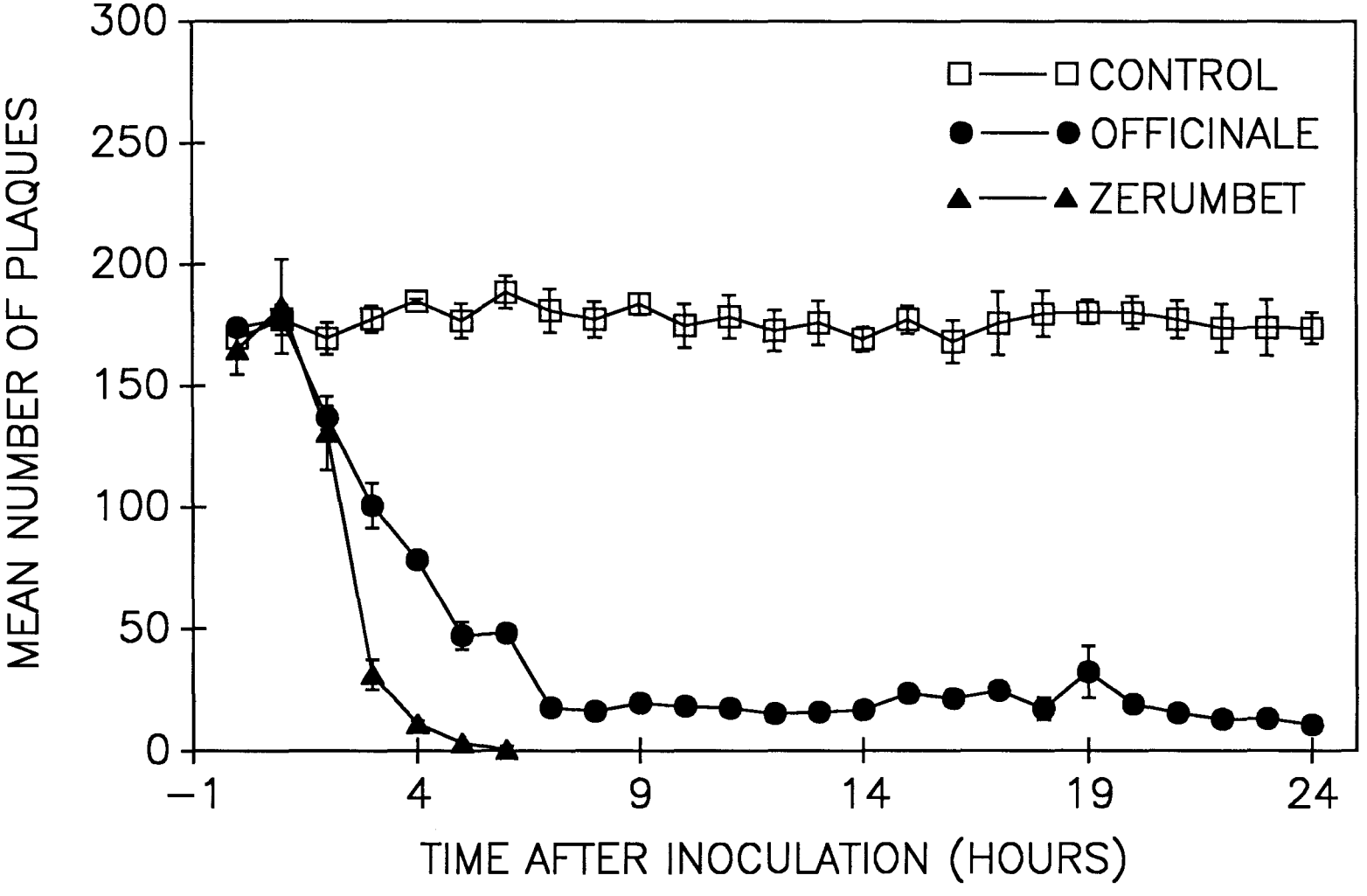
I. Viral Inhibition Test:

The results for the viral inhibition test indicate many important findings. One is that the untreated LPP-1 viral concentration is extremely stable and no change in the number of plaque count occurs throughout the 24 hours (fig. 13 and table 9). Other important fact is that there is clearly inhibitory activity occurring against the virus (fig. 13, table 10 and table 11). In other words, the number of plaques becomes drastically reduced during the 24 hour period of testing.

At a first glance at fig. 13, the difference between the control and the experimental groups can be appreciated. This difference begins at second hour of incubation and becomes

Fig. 13 Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells. Note that there is a decrease in the number of plaques in the experimental groups which begins two hours after the cells are incubated with the extracts.

VIRUS INHIBITION

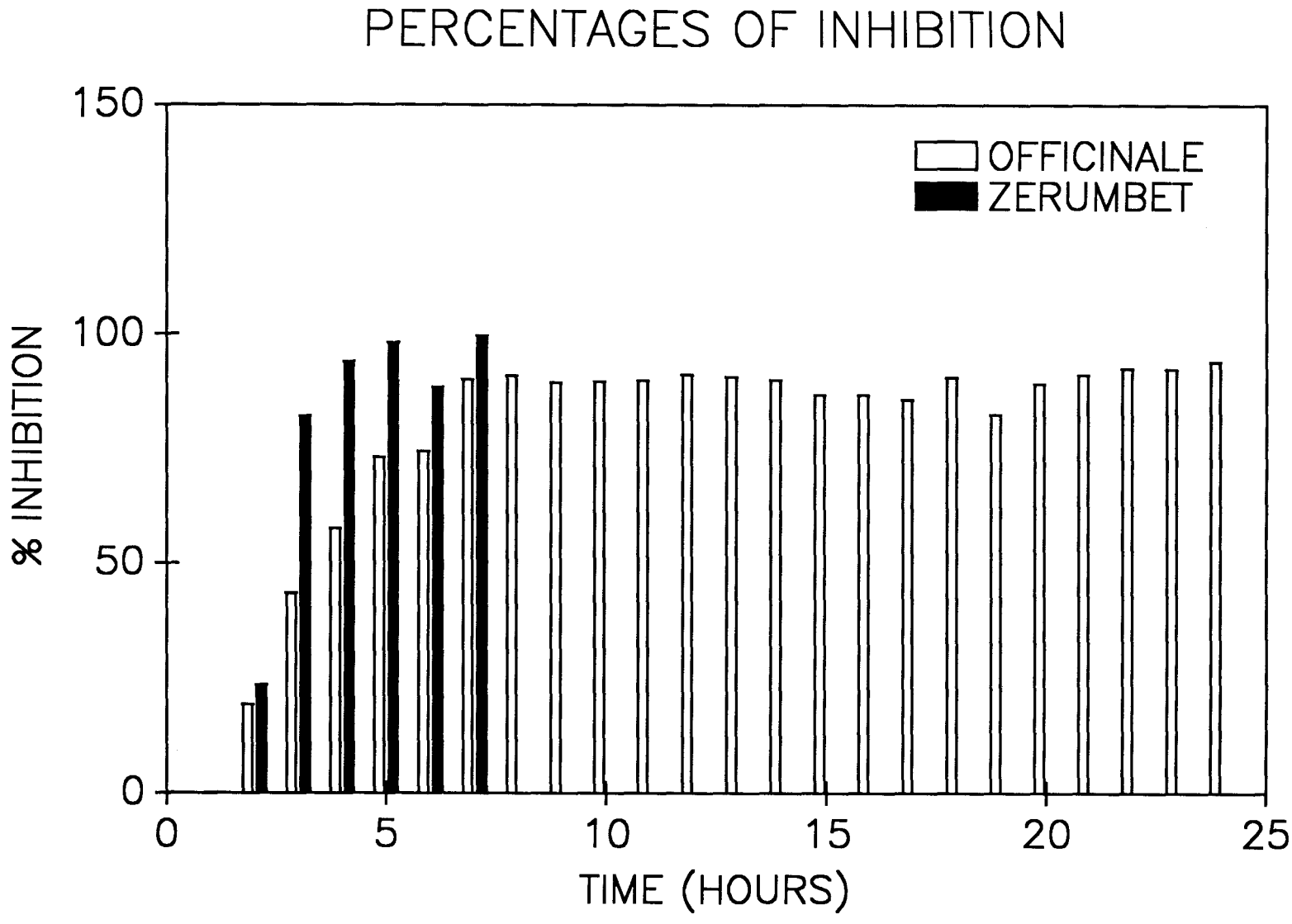


greater until about the 6th hour after which it remains the same. The activity of the Z. Zerumbet however, could not be measured after the sixth hour due to its extreme toxic effects on the host (fig. 13). The toxic effect prevented the growth of the host in the petri plates, therefore, not allowing the formation of the plaques in the petri plates to occur.

The data were also transformed into % inhibition so that the reader would be better able to distinguish between the potency of the two extracts. According to fig. 14, it seems that there is a difference between the potency of officinale and zerumbet. Zerumbet extract seems to be more potent.

To analyze the significance of the viral inhibition test data many statistical procedures were performed. Since there are three categories that are being compared to each other (control, officinale, zerumbet), single factor analysis of variance (ANOVA) was performed, instead of a student t-test. The student t-test can only be reliable when maximum number of categories being analyzed is 2. The ANOVA showed that there is no significant difference at the 0 and at the first hour. However during the time intervals 2nd, 3rd, 4th, 5th, and 6th hour, the control, officinale, and zerumbet treatment showed a significant difference, indicating a change in the pattern of the number of plaques in the petri plates (table 1). This

Fig. 14 Bio-assay of LPP-1 replication in the extract treated Plectonema cells. Numbers of plaques produced were transformed into % inhibition in order to compare the inhibitory activity of Zingiber officinale and Zingiber zerumbet.


PERCENTAGES OF INHIBITION
150
100
50
0
% INHIBITION
OFFICINALE
ZERUMBET
0
5
10
15
20
25
TIME (HOURS)

shows that the extracts begin to exert their effects after being mixed with the host for two hours. Furthermore, the inhibition seems to be an irreversible phenomenon. Once the decrease in the number of plaques occurs, it persists throughout the 24 hour period.

Using an ANOVA, one can test the null hypothesis to conclude $U_1=U_2=U_3$, in other words, the means of the control, officinale and zerumbet data are all the same at each point of time. However the rejection of the null hypothesis does not imply that all statistical means of the three categories (control, officinale, and zerumbet) are different from one another. One can know neither how many differences there are nor where differences are located among the population groups. For example, if there are three categories, as in viral inhibition test data, and the null hypothesis is rejected, then usually it can not be determined whether control population behaves differently than the zerumbet, whether control behaves differently than officinale, whether officinale behaves differently than the zerumbet, or whether they all behave differently. Since it is invalid to employ multiple t-tests to examine the differences between all possible pairs of means (Zar, 1984), the "Tukey Test" was performed on the data.

The Tukey test was done for the 3rd, 4th, 5th, and the 6th hour, since the null hypothesis was rejected by ANOVA at these points (table 2). At the hour number 3 to 5, the test

showed that all of the groups are statistically different from each other. Tukey test explains how the populations are behaving at each particular unit of time. To analyze the whole picture of six hours and to measure if the treatment with zerumbet is more potent than the officinale throughout the six hour period, a linear regression analysis was performed to determine if the slopes and the elevations (y-intercepts) of the officinale and zerumbet plot in fig. 13 is statistically different (Appendix A).

Both, the slopes and the elevations of the officinale and zerumbet plots, ended up being statistically the same. Whether this analysis was performed from 0 hour to the 6th hour, or 1st to the 3rd hour made no difference. Even though the officinale treated population might be different than zerumbet treated population, once the whole picture is analyzed the amount of reduction in the number of plaques or the amount of inhibition is the same. The important factor is that if each individual period of time is taken into account, then zerumbet treatment provides a greater reduction in the number of plaques.

II. Transmission Electron Microscope Experiment:

Once it was confirmed that the two plant extracts had inhibitory activities, it was necessary to study the nature of the inhibition. For instance, further study had to be done to find out whether the inhibitory activity occurred in the

cytoplasm or on the outside surface of the host. If inhibition was occurring outside of the cytoplasm, the possibility of interference with viral adsorption to the host existed. This interference could occur in many ways. For instance, the plant extract could cover the surface of the host therefore preventing the collision of the viral particles with the host surface. Another way that the interference could occur would be through providing a change in the nature of the receptor sites existing on the surface of host. Previously it had been shown that many chemical agents change the nature of these receptor sites leading to the prevention of adsorption of virus to the host (Garen and Puck, 1951 and Reiter, 1956).

The usage of cationic ferritin (CF) provided a rather interesting results in the TEM experiment. This probe indicated that the nature of the charges existing outside of the host surface is negative since CF strongly bound to the cell wall of the host in the control group (fig. 15). No CF binding was observed within the host cells in the experimental and control populations, indicating the inability of the CF to penetrate the cell wall and cell membrane. Furthermore, no evidence of the plant extract covering the surface of the host could be seen in the experimental group. The extract treated population, however, differed qualitatively from the control group.

This qualitative difference was due to the fact that CF

Fig. 15 Distribution of CF applied to living cells of Plectonema boryanum without the presence of the officinale rhizome extract. X22,500

Fig. 16 Distribution of CF applied to living cells of Plectonema boryanum with the presence of the

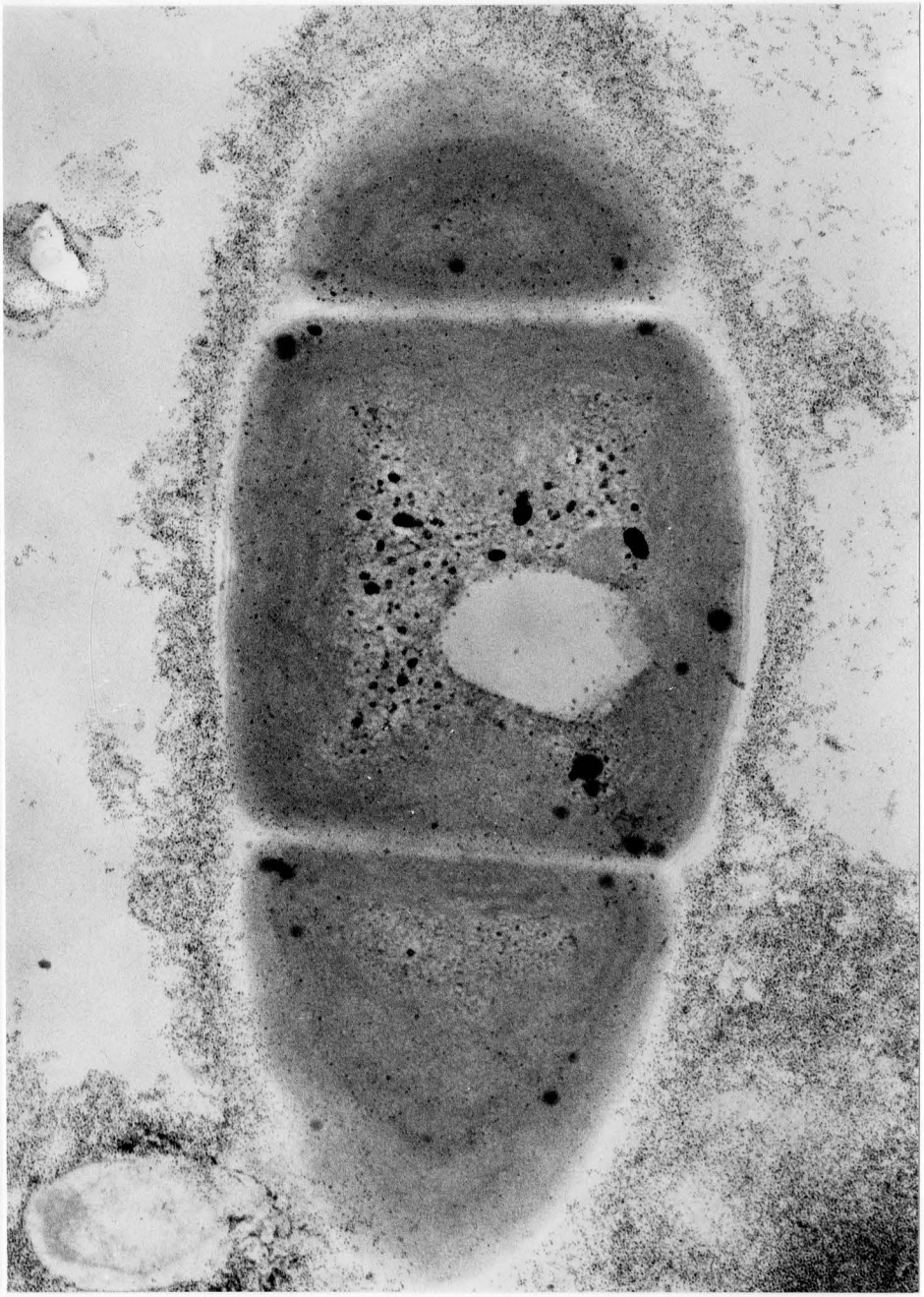


Fig. 16 Distribution of CF applied to living cells of Plectonema boryanum with the presence of the officinale rhizome extract. X15,000

Fig. 17 TEM micrograph of the same Plectonema cell as in figure 16 but with greater magnification. Note the

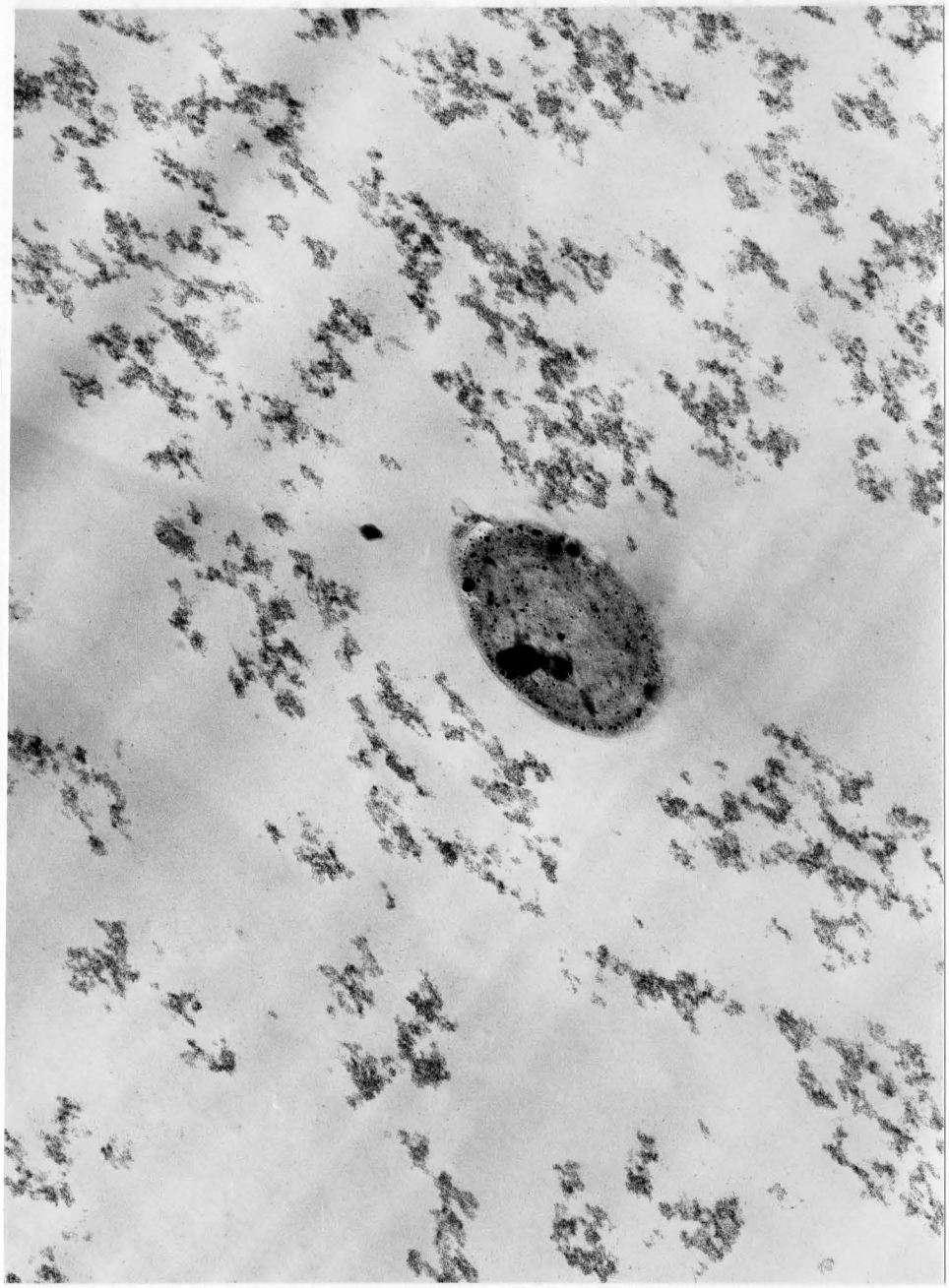
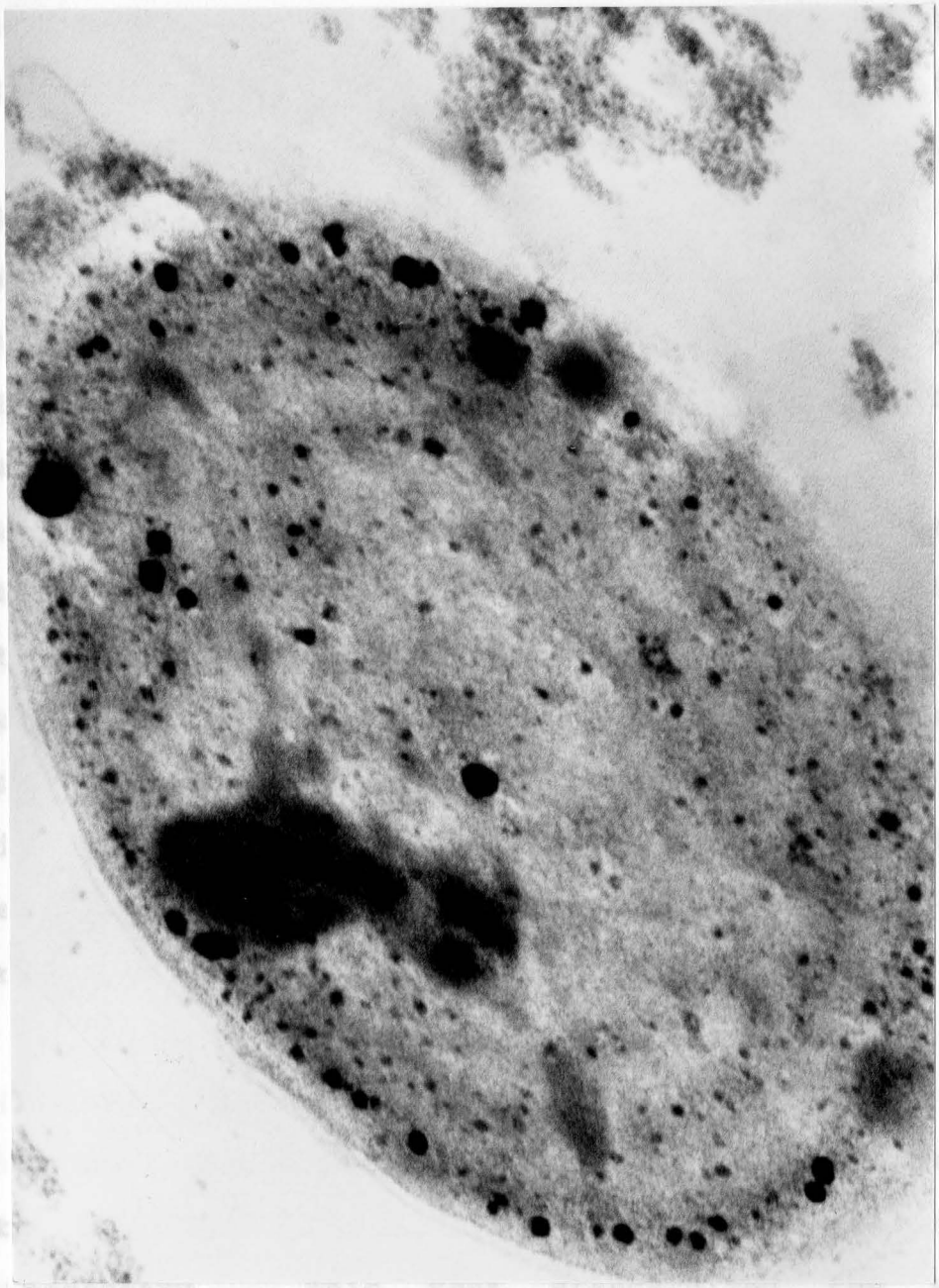


Fig. 17 TEM micrograph of the same Plectonema cell as in figure 16 but with greater magnification. Note the absence of the CF on the cell wall. X75,000



did not bind on the surface of the treated host as it did in the control group (fig. 16 and 17). This indicated that the extract had produced a change in the nature of the surface charges existing on the host. In many of the studies done on the bacteriophages, it was observed that changes in the charges exist on the host's surface which lead to the prevention of the adsorption step of the viral infection (Hershey, Kalmanson, and Bronfenbrenner, 1944). These authors found that the relative efficiency of plating (EOP) of phage T2 varies with the concentration of electrolytes added to the medium. Therefore, there is a great possibility that the electrolytes existing in the extract compete and bind electrostatically to the outside surface of the host, producing a difference in the charge characteristics existing on the host surface thereby interfering with the viral particles' ability to adsorb. It was at this point that the usage of more quantitative techniques for the study of the adsorption process seemed necessary.

III. Toxicity Test:

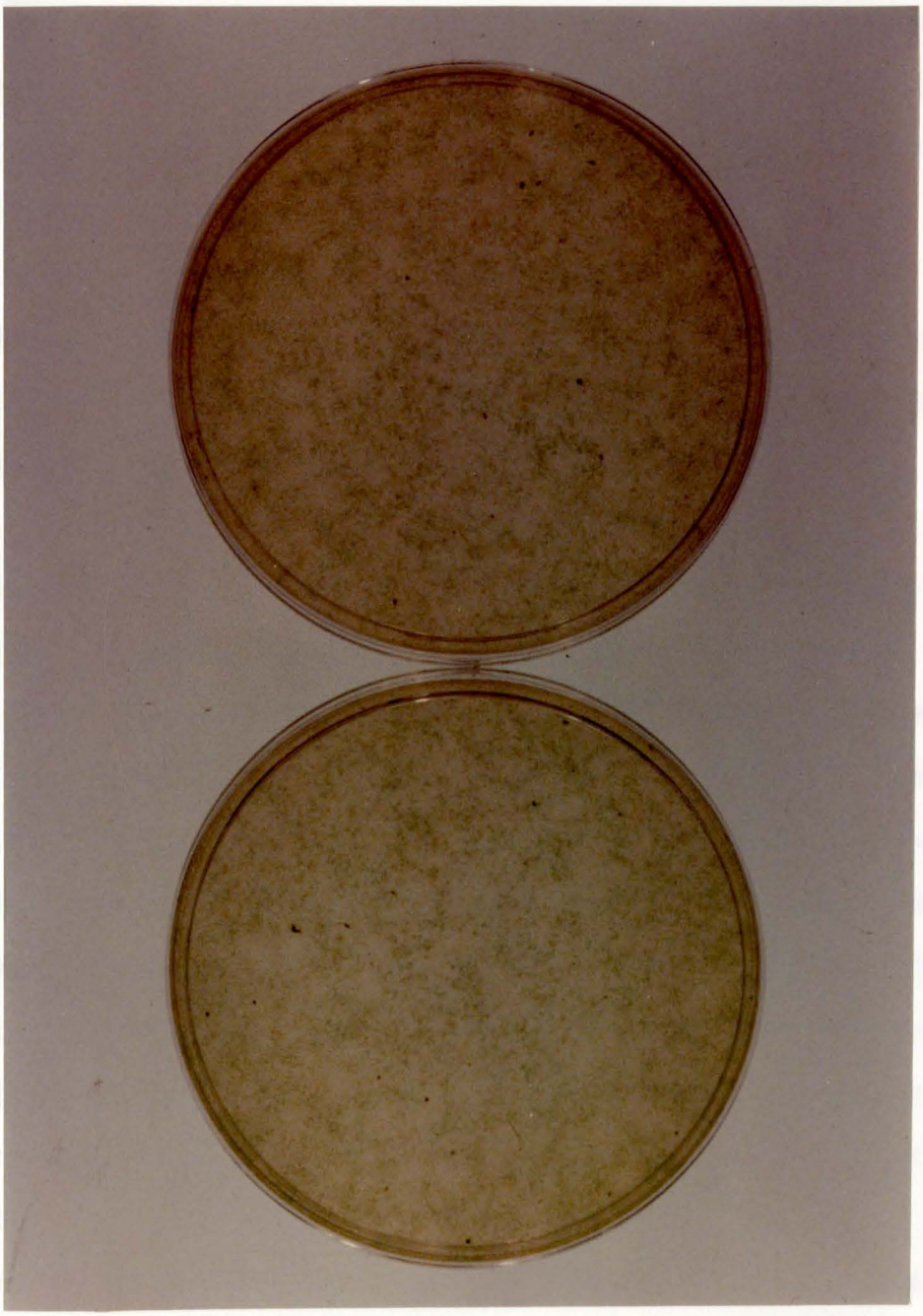
Before using more quantitative approach to study the inhibitory activities of the rhizome extracts, a toxicity test was necessary. This test standardized the maximum concentration of the extract that was to be used for the determination of the rate of adsorption, and the burst size of the virus.

Without a toxicity test no meaningful antiviral activity could be measured. This is because a cell has to be healthy metabolically in order for the viral infection to proceed. If the extracts were to be extremely toxic to the cyanobacteria, then no measurement of viral growth could be achieved, and in turn no measurement of the antiviral activity could be possible.

Metabolic activity of a cell is important also in terms of the effective viral adsorption. In his paper Schlesinger (1932b) showed that the adsorption rate on living bacteria is many times faster than the non-living bacteria. This difference in the rate of adsorption was due to loss and destruction of some of the receptor sites in the non-growing cultures of the bacteria. As a result, there was a possibility that the cyanobacterial cultures that received a too high concentration of the extract would stop growing actively and would lose some of their extracellular features like the receptor sites.

The toxicity test revealed that the host, Plectonema boryanum can tolerate concentrations as high as 20% (v/v) with incubation timing of up to 4 hours. On the solid media (agar plates), the number of colonies of the treated cultures was the same as the control cultures (fig. 18). Therefore, it was decided that any further experimentation procedures for the measurement of the viral inhibition would be done using 20% (v/v) concentration with the incubation time of four hours.

Fig. 18 Colonies of Plectonema boryanum grown in the solid media containing the zerumbet extract (left) and in the solid media containing Bold's medium (right). No difference was observed between the control (right) and the experimental group (left).



IV. Quantitative Examination of the Antiviral Activity

A. Determination of the Rate of Adsorption:

Krueger (1931) investigated the adsorption of a phage to living and heat-killed staphylococci. He found out that with an excess of host, the adsorption follows the kinetics of a first order reaction. The rate of adsorption is

$$-dP/dt = kBP$$

in which k is the velocity constant, B is the bacterial concentration, and P is the phage concentration. The solution of this equation yields

$$k = 2.3/Bt \log P_o/P$$

in which P_o is the concentration of unadsorbed phage at the beginning, and P at the end, of the time interval t.

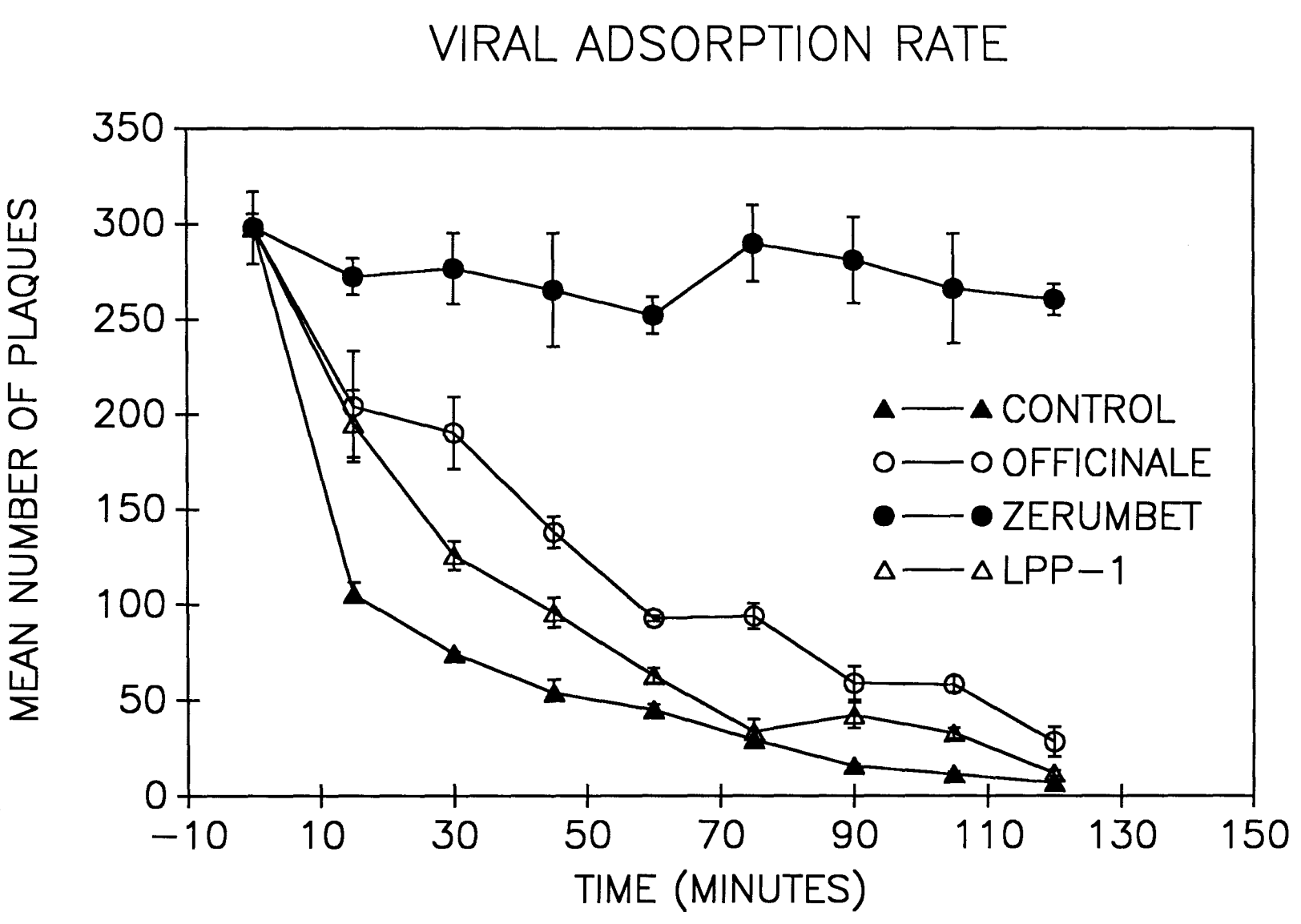
In cyanophage-cyanobacteria system, adsorption step is more complicated. This complication is due to the fact that many of the hosts are filamentous. Most techniques and statistical analyses used in the studies of phage infection systems in unicellular hosts like E. coli can not be strictly applied in the case of multicellular hosts. For example since Plectonema boryanum is a filamentous cyanobacteria, the adsorption kinetics of its virus, LPP-1, is non-linear. As a result, no meaningful k value (velocity constant) can be measured (Padan and Shilo, 1969) unless the host is a mutant which has only 1 to 6 cells per trichome. The host organism used for the entire experimentation in this thesis was the wild type which had an average of 50 cells per trichome.

Consequently, the adsorption step of the LPP-1 virus infection was studied entirely by the assay of the unadsorbed phage particles in the supernatant fluid media. Whether the extracts had any interference with the adsorption of the viral particles was studied by the assay of the unadsorbed phage and by centrifuging out the adsorbed particles. Assay of the unadsorbed phage was done for four different populations: officinale treated host, zerumbet treated host, officinale treated virus, and control (Fig. 19 and tables 12,13,14,15).

As it can be seen, zerumbet treatment of the host seemed to be more effective in the interference with viral adsorption than the officinale treatment. Furthermore the duration of the action of zerumbet treatment seems to be much longer because the number of free phage particles hardly decreased when the host and the virus were incubated together for 2 hours. Also, treatment of the host with officinale extract seemed to be more effective than the treatment of virus alone because a greater number of free phage particles existed at each time when host was treated compared to when virus was treated. To test the significance of the observations described above, one-way analysis of variance and Tukey tests were performed.

The ANOVA was done for each and every plot seen in fig. 19 (table 3). All of the data were significant statistically and indicated that the extract treatment provides significant change in the ability of the LPP-1 virus to adsorb to its

Fig. 19 Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells. Condition for the infection was prepared by an input of 0.1 PFU/trichome. Note the constancy of the number of unadsorbed LPP-1 virus particles when the Plectonema cells are treated with the extract of Zingiber zerumbet.


VIRAL ADSORPTION RATE
MEAN NUMBER OF PLAQUES
350
300
250
200
150
100
50
0
−10
10
30
50
70
90
110
130
150
TIME (MINUTES)
CONTROL
OFFICINALE
ZERUMBET
LPP−1

host, P. boryanum.

The Tukey test (table 4) showed that there was a significant difference between the zerumbet treatment of the host and the officinale treatment, starting at t=30 minutes after the incubation of the host with virus. This indicates that zerumbet extract is more effective in halting the adsorption step of the infection.

Whether the officinale treated host behaves differently than the officinale treated virus group is difficult to assess. These two groups only showed significant difference at 30, 60, and 75 minutes after the incubation of virus and the host. As it can be seen from table 4, this significant difference is not a great difference either, even though it is valid statistically.

The comparison of the officinale treated virus versus the control, showed that there was a statistical difference only at t=15 minutes. No further difference was observed statistically. This is interesting because it can be an indication that whatever the nature of the interaction of the extract and the virus, this interaction interferes with the adsorption for about 15 minutes. In other words, it is a reversible interaction. It seems therefore that the extract treatment can not exert a prolonged effect on the virus directly. Comparison of the officinale treated group with control revealed a significant difference up to t=90 minutes. Therefore, it can be postulated that the interaction of the

extract and the cell surface, which leads to interference with the adsorption of virus (possibly by producing changes in the receptor sites of the host), is reversible like the phenomenon observed in the virus treated population. One difference however between these two groups is the duration of the action of the extract. The officinale extract's effect is longer lasting when the host is treated as opposed to when the virus is treated.

The zerumbet treated host group was significantly different all throughout the 2 hour period than the control group and the effect of the extract remained the same throughout the two hours time for which the experiment was planned. This indicates that zerumbet is extremely potent in terms of its ability to interfere with viral adsorption.

The adsorption rate experiment revealed one reason why there was a significant decrease in the number of plaques in the agar media of the viral inhibition test, described above. In phage research, it is known that the plaque size is limited by many factors. One of these factors is the adsorption step. If LPP-1 virus particle does not adsorb to its host to start the first cycle of infection until late in the development of the lawn of cyanobacteria, the result will be a small plaque or no visible plaque. This is why slowly adsorbing viruses like LPP-1 produce plaques that have a great difference in their sizes. The particles adsorbing early produce much larger plaques than those adsorbing late (Sagik, 1954). If

LPP-1 virus is allowed to adsorb to its host in the fluid medium, and the free phage particles are removed and the infected cyanobacteria are plated, a greater uniformity of plaque size will be achieved within the petri plates. Adsorption rate therefore has a great affect on the number of plaques that appear in the petri plates.

Other factors that can reduce and possibly did reduce the number of plaques in viral inhibition experiment are the latency period and the burst size, described below. To determine whether the inhibition of viral reproduction was also due to intracellular inhibition, measurement of the latency period and of the burst size of LPP-1 virus was an absolute necessity.

B. The Single Step Growth Experiment:

1. Determination of the Burst Size In *P. boryanum* _ As mentioned earlier, the one-step growth experiment allows one to determine both the latent period and the burst size of the infective viral particles.

The results of this experiment are presented in figure 20, 21, and 22. To facilitate the reader's understanding of the one-step growth experiment, the results from the control group will be discussed first and then the results of the experimental groups will be compared to the control group.

It is apparent from table 20 that in this experiment the plaque counts from the First Growth Tube (F.G.T.) were constant through 7 hours but increased suddenly by the 8th

Fig. 20 Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media. Infection was started with an input of 0.001 PFU/cell. Latent period was considered to begin at 0 hour (after 1 hour adsorption time where further adsorption of the virus was prevented by dilution). B0V0=control; B1V0 (empty circle)=officinale treated host; B1V0 (filled circle)=zerumbet treatedhost; B0V1 = officinale treated virus.

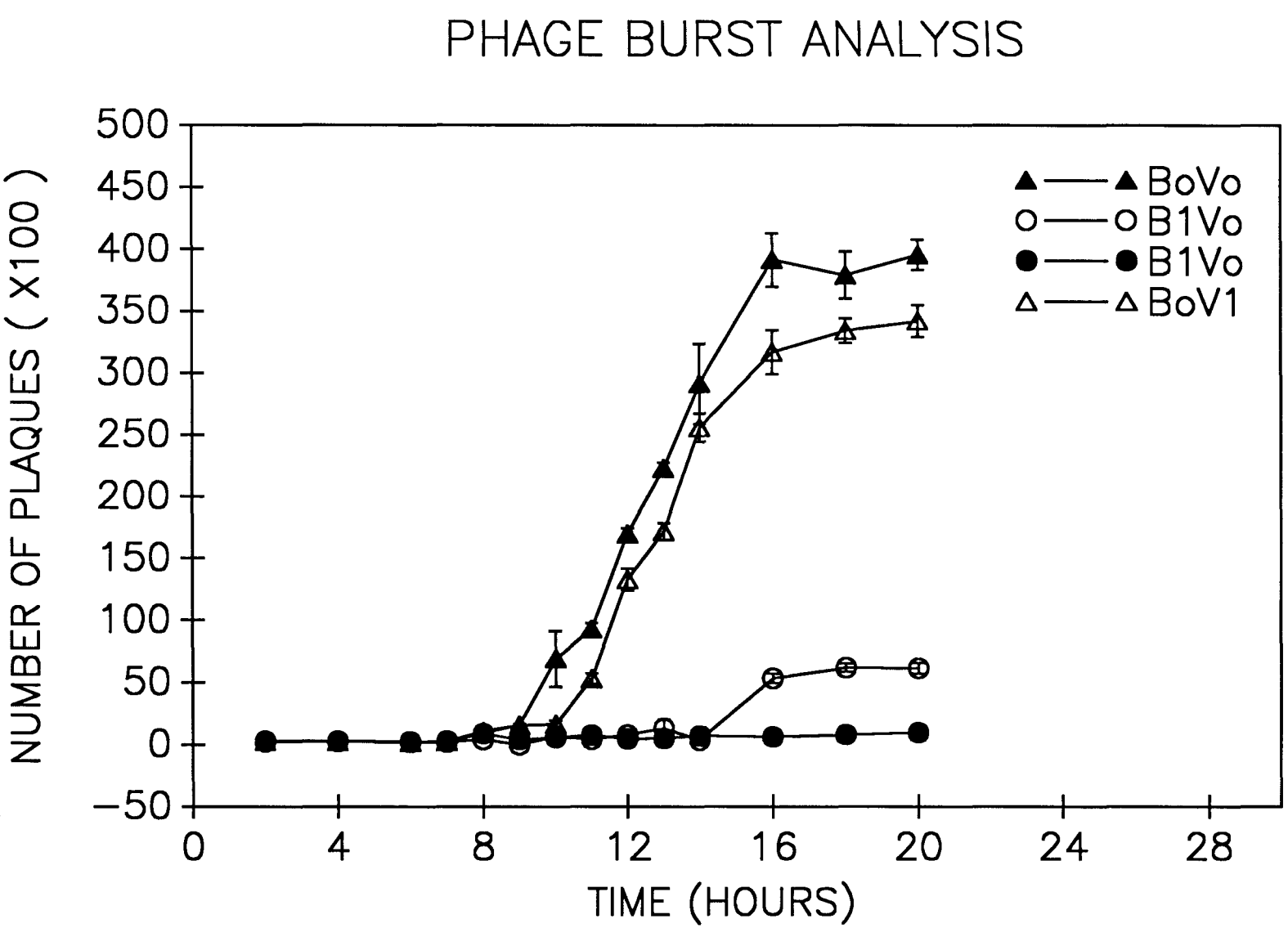

PHAGE BURST ANALYSIS
NUMBER OF PLAQUES (X100)
500
450
400
350
300
250
200
150
100
50
0
−50
0
4
8
12
16
20
24
28
TIME (HOURS)
BoVo
B1Vo
B1Vo
BoV1

Fig. 21 Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media. Infection was started with an input of 0.001 PFU/cell. Note that the number of phage progeny is greater when the host is treated with the officinale extract before the adsorption of the virus.

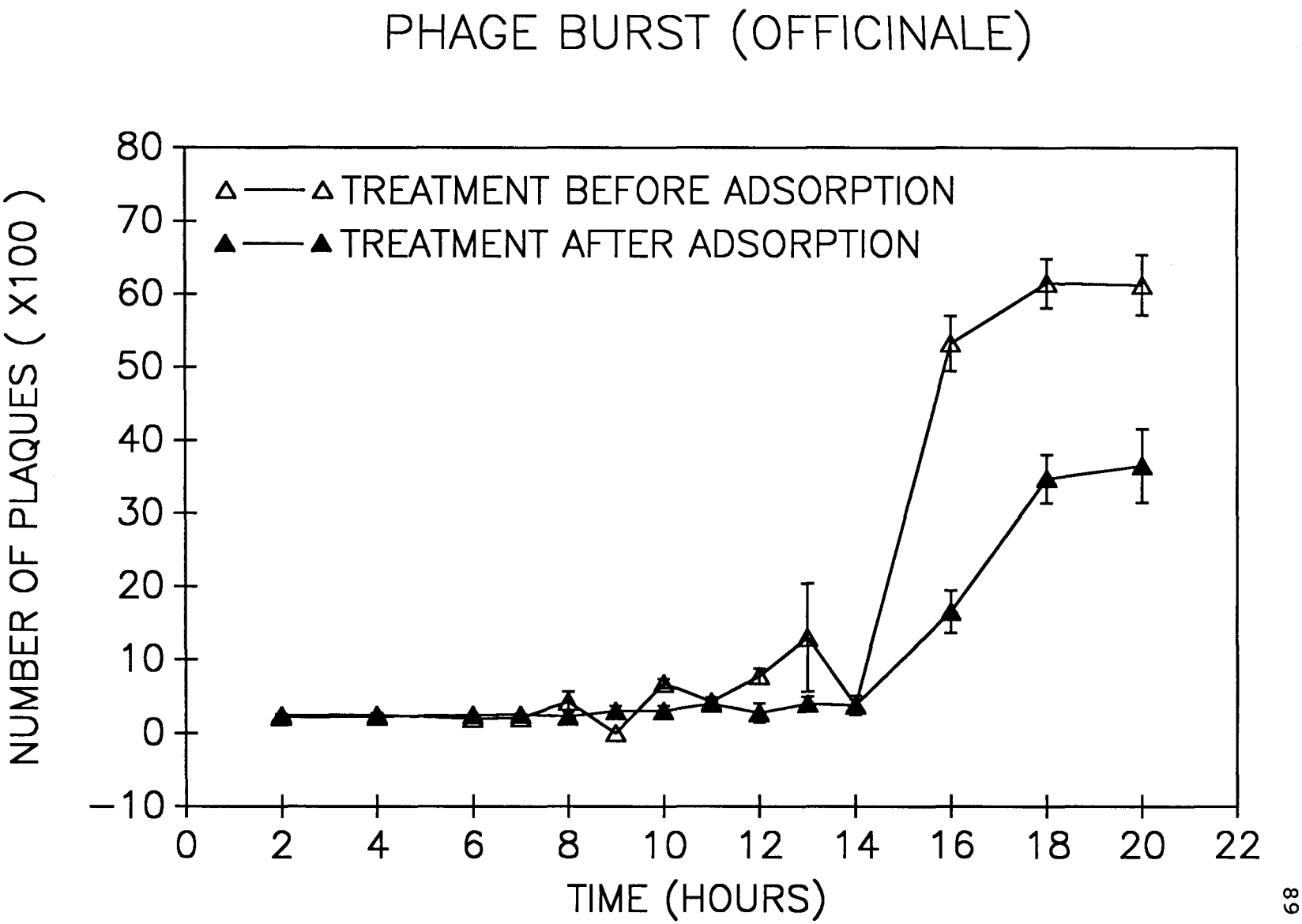
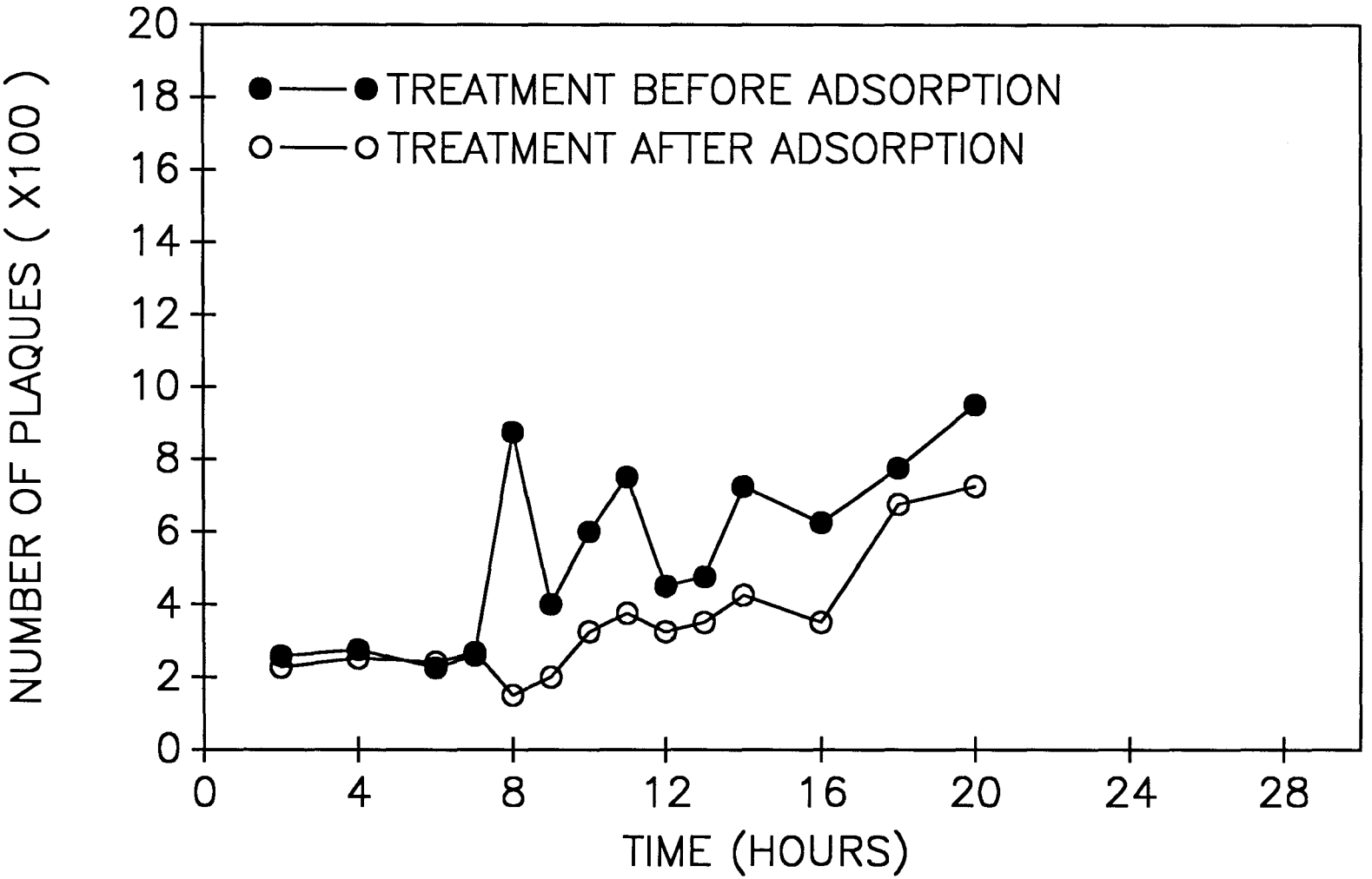

PHAGE BURST (OFFICINALE)
TREATMENT BEFORE ADSORPTION
TREATMENT AFTER ADSORPTION
NUMBER OF PLAQUES (X100)
80
70
60
50
40
30
20
10
0
-10
0
2
4
6
8
10
12
14
16
18
20
22
TIME (HOURS)

Fig. 22 Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media. Plectonema cells were treated with zerumbet extract before and after the adsorption of the LPP-1 virus.

PHAGE BURST (ZERUMBET)



hour. Therefore, the end of the latent period comes between the 7th and the 8th hour after the infection. The counts from the Second Growth Tube (S.G.T.) increased up to about 16th hour and remained constant from then on. Average count during the latent period was 242 which, multiplied by the dilution factor of 100, indicates 2.42×10^4 infected cells/ml. of the adsorption tube. This number, however, is not the actual number of infected cells per ml..

Usually, in the one-step growth experiments, particularly those of the colliphages, antiphage serum is used to inactivate the unadsorbed phage particles in the sample. This then allows the plaques at the beginning of the experiment to reveal the number of infected cells/ml. and the plaques at the end of the experiment to reveal the phage progeny/ml. But in this experiment, the use of anti-LPP-1 serum was avoided because there was a possibility that the serum would affect the ability of the infected cell to produce plaques in the petri plates. As a result, the plaques in the early phase of the experiment indicated the number of infected Plectonema cells plus the number of unadsorbed LPP-1 phage particles. Similarly, plaques at the late phase of the experiment indicated the phage progeny plus the unadsorbed phage particles. The plaques caused by the infected host cells can not be distinguished from plaques caused by free virus particles. Therefore, to be able to measure the number of infected cells and the number of progeny virus, assay of the

unadsorbed viruses was done early in the experiment and the number of free, unadsorbed phage particles was determined. Then this number was subtracted from the actual plaques counted throughout the experiment in order to measure the correct number of infected cells and progeny phage.

Assay of the unadsorbed phage particles revealed a value of 600 p.f.u. per ml. of the adsorption tube. This value in terms of the F.G.T. becomes 6 p.f.u. per ml. Therefore, the number of infected cells of the adsorption tube becomes $(2.42x10^4)-(6x10^2)$ or $2.36x10^4$ per ml.. The multiplicity of infection, the average number of phage particles adsorbed per cell in the adsorption mixture, is 0.0018. This was calculated by dividing the number of infected cells per ml. $(2.36x10^4)$ by the total number of cells per ml. $(1.31x10^7)$. Notice that since a low m.o.i is used, the number of infected cells equals the number of virus particles adsorbed. The per cent adsorption in one hour is 92. This is because the assay of the original virus concentration at time t=0 revealed $2.57x10^4$ p.f.u. per ml..

Average count after the completion of lysis is 389 which multiplied by the dilution factor of 10^2 in going from F.G.T. to S.G.T., gives a count of 38,900 in terms of the F.G.T.. Average burst size is therefore 38,900/236 or 165. Total time period from the end of the latent period to the completion of the lysis is called the rise period which in this case was about 8 hours. Notice that the rise period of the LPP-1

cyanophage infection takes a much longer time than the bacteriophages like T2 or T4 which take only about 5 to 10 minutes (Ellis and Delbruck, 1939).

Results of the control and the experimental populations are expressed graphically in fig. 20, 21, and 22. In these graphs, phage assays are plotted against the time after initiation of the adsorption step. The latent period, rise period, stationary period and burst size can clearly be seen.

The phage burst experiment was designed so that the conditions of single infection would exist. In other words, the probability and proportion of a cyanobacterial cell being infected by more than one virus would be very low. In fact since the host organism is filamentous, the multiplicity of infection was chosen to be very low in order to decrease the probability of more than one virus particle adsorbing to a trichome. If more than one virus particle adsorbs to a trichome, then a systematic error occurs in the interpretation of plaques in the petri plates. For instance, if two virus particles adsorb to one trichome, the result would be one plaque instead of two which will lead the experimenter into thinking that one virus particle has successfully adsorbed and begun the process of infection.

The input ratio of phage particles to cyanobacteria cells was 0.001, but with 92% adsorption in one hour the multiplicity of infection was 0.0018. The distribution of phage particles among the host cells follows the Poisson

distribution as long as multiplicity of infection of less than 2 is used (Ellis and Delbruck, 1939). The general Poisson formula is $P(r) = (n^r/r!) \times e^{-n}$ in which P(r) is the proportion of the cyanobacteria adsorbing r phage, and n is the multiplicity of infection. Consequently, the proportion of cyanobacteria not infected at all is P(0) which is $(n^0/0!) \times e^{-n}$ and if simplified it becomes e^{-n}. Therefore, the P(0) for the experiment becomes $e^{-0.0018}$ which equals 99.8%. The proportion infected with 1 phage particle is P(1) which if calculated, $(0.0018^1/1!) \times e^{-0.0018}$, equals 0.0018. The proportion infected with 2 or more phage particles is the remainder, which is 0.000002. This is hardly significant and it indicates that hardly any of the cells had two or more virus particles adsorbing to them.

The adsorption period of 1 hour was chosen because the LPP-1 virus is a slow adsorbing phage (Goldstein, 1967). The input ratio of 0.001 was selected to give a low probability of a trichome being infected by more than 1 virus particle. With slowly adsorbing phages like LPP-1, it must be kept in mind that there is a limit to the time of the incubation of virus and its host. This is because if the latent period is over before the adsorption is complete, due to lysis some of the progeny virus will end up adsorbing to the host cells. This leads to a longer than usual rise period which then will not indicate the true rise period.

The dilution of the original adsorption mixture 1:100

into the F.G.T. tube was done because of two reasons. One is that the dilution stops the adsorption process, therefore, allowing one single infection process to proceed and to be measured. The dilution also allows a countable number of plaques to be present in the petri plates. The dilution factor of 1:100 in going from F.G.T. to S.G.T. was chosen because of the expected rise in the plaque count as lysis of cells proceeded.

Another important consideration that must be kept in mind in going from the F.G.T. to S.G.T. is the sampling error. The 1 ml. sample from the F.G.T. that was diluted 1:100 in S.G.T. had about 100 infected cells plus some free phage particles. A sample of this size has an expected sampling error of 10 per cent (Ellis and Delbruck, 1939). As a result the determination of the burst size has at least this much error assuming every other step has been done perfectly. The larger the sample taken out of F.G.T. in preparing S.G.T., the lower the sampling error will be.

In this experiment, the over all dilution in going from the adsorption tube to the S.G.T. was 10^{-4}. This is important because it prevents the released phage particles from readsorbing to the surviving cyanobacterial filaments. This allows the stationary period in the graph to be a true stationary period. If the host organism was a faster growing organism, like E. coli, the dilution from the adsorption tube to the S.G.T. must be several times higher. This is because

the host cells would continue to reproduce also so that the probability of a released phage particle adsorbing to the host cells increases.

One interesting observation made in this experiment was noticed in the 7th hour sample from the S.G.T.. This plate had 88 plaques, but when looking at the F.G.T. it should have had between 2 to 10 plaques. Most likely, the reason for this high plaque count is that the 4 ml. sample that was pipeted from the S.G.T. contained an infected cell which probably burst and released its phage progeny in the pipet while plating was being done. The 88 phage particles should have been distributed uniformly through the 100 ml. volume of the S.G.T.. If this were to happen, the actual contribution of plaques would have been about 3 plaques, not 88. Because of the fact that the infected cells become more fragile, there was a possibility that the burst occurred within the pipet while removing out the samples for plating.

2. Comparison of the Experimental and Control Groups _ In cyanophage/cyanobacteria system, not only the measurement of the adsorption but also the measurement of viral growth is a more complicated process. This again is due to the fact that many of the hosts are filamentous. This filamentous nature may trigger a great change in the way viral reproduction occurs as opposed to the bacteriophages. Apart from being filamentous, some cyanobacteria have heterocyst cells or spore cells which represent cellular differentiation. These

differentiated cells can clearly provide a better or a worse environment for the viral growth. As a result the techniques used in studying host-virus interactions in unicellular cells can not be strictly applied in the case of cyanophage/ cyanobacteria system. Plectonema boryanum, although filamentous, is not a heterocystous-spore containing organism, so it represents a good model system for this study.

The technique for the determination of the burst size is extremely adaptable. It allows one to change the physical and biochemical environment of the host cell in order to measure the effects that these changes will have on the infectious process. In this study, the physical and biochemical environment of the host cell was changed by the application of the extract. Five experimental groups and one control group were set up (tables 21,22,23,24,25). The experimental groups consisted of officinale treatment of the host before adsorption, officinale treatment of the host after adsorption, zerumbet treatment of the host before adsorption, zerumbet treatment of the host after the adsorption, and the officinale treatment of the virus.

As it can be seen from figure 20, when the host is treated with either of the extracts, the latent period becomes prolonged. This prolongation is also seen when the virus is treated with the extract, but to a lesser extent. Hardly any reduction of the burst size occurred when the virus was treated with the extract. On the other hand there seemed to

be a tremendous reduction of the burst size when the host was treated. Also there seemed to be a difference between the officinale treatment and zerumbet treatment before the adsorption. The zerumbet extract seems to be much more inhibitory in terms of its reduction of the burst size. Statistical tests that were performed for the data in figure 20, 21, and 22 were the analysis of variance and the Tukey Test (table 5,6,7, and 8).

The ANOVA was done for the plots of the 9th, 10th, 11th, 12th, 14th, and 16th hour after the infection. All of the data in these plots were statistically significant and indicated that the extract treatment provides a significant change in the ability of the LPP-1 virus to replicate in its host.

The Tukey analysis showed that except for the slight significance for the 9th hour plot, there was no statistical difference between the number of progeny virus in the preadsorption officinale treated Plectonema cells and the preadsorption zerumbet treated Plectonema cells. But in reality there is a difference and it is indeed a great difference. The Tukey analysis could not measure the difference because the numbers used for this analysis were the plaque count received from the S.G.T. and the numbers were not expressed in terms of the F.G.T.. A difference of one plaque between the petri plates from S.G.T. can mean a difference of 100 plaques if the data in S.G.T. is expressed in terms of the

F.G.T.. This is because the preparation of the S.G.T. from F.G.T. required 1:100 dilution. As a result of this consideration, it can concluded that indeed a great difference existed between the number of progeny virus from officinale treatment as opposed to zerumbet treatment (table 26). The latent period of infection was the same for both of the extract treatments.

The officinale treated host data were shown to be significantly different than the officinale treated virus data when Tukey test was performed. This difference was observed for the 9th, 11th, 12th, 14th, and the 16th hour. Again as expected, the treatment of the host seems to be a better and a more effective way of inhibiting the viral growth than treating the viral particles alone. When the host was treated, the latent period was prolonged more and the burst size was reduced more.

The comparison of the control and officinale treated virus showed that these two populations were different statistically only at t=10,11,12 and 16. This means that when the virus is treated, the latent period is prolonged (fig. 20). The rise period starts later in the virus treated group than the control but it ends at the same time with lesser number of progeny virus. Most likely then, the viral treatment results in a later than usual infection cycle. There is a great deal of support for this hypothesis from the adsorption rate experiment discussed above. Recall that when

the LPP-1 virus was treated with the extract, there was a reversible interference with its adsorption to the host cells. The later the adsorption occurs, the later is the penetration, uncoating, and replication. In other words, the later the adsorption, the later is the beginning and the end of the latent period of viral growth. The fact that the officinale treatment of the virus resulted in lower number of phage progeny (table 26 and fig. 20) must be interpreted cautiously. The lower number of phage progeny after the virus treatment alone does not mean that the extract is exerting inhibitory effects on the virus. This reduction in the number of plaques would most likely not occur if the officinale extract were to be removed from the virus particles by washing them with saline. Washing of the treated virus particles was avoided so that more experimental categories could be tested along with the control series.

The Tukey analysis of control v.s. officinale treatment of host and control v.s. the zerumbet treatment of host showed a significant difference at all plots considered above. The conclusion drawn from this was that the extract treatment elicited an intracellular inhibition toward the growth of the virus within the host, both by increasing the latency period (time after which lysis occur), and decreasing the burst size.

As it was stated earlier, the host was also treated with both of the extracts <u>after</u> the adsorption of virus. This was done to see whether there was any dissimilarity in the number

of plaques when treatment was done before and after the adsorption. Figure 21 shows the results of P. boryanum treatment with the officinale extract before and after the viral adsorption. As seen here, there is no change in the latency period of the virus, yet there is a significant decrease in the progeny virus in the after adsorption treatment. Analysis of variance of the 16th, 18th, and 20th hour (table 7) however showed that indeed this difference is significant. It is difficult to speculate why there would be a difference in the number of phage progeny given off in both of the treatments. It seems, however, that treatment of cells with officinale after the adsorption of the virus makes the virus more susceptible to the antiviral effects of the extract. The assay of the infected cells from the F.G.T. revealed a reduction in number of infected trichomes in post adsorption treatment as opposed to preadsorption treatment. The assay of the unadsorbed phage particles in the adsorption sample revealed a greater number of free phage particles after the addition of the extract. This possibly means that the adsorbed virus particles became detached from the cell surface once the extract was applied. That these virus particles were still intact virions containing their DNA and not ghost particles was known from the fact that they produced plaques in the petri plates.

Treatment of the host with zerumbet extract after the adsorption of virus showed no difference. The analysis of

variance (table 8) revealed no significant difference in the total number of the progeny virus (fig. 22). Here also one ought to be skeptical in the results of the analysis of the Tukey test. Again the Tukey analysis failed short of measuring the difference between the preadsorption and postadsorption zerumbet treatment, since the plaque count was from the S.G.T.. A difference of one plaque between the petri plates from S.G.T. means actually a difference of 100 plaques since in going from the F.G.T. to S.G.T, hundredfold dilution takes place. Here also the number of infected cells per ml. of the solution was lower than the treatment with zerumbet before adsorption. When the assay of the free phage particles was done, it revealed more unadsorbed phage particles than the control group. So again evidently the adsorbed phage particles were detached from the cell surface when the extract was applied.

It was seen earlier that the reduction in the number of plaques in the viral inhibition test was partly due to reduction in the rate of adsorption. The phage burst experiment also showed that the reduction in the number of plaques was also due to the increase in the latent period and the decrease in the burst size. The longer the latent period is, the longer it will take for the plaques to appear in the petri plates. Moreover, the smaller the burst size is, the longer it will take for the plaques to appear in the plates.

C. The Single Cell Burst Experiment:

1. Determination of individual cell burst size in the control group _ The single cell burst experiment was performed to confirm and double check the results of the one-step growth experiment. Recall that in one-step growth experiment, the value of burst size is an average value of the whole population of the infected cells that lyse and release the progeny phage particles. In single cell burst experiment, however, the burst size value is measured directly from the lysis of a single infected cell.

This method is essentially diluting a suspension of the infected cyanobacteria cells to the point that small samples of the dilution contain no more than one infected cell within the trichome. The samples are incubated until the burst of the infected cells occurs. Then the samples are plated to determine the phage yield of the single infected cyanobacterium.

In the one-step growth experiment, Poisson distribution was used to measure the distribution of virus particles among the host cells. In single cell burst experiment, however, the Poisson distribution is used to measure the distribution of the infected cyanobacterium among the samples (Ellis and Delbruck, 1939). Recall that the Poisson formula is

$$P(r) = (n^r/r!) \times e^{-n}$$

in which P(r) is the proportion of samples containing r particles and n is the number of particles per sample. For

the single cell burst experiment, unlike the one-step growth experiment, r represents the number of infected host cells rather than the phage particles. Furthermore n represents the number of infected cyanobacteria cells (r) per sample, not the multiplicity of infection which is the number of adsorbed phage particles per number of host cells. For the single cell burst experiment, the value of n should be made so low that the proportion of samples containing 2 or more infected cyanobacteria will be small compared to the proportion containing 1. This is because if the samples containing 2 or more infected host cells are plated, the number of plaques in the petri plates will be too high and it will not represent the burst size from the single infected cell. The value of n used for this experiment was 0.35 since a higher number would increase the proportion of samples containing 2 or more infected cyanobacteria and a lower number would increase the amount of samples containing no infected cyanobacteria.

The multiplicity of infection used in this experiment was 0.1 per trichome. This number was chosen to be low so that the possibility of more than one infected cell within a trichome would decrease so that the plaque count would represent the phage progeny from one infected cell within the trichome, not 2 or more. Since the number of singly infected trichomes per ml. was very high, dilution was performed so that only 0.35 singly infected trichomes would exist per ml.. Recall that this was done in order to reduce the value n so

that the P(2), the proportion of the samples containing 2 infected trichomes would be low.

Time, min.	Tube	Procedure	Expected # of infected trichomes/ml.
0	1. Adsorption	Add 8 ml. of virus to 92 ml. of host	1.6×10^4
60	2. Dilution	Add 1 ml. of #1 to 9 ml. of Bold's media	1.6×10^3
65	3. Dilution	Add 1 ml. of #2 to 9 ml. of Bold's media	1.6×10^2
70	4. Sample flask	Add to 99.8 ml. of Bold's media 0.21 ml. of #3	0.35

This dilution was distributed into 50 samples of 1 ml. each. After the 16 hour incubation, all bursts were to occur and the samples were then plated according to the agar layer method.

The adsorption rate for LPP-1 is known to be about 90% in one hour (Goldstein, 1967) for Plectonema boryanum at room temperature grown in Bold's medium to a concentration of 5×10^5 trichomes per ml.. An input ratio of 0.1 LPP-1 per trichome would then result in adsorption of $0.1 \times 0.9 = 0.09$ LPP-1 per trichome in one hour. The proportion of uninfected trichomes would be equal to $e^{-0.09} = 0.914$ or 91.4 per cent. This means that 8.6 per cent of the trichomes will be infected. The concentration of unadsorbed phage in the adsorption tube would be $(2.07 \times 10^5 / 12.5) \times 0.10 = 1.66 \times 10^3$. The numerator is the concentration of LPP-1 virus prepared. The denominator is the dilution factor that is taken into account when the prepared

viral concentration is added to the host. The third factor above is the percent unadsorbed phage particles in one hour. Since only 8.6 per cent of the trichomes were to be infected, the concentration of infected trichomes per ml. was $2.07 \times 10^5 \times 0.086 = 1.8 \times 10^4$. This means there were less than 0.1 unadsorbed virus particle per infected cell which would lyse and release the progeny virus. This would be negligible in comparison with the average burst size of the infected cells. But this would mean that few of the petri plates would have 1 or 2 plaques due to free phage particles.

The Bold's medium suspension containing about 0.35 infected trichome was distributed in 1 ml. samples into 50 test tubes. Distribution was completed before the end of the latent period so that lysis of the infected cells within the trichomes would not cause error in the measurement of the number of plaques. The samples and the remainder of the suspension in the sample flask were incubated at room temperature under 150 ft-c fluorescent light until well after the end of the rise period (14 to 16 hrs.) to make sure that all bursts have taken place. The 50 samples were all plated. The sample flask containing the remainder of the diluted suspension was also plated several times to determine the average burst size in the suspension.

Of the 50 plates, 32 had no plaques, and 18 had a total of 2078 plaques, individual plate counts were as follows:

20	36	79	118	135	234
22	49	105	119	141	253
28	59	108	120	145	307

Average plaque count on the plates made from the sample flask after the end of the rise period was 48.

Thirty two of 50 plates had no plaques. This means that the samples from which these plates were prepared had no infected trichomes. P(0) = 32/50 = 0.64. Recall that at P(0) the Poisson formula reduces from $P(r) = (n^r/r!) \times e^{-n}$ to $P(0) = e^{-n}$. As a result $e^{-n} = 0.64$. So the average number of infected trichome per sample (in other words "n") was 0.45. Since the samples were prepared in 1 ml. aliquot, one can deduce that there were 0.45 infected trichomes per ml. in the sample flask also. As we can see, due to pipet error the value of 0.35 infected cells per ml. which was originally intended at the beginning of the experiment was not achieved due to the existence of systematic error (pipet error). This error, however, lies within an acceptable range. The dilution factor in going from the adsorption tube to the sample flask was 4.6×10^4, so the number of infected cyanobacteria cells in the adsorption tube was $(0.45) \times (4.6 \times 10^4) = 2.07 \times 10^4$ and not 1.6×10^4 which originally was intended. Again this can be explained as the systematic error (pipet error) which is within the acceptable range.

If 50 samples contained an average of 0.45 infected

trichomes/sample, the total number of infected cells in 50 samples should be 50 x 0.45 = 22.5 cells. But out of 50 plates only 18 plates had plaques in them. This means that 4 or 5 plates had plaques which came from more than 1 infected cells. Poisson distribution was very helpful in this instance since $P(0) = e^{-0.45} = 0.64$ or 32 out of 50 plates with no infected cyanobacterium.

P(1) = 0.29 or 14 plates out of 50 with one infected cyanobacterium.

P(2) = 0.06 or 3 plates out 50 with 2 infected cyanobacteria.

P(3) = 0.009 or 0 plates in 50 with 3 infected cyanobacteria.

The actual distribution however was 14 plates with 1 infected cyanobacterium and 4 plates with 2 infected cyanobacteria, or 22 infected cyanobacteria in total. These infected cyanobacteria produced a total of 2078 plaques, or an average burst size of 2078/22 = 95 phage particles per host cell.

Average plaque count per 1 ml. sample taken from the sample flask after the end of the rise period was 32. If burst size is calculated from the results of the sample flask it amounts to 48/0.45 = 107. As it can be seen, the average burst size from the single cell bursts is calculated from 22 infected cells. The average burst size in the sample flask however is calculated from about 23 infected cyanobacteria. This is because the original sample flask had 100 ml. containing 0.45 infected Plectonema cells per ml. (see above). Therefore this sample flask contained total of 100 x 0.45 =

45 infected cells of which 22 were sampled out into the 50 test tubes leaving 23 infected cells from which the average burst size is calculated.

Neither the sample flask method nor the single burst method can be a reliable estimate of the average burst size. This is due to the fact that the single cell burst sizes have a very wide distribution, which range from 20 to 307. Of course, 4 of the plates contained phage yields from 2 infected cyanobacteria each but it is impossible to tell which plates and there is no logical reasoning suggesting that the plates containing large number of plaques were the ones which contained more than one infected Plectonema cell. This broad range in individual burst sizes is an interesting fact. No good explanation for this great range in burst sizes exist and it can not be dependent upon the size of the host cell, since Delbruck (1945) found that the spread of the burst sizes is much greater than the spread of the cell sizes. In the cyanobacteria model system there may be a factor which can be taken into account for causing the wide distribution. This factor is the size of the trichome. In the wild type trichomes size range can vary from 20 Plectonema cells per trichome up to 200 Plectonema cells per trichome. As a result there is a possibility that the large trichomes can provide a local density effect for the infected cell within the trichome that lyses. This means that some of the phage progeny released might be able to adsorb to the neighboring Plectonema

cells within the trichome and therefore provide no plaques on the petri plates. This possibility diminishes if the size of the trichome containing the infected cell is small.

2. The comparison of control versus experimental groups_ when the single burst experiment was performed, it contained one control and five experimental categories. The experimental groups consisted of officinale treated LPP-1 virus, officinale treated Plectonema cells before viral adsorption, officinale treated Plectonema cells after viral adsorption, zerumbet treated Plectonema cells before viral adsorption, and zerumbet treated Plectonema cells after the viral adsorption. The individual plate counts revealed the following results.

control:

9	26	75	124	215
15	40	108	131	272
18	71	120	210	324

officinale treatment of the LPP-1 virus:

4	11	58	105	186	233
7	29	79	144	204	307
9	40	101	145	213	

officinale treatment of the host before viral adsorption:

1	21	38	53	79
4	28	41	58	102
12	34	44	60	126

officinale treatment of the host after viral adsorption:

2	13	47	68	99
2	19	48	70	122
8	31	55	93	132

zerumbet treatment of the host before viral adsorption:

0	0	2	11	19
0	0	5	13	25
0	0	9	13	29

zerumbet treatment of the host after viral adsorption:

0	0	10	20	31
0	1	15	23	32
0	7	18	28	40

Like the one-step growth experiment, the single cell burst experiment also showed that when virus is treated with the extract, no reduction of the burst size occurs. Hardly any difference can be seen in the range of values of control versus the virus treatment. The range however decreases when the Plectonema cells are treated with the officinale extract

indicating reduction of the burst size. The zerumbet treatment of the host cells reduced the burst size and it reduced the distribution of the individual burst size values even more. This indicates and confirms the results of the one-step growth experiment that the zerumbet extract is more potent for the burst size reduction. Whether the extract treatment was before or after the viral adsorption showed no difference in the distribution of the individual burst size values for officinale and zerumbet. This is indeed surprizing since a difference between the two ways of the treatment was measured in the one-step growth experiment. Greater amount of data in the single burst experiment is necessary to oppose the results of the one-step growth experiments.

The single cell burst experiment therefore confirmed that it is the treatment of the host cells that causes the viral inhibition, not the treatment of the LPP-1 virus. The experiment also showed that the zerumbet extract is more inhibitory compared to the officinale extract.

V. Efficiency of Plating

Throughout this study, the agar layer method described by Gratia (1936c) was used for the counting of the plaques. One of the advantages of the agar layer method is that it increases the efficiency of plating. The EOP is that proportion of viable virus particles which form plaques in the

petri plates when plated. The EOP depends first on the ability of the virus to adsorb to the host cell and second on the ability of the virus to reproduce in the host causing lysis and liberation of the progeny virus until a plaque is formed. The plaques within the petri plates grow until the host cells are no longer in their log phase of growth. At this point, the host cells that are infected no longer liberate large number of virus particles due to their slowed down metabolic activity and the accumulation of toxic wastes.

In some cases a decrease in the concentration of the amino acids existing in the medium within which adsorption of phage to its host takes place reduces the EOP considerably (Anderson, 1945). In some phages, the addition of salts like NaCl to the adsorption media increases the EOP a great deal (Hershey, Kalmanson, and Bronfenbrenner, 1944). The LPP-1 virus for instance requires Mg^{+2} ions to be present without which the EOP will be extremely low (Goldstein, 1967).

The absolute EOP can not be measured. With the agar layer technique no method for determining the total number of infective virus particles exist. The relative EOP, however, can be measured easily (Ellis and Delbruck, 1939). For this study, the comparison between the infected cells at their log phase of growth and infected cells at their stationary phase of growth was used to determine the relative efficiency of plating. In this method, the LPP-1 phage is diluted so that there is less than 1 infectious particle per ml. and then it

is distributed in test tubes to give 50 samples of 1 ml. each. Each of the 50 samples then is inoculated with the concentrated Plectonema cells and incubated underneath fluorescent light of 150 ft-c for about 16 hours to make sure the lysis of the cells that are infected has occurred. Then each of the 50 samples are plated to see whether virus particle was present in the original samples.

The proportion of samples containing no virus, P(0), is determined and the average number of virus particles per sample, n, is calculated from the Poisson distribution

$$P(r) = (n^r/r!) \times e^{-n}$$

which for P(0) it simplifies to e^{-n}. For the cell population in the log phase of growth 18 petri plates out of 50 had no plaques; therefore, $P(0) = 18/50 = 0.36 = e^{-n}$. The number of phage particles per sample, n, was calculated to be 1.02. For the population of Plectonema cells in the stationary phase 28 petri plates out of 50 had no plaques in them. P(0) for this group was 28/50 or 0.56. The value of n therefore was 0.58. The relative EOP is therefore 0.58/1.02 = 0.57. This means that in the population of the cells in the stationary phase, only 57 virus particles out of 100 will form plaques.

Part of the reason for this decrease in the ability of the LPP-1 to produce plaques is due to the fact that the cells in the stationary phase are not actively growing and metabolizing. Furthermore, when the host cells are not in the log phase of growth, the proportion of dead cells, and

cellular debris is high. A phage particle adsorbed to a dead cell or cellular debris can not multiply and produce a plaque within a petri plate. It was for this reason that throughout the whole study of the antiviral activities of Z. officinale and Z. zerumbet, Plectonema cells in their log phase of growth were used.

SUMMARY AND CONCLUSION

The focus of this research was to determine whether the rhizome extract of Zingiber officinale and Zingiber zerumbet plants exhibited any antiviral activities upon a virus infecting a cyanobacterium, Plectonema boryanum. The research was then geared toward investigation of the cause of this antiviral property. The experiments were divided into the following categories.

I. Viral Inhibition Test

This test showed that significant reduction of viral plaques occurs in the petri plates when the cyanobacteria cells are incubated with the individual plant extracts, indicating that the extracts provide a less suitable environment for the life cycle of the virus. The test also showed that the zerumbet extract was toxic to the host cells since it prevented their growth and multiplication after the host cells were incubated with it for six hours. Furthermore, the inhibitory activities of the two extracts was irreversible throughout the 24 hour and the 6 hour periods when the officinale and zerumbet extracts respectively were incubated with the host cells. Once the decrease in the number of

plaques had occurred, the reduction persisted throughout the period of incubation.

II. Transmission Electron Microscopy

When cationic ferritin, a probe used to determine qualitative changes occurring on the host cell surface, was used in the TEM study, the results indicated that the nature of the surface charges existing on the Plectonema cells is negative since the probe strongly attached to the cell wall of the control population. The probe molecules were also not permeable to the cell wall and cell membrane of the Plectonema cells since no cationic ferritin binding was observed within the host cells.

This study also showed that the probe did not bind to the surface of the host cells treated with officinale extract as it did to the untreated cells. The extract treatment had produced a change in the negative nature of the surface charges of the cells resulting in change in the binding pattern of the cationic probe.

III. Adsorption Rate Experiments

Measurements of the rate of adsorption of the LPP-1 virus revealed some significant results that could explain part of the nature of the viral inhibition seen in preliminary experiments. At first, the data indicated that LPP-1 is a slow adsorbing virus since it took <u>one</u> hour for almost 90% of

the virus particles to adsorb to the Plectonema cells (table 16). The rate of adsorption was reduced down to 77% when the cell population was treated with the officinale extract (table 17). Zerumbet treatment, however, reduced the rate further down to 38% and did not allow any further adsorption to occur as the time of incubation of the host cell population and virus particles was increased (table 18). The data also indicated no significant effect on the rate of adsorption of the virus particles that were treated with the officinale extract (table 19). This showed that the inhibitory effects on the adsorption phase of the virus occur only when the cells are treated with the extracts, not the virus particles.

IV. One-Step Growth Experiments

When the host cells were treated with the officinale and zerumbet extracts, the latent period of viral infection was prolonged and the burst size was reduced. Here also, the zerumbet extract exhibited a greater potency in the reduction of the burst size and inhibition of viral reproduction within the cell. Treatment of the virus with the officinale extract only prolonged the latent period of the infectious cycle and did not reduce the burst size. What this means is that most likely the extract treatment of the virus results in a later than usual infection, not intracellular inhibition.

Significant reduction in the number of phage progeny occurred when the host cells were treated with the officinale

extract after the adsorption of the virus, compared to the officinale treatment of the cells before the adsorption of the virus. This means that there was a greater reduction of the burst size in the post adsorption treatment as opposed to preadsorption treatment. Such difference between the preadsorption and postadsorption treatment was also observed when the cells were treated with zerumbet extract.

V. The Single Cell Burst Method

When the burst size was measured in each infected cell individually as opposed to the one-step growth method, a very wide distribution of the single cell burst sizes was observed in both the control and the extract treated groups. Nevertheless, the range of the values did not differ from the control series when the virus was treated with the extract. This confirmed the results of the treatment of the virus with the officinale extract in the one-step growth experiment.

Treatment of the cells with the extracts reduced the range of values indicating reduction of burst size occurring for both officinale and zerumbet treatment. Moreover, the zerumbet treatment showed more potency and more effectiveness in its reduction of the burst size. Whether the extract treatment was done before or after the viral adsorption showed no difference in the distribution of the burst size values for both officinale and zerumbet extracts. The single cell burst experiment agreed with the one-step growth experiment in

showing that it is the treatment of the host cells that provides effective viral inhibition and not the treatment of the virus. The data also agreed in exhibiting the more potent nature of the zerumbet extract in fighting the viral infection.

VI. Efficiency of Plating

Determination of the relative efficiency of plating indicated the reason for using the Plectonema cell population in log phase of growth as opposed to other phases in cell growth throughout all experimentations measuring host-virus interactions.

Determination of the relative efficiency of plating indicated that the LPP-1 virus reproduces better in a Plectonema cell that is in the log phase growth as opposed to the stationary phase. When the cells are not in their log phase of growth, they are not actively growing and actively metabolizing. These cells, therefore, do not provide a good environment for viral growth and replication within its host.

VII. Final Remarks

As it has been demonstrated several times during this study, the rhizome extracts of Zingiber officinale and Zingiber zerumbet possess antiviral properties. It has been known for many years that many plants contain "proteins" which protect the plants from fungal, bacterial, viral, and many

other parasitic infections. If these proteins do exist in the rhizomes of Z. officinale and Z. zerumbet, then the mechanism of viral inhibition by the extracts of these plants can possibly be explained.

Tremendous amount of studies have been done on these plant proteins and it has been suggested that they are composed of two chains one of which is a lectin which binds to the carbohydrates on the cell membranes and the other a toxin which enters the cell and exerts its action after the bonding between lectin and the cell membrane has been accomplished (Kocourek and Horejsi, 1981).

These proteins inactivate the ribosome machinery of the cell (Stirpe, 1982) and therefore they are designated as Ribosome Inactivating Proteins (RIP). The antiviral effects of the RIP could be due to inactivation of ribosomes of the virus infected cells (Owens, 1973). Several studies have also concluded that the RIP enters more easily into virus infected cells because of the increased permeability of virus infected cells (Carrasco, 1978; Yamaizumi et al., 1979).

If indeed the existence of RIP can be demonstrated in the rhizome extracts of Z. officinale and Z. zerumbet, then it is possible that the RIP penetrates the infected Plectonema boryanum cell and exerts its antiviral action by inhibiting specifically the ribosomes of the host cells that are involved in producing viral proteins. The support for this hypothesis comes from the observation that both of the rhizome extracts

exert their effect when the host is treated as opposed to the virus.

Speculation on the existence of the RIP in the Zingiber plants also can explain why a greater number of phage progeny existed in the preadsorption officinale treated Plectonema cells than postadsorption officinale treated cells. Since the cell membranes of the virus infected cells are known to be more permeable to RIP than the cell membranes of the uninfected cells, the RIP can more readily enter an infected cell and therefore interfere sooner with the virus production machinery, leading to a greater reduction of the burst size.

Interestingly enough, Kassanis (1981) compares this resistance system in plants against infection to the immune system of the animals and explains many similarities that indeed exist between the two systems.

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Appendix: A

Linear Regression; Comparing Linear Regression Equations

A. Determination of the linear regression equation _ In the linear regression two variables exist in which the magnitude of one of the variables (the dependent variable) depends on the magnitude of the second variable (the independent variable). For example, in the relationship between viral plaques appearing in the petri plates and time, plaque appearance is the dependent variable and time the independent variable. The simplest functional relationship of one variable to another in a sample taken from a population is simple linear regression,

$$Y_i = a + bX_i$$

where a is the y-intercept and b is the slope of the linear equation. To calculate the linear regression equation, equation of a straight line, the following which is taken from the officinale extract data (fig. 13) will be considered.

Time (hours) (X)	Number of Plaques (Y)
0	174
1	178
2	137
3	101
4	78
5	47
6	48

The simple linear regression equation can be claculated by the method of least squares for this example of data.

$n = 7$ $\qquad$ $\Sigma Y = 763$

$\Sigma X = 21$ $\qquad$ $\bar{Y} = 109$

$\bar{X} = 3$ $\qquad$ $\Sigma XY = 1590$

$\Sigma X^2 = 91$ $\qquad$ $\Sigma xy = \Sigma XY - \frac{(\Sigma X)(\Sigma Y)}{n}$

$\Sigma x^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{n}$ $\qquad$ $= -699$

$= 28$

$b = (\Sigma xy)/(\Sigma x^2) = -699/28 = -24.96$

$a = \bar{Y} - b\bar{X} = 109 - (-24.96)(3)$

$= 183.88$

So, the simple linear regression equation is

$$Y = 184.42 - 25.14X \ .$$

B. Comparison of the slopes of two linear regression equations _ A simple method for testing whether two samples taken from two different populations have the same regression equation slopes statistically is the use of Student's t-test.

The test statistic is

$$t = \frac{b1 - b2}{S_{b1-b2}}$$

where the standard error of the difference between regression coefficients is

$$S_{b1-b2} = \sqrt{\frac{(S^2_{y.x})_p}{(\Sigma x^2)_1} + \frac{(S^2_{y.x})_p}{(\Sigma x^2)_2}}$$

and the pooled residual mean square is calculated as

$$(S^2_{y.x})_p = \frac{(\text{residual SS})_1 + (\text{residual SS})_2}{(\text{residual DF})_1 + (\text{residual DF})_2},$$

the subscripts 1 and 2 refer to the two regression lines being analyzed. Suppose one wants to test the difference between two sample regression coefficients.

Sample 1:

$\Sigma x^2 = 1470.8712$

$\Sigma xy = 4363.1627$

$\Sigma y^2 = 13299.5296$

$n = 26$

$b = \frac{4363.1627}{1470.8712} = 2.97$

residual SS $= 13299.5296 - \frac{(4363.1627)^2}{1470.8712}$

$= 356.7317$

residual DF $= 26 - 2 = 24$

Sample 2:

$\Sigma x^2 = 2272.4750$

$\Sigma xy = 4928.8100$

$\Sigma y^2 = 10964.0947$

$n = 30$

$b = \frac{4928.8100}{2272.4750} = 2.17$

residual SS $= 10964.0947 - \frac{(4928.8100)^2}{2272.4750}$

$= 273.9142$

residual DF $= 30 - 2 = 28$

$$(S^2_{y.x})_p = 12.1278$$

$$S_{b1-b2} = 0.1165$$

$$t = \frac{2.97 - 2.17}{0.1165} = 6.867$$

$$v = 24 + 28 = 52$$

$$t_{0.05(2),52} = 2.007$$

So it can be concluded that the samples are not equal and they are different.

C. Comparison of the Elevations of two linear Regression Equations _ If two sample regression lines are not concluded to have different slopes, then the two lines are assumed to be parallel. If this is indeed the case, one can determine whether the two sample regressions have the same elevations (i.e. the same vertical position on a graph) and therefore coincide.

To test the hypothesis that states that the elevations of the two sample regression lines of officinale and zerumbet extract treatments (fig. 13) are the same, the following quantities for use in a t-test must be defined:

Sum of squares of X for "common regression"

$$= A_c = (\Sigma\, x^2)_1 + (\Sigma\, x^2)_2 ,$$

Sum of crossproducts for "common regression"

$$= B_c = (\Sigma\, xy)_1 + (\Sigma\, xy)_2 ,$$

Sum of squares of Y for "common regression"

$$= C_c = (\Sigma\, y^2)_1 + (\Sigma\, y^2)_2 ,$$

Residual SS for "common regression"

$$= SS_c = C_c - \frac{B_c^2}{Ac}$$

Residual DF for "common regression"

$$= DF_c = n_1 + n_2 - 3 ,$$

Residual MS for "common regression"

$$= (S^2_{y.x})_c = \frac{SSc}{DFc}$$

Then, the t value for the t-test can be calculated as

$$t = \frac{(Y_1 - Y_2) - b_c(X_1 - X_2)}{\sqrt{(S^2_{y.x})_c \left[\frac{1}{n_1} + \frac{1}{n_2} + \frac{(X_1 - X_2)^2}{A_c}\right]}}$$

For officinale Sample (1)

$n = 3$

$\bar{X} = 2$

$\bar{Y} = 138.25$

$\Sigma x^2 = 2$

$\Sigma xy = -77$

$\Sigma y^2 = 2967.87$

$b = -38.5$

For Zerumbet Sample (2)

$n = 3$

$\bar{X} = 2$

$\bar{Y} = 114.83$

$\Sigma x^2 = 2$

$\Sigma xy = -151.5$

$\Sigma y^2 = 11844.29$

$b = -75.75$

$$(S^2_{y.x})_p = 185.77$$

$$S_{b1-b2} = 13.63$$

$$t = 2.733$$

$$t_{0.05(2),2} = 4.303$$

conclusion: slopes are the same,

$$A_c = 2 + 2 = 4$$

$$B_c = -77 + (-151.5) = -228.5$$

$$C_c = 2967.88 + 11844.29 = 14812.17$$

$$SS_c = 14812.17 - \frac{(-228.5)^2}{4} = 1759.11$$

$$DF_c = 3 + 3 - 3 = 3$$

$$(S^2_{y.x})_c = \frac{1759.11}{3} = 586.37$$

$$b_c = -\frac{228.5}{4} = -57.125$$

$$t = 0.0599$$

$t_{0.05(2),3} = 3.182$ conclusion: Elevations are the same.

APPENDIX: B

Single Factor Analysis of Variance - ANOVA

Since in this study measurements of a variable were collected from at least three samples (control, officinale treatment, zerumbet treatment), ANOVA had to be used instead of the student's t-test. Suppose we wish to test 4 different categories (i.e. control, officinale, zerumbet, etc.) and we want to see if the average plaque count from these categories are equal or not.

1	2	3	4
60.8	68.7	102.6	87.9
57.0	67.7	102.1	84.2
65.0	74.0	100.2	83.1
58.6	66.3	96.5	85.7
61.7	69.8	----	90.3
n_i = 5	5	4	5	N = 19
ΣX_{ij} = 303.1	346.5	401.4	431.2	
$\bar{X}_i$ = 60.62	69.30	100.35	86.24	
$\frac{(\Sigma X_{ij})^2}{n_i}$ = 18373.922	24012.450	40280.490	37186.688	

$$\Sigma \frac{(\Sigma X_{ij})^2}{n_i} = 119853.550$$

$\Sigma\Sigma X_{ij} = 1482.2$

$\Sigma\Sigma X_{ij}^2 = 119981.900$

total Df = N - 1 = 19 - 1 = 18

groups Df = K - 1 = 4 - 1 = 3

error Df = N - K = 19 - 4 = 15

$$C = \frac{(\Sigma\Sigma X_{ij})^2}{N} = 115627.202$$

total sum of squares = $\Sigma\Sigma X_{ij}^2 - C = 4354.698$

group sum of squares = $\Sigma \frac{(\Sigma X_{ij})^2}{n_i} - C = 4226.248$

error sum of squares = total SS - groups SS = 128.350

groups mean square = $\frac{\text{groups SS}}{\text{groups DF}} = 1408.783$

error mean square = $\frac{\text{error SS}}{\text{error DF}} = 8.557$

$F = \frac{\text{groups MS}}{\text{error MS}} = 165$

$F_{0.05(1),3,15} = 3.29$

conclusion is that the four samples are not the same.

APPENDIX: C

The Tukey Analysis

Using a single factor analysis of variance, we can test whether the sample means for officinale treatment, zerumbet treatment, and control groups are equal or not. If the ANOVA indicates that indeed they are not, this does not indicate that all of the three groups of samples are different from each other. We can not tell if it is the officinale treatment that is unequal to zerumbet tretment or zerumbet treatment unequal to control or officinale treatment unequal to control or all three unequal to each other. Since the Student's t-test can not be applied to examine the differences between all possible pairs of means, a multiple comparison test like Tukey test needs to be performed.

Suppose that the ANOVA indicated that it can not be concluded that the sample means of control (1), officinale treatment (2), and zerumbet treatment (3) are equal and and we wish to see if the control sample is equal to officinale, or if the officinale is equal to zerumbet, or if control is equal to zerumbet.

Given:

$\bar{X}$ (1) = 184.75 $\bar{X}$ (2) = 78.25 $\bar{X}$ (3) = 11.00

Error MS = 6.611 (Derived from the ANOVA)

To test each sample mean against the other the standard error (SE) has to be determined.

$$SE = \sqrt{\frac{\text{Error MS}}{n}} = \frac{6.611}{4} = 1.29$$

where n represents number of measurements taken from each sample alone. Since four measurements were taken (four petri plates) in each sample, n = 4. We also need to calculate q,

$$q = \frac{\bar{X}_B - \bar{X}_A}{SE} .$$

For example, if we want to test control sample v.s. officinale treated sample,

$$\bar{X}_B - \bar{X}_A = 184.75 - 78.25 = 106.5$$

$$q = \frac{106.5}{1.29} = 82.56$$

$$q_{0.05,9,3} = 3.95$$

Therefore, the conclusion is that they are unequal.

APPENDIX: D

CHEMICAL COMPOUNDS USED FOR GROWTH AND MAINTAINANCE OF Plectonema boryanum IN LIQUID AND SOLID MEDIA (BOLD'S MEDIUM).

Six salt solutions, 400 ml. in volume, are employed with each containing one of the following salts in the amount listed:

$NaNO_3$	10g/400 ml. H_2O
$MgSO_4.7H_2O$	2g/400 ml. H_2O
K_2HPO_4	4g/400 ml. H_2O
KH_2PO_4	6g/400 ml. H_2O
$CaCl_2$	1g/400 ml. H_2O
NaCl	1g/400 ml. H_2O

To 936 ml. of distilled and deionized water, add 10 ml. of each stock solution above and 1.0 ml. of each of the following trace element solutions:

A. EDTA Stock. To 1000 ml. distilled and deionized water, add 50g EDTA and 31g KOH.

B. H-Fe Stock. To 1000 ml. "acidified water (see below), add 4.98g $FeSO_4.7H_2O$.

C. Boron Stock. To 1000 ml. distilled and deionized water, add 11.42g H_3BO_3.

D. $H-H_5$ Stock. To 1000 ml. acidified water, add 8.82g $ZnSO_4$, 0.71g MoO_3, 0.49g $Co(NO_3)_2.6H_2O$, 1.44g $MnCl_2$, and 1.57g $CuSO_4.5H_2O$.

Acidified Water is prepared by adding 1 ml. concentrated H_2SO_4 to 999 ml. of distilled and deionized water.

Once prepared, the Bold's medium can be sterilized by autoclaving for 15 minutes at 15 pounds pressure. The solid Bold's medium can be prepared by adding 17g of agar per liter of medium.

APPENDIX: E

DETERMINATION OF THE CONCENTRATION OF CYANOBACTERIA IN THE BOLD'S MEDIUM.

Population count of the Plectonema boryanum was done by using hemacytometer, 0.1 mm deep with no. 1.5 cover glasses. The counting was done using a phase contrast microscope. The hemacytometer has 2 chambers each consisting of 9 squares 1 mm on a side. The chambers were filled several times with 1:10 (v/v) dilution of the original culture of Plectonema cells due to the great density of the culture. A total of 100 squares were counted and an average value Q was calculated.

Given the average value Q, the density of the suspension was measured:

$$d = Q \times 10^4$$

then, the density value was multiplied by 10, since the chamber fillings were 1:10 dilutions of the original culture.

APPENDIX: F

DETERMINATION AND PREPARATION OF SPECIFIC CONCENTRATION OF PHAGE LPP-1.

A. Determination:

The determination of the viral concentration is absolutely one of the most necessary steps in the virological techniques used in virus research. To accomplish this task, a solution of virus must be titrated at first.

Titration of the LPP-1 virus is done by making several tenfold dilutions of the virus. Specific volumes of the tenfold dilutions are then separately added to a culture media containing the susceptible host followed by plating. The petri plates are then incubated at room temperature underneath 150 ft-c fluorescent light, since the host in this case does not grow without suitable light conditions. Plaques apear at about 3 to 4 days.

Plates that show approximately 100 to 200 plaques are chosen for counting. Plaque count is then multiplied by the total dilution factor which is the final dilution times the plating dilution. For example,

Plaque count = 170 = 1.7×10^2

Dilution containing 170 plaques = 10^{-6}

plaing dilution (0.1 ml. used) = 10^{-1}

Titer of LPP-1 virus = $\frac{1.7 \times 10^2}{10^{-6} \times 10^{-1}}$

= 1.7×10^9 PFU per ml.

B. Preparation:

Throughout this study, it was many times necessary to prepare a specific concentration of the LPP-1 virus from a known concentration of the phage stock in order to have a correct input ratio of phage to Plectonema cells. Suppose that from an original viral concentration of 1.7×10^6 PFU/ml., a concentration of 1.82×10^5 PFU/ml. is to be prepared. First calculation should be the determination of the dilution factor in going from the old to the new desired viral concentration.

Dilution factor = $\frac{\text{Old virus Conc.}}{\text{new virus Conc.}}$

= $1.70 \times 10^6 = 9.34$

This means that a sample of the old virus concentration ought to be diluted 1:9.34 (v/v). Next, the volume of the new virus concentration is to be considered. Suppose,

X = volume of new virus concentration needed

Y = dilution factor

$\frac{X}{Y}$ = volume of old concentration to be used

$$X - \frac{X}{Y} = \text{volume of the solvent that is to be added}$$

then if 100 ml. of 1.82×10^5 PFU/ml. is to be prepared and the dilution factor is 9.34, then 100/9.34 or 10.7 ml. of 1.70×10^6 PFU/ml. concentration should be added to 100 -(100/9.34) or 89.3 ml. of the solvent.

TABLE:1

ANALYSIS OF VARIANCE: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the first hour.(fig. 13)

SOURCE	SS	d.f.	MS	F	p
Groups	77.167	2	38.583	0.068*	0.935
Error	5110.500	9	567.833	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the second hour.(fig. 13)

SOURCE	SS	d.f.	MS	F	p
Groups	3510.17	2	1755.083	4.358*	0.047
Error	3624.75	9	402.750	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the third hour. (fig. 13)

SOURCE	SS	d.f.	MS	F	p
Groups	42818.17	2	21409.08	105.94*	0.000...
Error	1818.75	9	202.083	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the fourth hour. (fig. 13)

SOURCE	SS	d.f.	MS	F	p
Groups	61405.2	2	30702.58	4644.1*	0.000...
Error	59.50	9	6.611	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the fifth hour. (fig. 13)

SOURCE	SS	d.f.	MS	F	p
Groups	65078.0	2	32539.00	289.31*	0.000...
Error	1012.25	9	112.472	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the sixth hour. (fig. 13)

SOURCE	SS	d.f.	MS	F	p
Groups	76392.2	2	38196.08	528.26*	0.000...
Error	650.75	9	72.31	_	_

* p 0.05

TABLE:2

TUKEY ANALYSIS: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the third hour. 1 = control; 2 = officinale treatment; 3 = zerumbet treatment. Source is fig. 13.

Comparison	Difference	SE	q	$q_{0.05,9,3}$
1 vs. 3	146.25	7.11	20.57	3.95
1 vs. 2	77.00	7.11	10.83	3.95
2 vs. 3	69.25	7.11	9.74	3.95

TUKEY ANALYSIS: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the fourth hour. 1 = control; 2 = officinale treatment; 3= zerumbet treatment. Source is fig. 13.

Comparison	Difference	SE	q	$q_{0.05,9,3}$
1 vs. 3	173.75	1.29	135.15	3.95
1 vs. 2	106.50	1.29	82.84	3.95
2 vs. 3	67.25	1.29	52.31	3.95

TUKEY ANALYSIS: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the fifth hour. 1 = control; 2 = officinale treatment; 3 = zerumbet treatment. Source is fig. 13.

Comparison	Difference	SE	q	$q_{0.05,9,3}$
1 vs. 3	173.50	5.30	32.74	3.95
1 vs. 2	129.50	5.30	24.43	3.95
2 vs. 3	44.00	5.30	8.30	3.95

TUKEY ANALYSIS: Bio-assay of LPP-1 virus replication in the extract treated and extract untreated Plectonema cells at the sixth hour. 1 = control; 2 = officinale treatment; 3= zerumbet treatment. Source is fig. 13.

Comparison	Difference	SE	q	$q_{0.05,9,3}$
1 vs. 3	188.00	18.08	10.40	3.95
1 vs. 2	140.25	18.08	7.76	3.95
2 vs. 3	47.75	18.08	2.64	3.95

TABLE:3

ANALYSIS OF VARIANCE: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t = 15 minutes. (fig. 19)

SOURCE	SS	d.f.	MS	F	p
Groups	56442.5	3	18814.2	14.584*	0.000...
Error	15540.5	12	1290.04	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=30 mintues. (fig. 19)

SOURCE	SS	d.f.	MS	F	p
Groups	91338.2	3	30446.06	40.303*	0.000...
Error	9065.25	12	755.44	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=45 minutes. (fig. 19)

SOURCE	SS	d.f.	MS	F	p
Groups	100302.2	3	33434.1	31.452*	0.000...
Error	12756.3	12	1063.02	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=60 mintues. (fig. 19)

SOURCE	SS	d.f.	MS	F	p
Groups	107520.3	3	35840.08	300.44*	0.000...
Error	1431.50	12	119.29	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=75 minutes. (fig. 19)

SOURCE	SS	d.f.	MS	F	p
Groups	179713.3	3	59904.4	116.12*	0.000...
Error	6190.50	12	515.875	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=90 mintues. (fig. 19)

SOURCE	SS	d.f.	MS	F	p
Groups	179496.5	3	59832.17	94.156*	0.000...
Error	7625.50	12	635.46	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=105 minutes. (fig. 19)

SOURCE	SS	d.f.	MS	F	p
Groups	165784.7	3	55261.6	64.07*	0.000...
Error	10350.3	12	862.521	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=120 mintues. (fig. 19)

SOURCE	SS	d.f.	MS	F	p
Groups	180117.7	3	60039.229	408.6*	0.000...
Error	1763.25	12	146.94	_	_

* p 0.05

TABLE:4

TUKEY ANALYSIS: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=15 minutes. 1 = zerumbet treatment of the host; 2 = officinale treatment of the host; 3 = officinale treatment of the virus; 4 = control. Source is fig. 19.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	67.75	17.96	3.772	4.199
1 vs. 3	77.25	17.96	4.301	4.199
1 vs. 4	167.0	17.96	9.298	4.199
2 vs. 3	9.5	17.96	0.529	4.199
2 vs. 4	99.25	17.96	5.526	4.199
3 vs. 4	89.75	17.96	4.997	4.199

TUKEY ANALYSIS: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=30 minutes. 1 = zerumbet treatment of the host; 2 = officinale treatment of the host; 3 = officinale treatment of the virus; 4 = control. Source is fig. 19.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	86.25	13.74	6.276	4.199
1 vs. 3	150.75	13.74	10.97	4.199
1 vs. 4	202.25	13.74	14.72	4.199
2 vs. 3	64.50	13.74	4.693	4.199
2 vs. 4	116.0	13.74	8.441	4.199

TUKEY ANALYSIS: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=45 minutes. 1 = zerumbet treatment of the host; 2 = officinale treatment of the host; 3 = officinale treatment of the virus; 4 = control. Source is fig. 19.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	127.3	16.30	7.807	4.199
1 vs. 3	169.5	16.30	10.40	4.199
1 vs. 4	211.5	16.30	12.98	4.199
2 vs. 3	42.25	16.30	2.592	4.199
2 vs. 4	84.25	16.30	5.169	4.199
3 vs. 4	42.00	16.30	2.577	4.199

TUKEY ANALYSIS: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=60 minutes. 1 = zerumbet treatment of the host; 2 = officinale treatment of the host; 3 = officinale treatment of the virus; 4 = control. Source is fig. 19.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	159.25	5.46	29.16	4.199
1 vs. 3	189.25	5.46	34.66	4.199
1 vs. 4	207.00	5.46	37.91	4.199
2 vs. 3	30.00	5.46	5.494	4.199
2 vs. 4	47.75	5.46	8.744	4.199
3 vs. 4	17.75	5.46	3.250	4.199

TUKEY ANALYSIS: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=75 minutes. 1 = zerumbet treatment of the host; 2 = officinale treatment of the host; 3 = officinale treatment of the virus; 4 = control. Source is fig. 19.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	195.8	11.36	17.24	4.199
1 vs. 3	256.3	11.36	22.57	4.199
1 vs. 4	260.5	11.36	22.94	4.199
2 vs. 3	60.50	11.36	5.328	4.199
2 vs. 4	64.75	11.36	5.702	4.199
3 vs. 4	4.25	11.36	0.374	4.199

TUKEY ANALYSIS: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=90 minutes. 1 = zerumbet treatment of the host; 2 = officinale treatment of the host; 3 = officinale treatment of the virus; 4 = control. Source is fig. 19.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	222.00	12.60	17.61	4.199
1 vs. 3	238.75	12.60	18.94	4.199
1 vs. 4	265.25	12.60	21.05	4.199
2 vs. 3	16.75	12.60	1.329	4.199
2 vs. 4	43.25	12.60	3.432	4.199
3 vs. 4	26.50	12.60	2.103	4.199

TUKEY ANALYSIS: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=105 minutes. 1 = zerumbet treatment of the host; 2 = officinale treatment of the host; 3 = officinale treatment of the virus; 4 = control. Source is fig. 19.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	207.8	14.68	14.15	4.199
1 vs. 3	233.25	14.68	15.89	4.199
1 vs. 4	254.8	14.68	17.35	4.199
2 vs. 3	25.50	14.68	1.737	4.199
2 vs. 4	47.00	14.68	3.201	4.199
3 vs. 4	21.50	14.68	1.464	4.199

TUKEY ANALYSIS: Bio-assay of the unadsorbed LPP-1 virus replication in Plectonema cells at t=120 minutes. 1 = zerumbet treatment of the host; 2 = officinale treatment of the host; 3 = officinale treatment of the virus; 4 = control. Source is fig. 19.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	231.75	6.06	38.24	4.199
1 vs. 3	248.25	6.06	40.96	4.199
1 vs. 4	253.25	6.06	41.78	4.199
2 vs. 3	16.50	6.06	2.722	4.199
2 vs. 4	21.50	6.06	3.547	4.199
3 vs. 4	5.00	6.06	0.825	4.199

TABLE:5

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 9th hour. (fig. 20)

SOURCE	SS	d.f.	MS	F	p
Groups	788.25	3	262.75	94.12*	0.000...
Error	33.5	12	2.792	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 10th hour.(fig. 20)

SOURCE	SS	d.f.	MS	F	p
Groups	10822.5	3	3607.500	7.012*	0.006
Error	6173.5	12	514.458	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 11th hour. (fig. 20)

SOURCE	SS	d.f.	MS	F	p
Groups	21110.2	3	7036.7	138.6*	0.000...
Error	609.25	12	50.771	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 12th hour.(fig. 20)

SOURCE	SS	d.f.	MS	F	p
Groups	87004.2	3	29001.4	287.8*	0.000...
Error	1209.3	12	100.771	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 14th hour. (fig. 20)

SOURCE	SS	d.f.	MS	F	p
Groups	290073.7	3	96691.2	82.15*	0.000...
Error	14124.3	12	1177.02	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 16th hour.(fig. 20)

SOURCE	SS	d.f.	MS	F	p
Groups	436719.5	3	145573.2	295.5*	0.000...
Error	5912.5	12	492.71	_	_

* p 0.05

TABLE:6

TUKEY ANALYSIS: Bio-assay of the "total infective centers" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 9th hour. 1 = control; 2 = officinale treatment of the virus; 3= officinale treatment of the host; 4= zerumbet treatment of the host. Source is fig. 20.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	0	0.84	0	4.199
1 vs. 3	15.75	0.84	18.85	4.199
1 vs. 4	11.75	0.84	14.06	4.199
2 vs. 3	15.75	0.84	18.85	4.199
2 vs. 4	11.75	0.84	14.06	4.199
3 vs. 4	4.00	0.84	4.790	4.199

TUKEY ANALYSIS: Bio-assay of the "total infective centers" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 10th hour. 1 = control; 2 = officinale treatment of the virus; 3 = officinale treatment of the host; 4 = zerumbet treatment of the host. Source is fig. 20.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	52.75	11.34	4.65	4.199
1 vs. 3	62.25	11.34	5.49	4.199
1 vs. 4	63.00	11.34	5.55	4.199
2 vs. 3	9.50	11.34	0.84	4.199
2 vs. 4	10.25	11.34	0.90	4.199
3 vs. 4	0.75	11.34	0.07	4.199

TUKEY ANALYSIS: Bio-assay of the "total infective centers" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 11th hour. 1 = control; 2 = officinale treatment of the virus; 3 = officinale treatment of the host; 4 = zerumbet treatment of the host. Source is fig. 20.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	40.00	3.56	11.23	4.199
1 vs. 3	88.50	3.56	24.84	4.199
1 vs. 4	85.25	3.56	23.93	4.199
2 vs. 3	48.50	3.56	13.61	4.199
2 vs. 4	45.25	3.56	12.70	4.199
3 vs. 4	3.25	3.56	0.912	4.199

TUKEY ANALYSIS: Bio-assay of the "total infective centers" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 12th hour. 1 = control; 2 = officinale treatment of the virus; 3 = officinale treatment of the host; 4 = zerumbet treatment of the host. Source is fig. 20.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	37.00	5.02	7.37	4.199
1 vs. 3	162.00	5.02	32.3	4.199
1 vs. 4	165.25	5.02	32.0	4.199
2 vs. 3	125.00	5.02	24.9	4.199
2 vs. 4	128.25	5.02	25.6	4.199
3 vs. 4	3.25	5.02	0.65	4.199

TUKEY ANALYSIS: Bio-assay of the "total infective centers" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 14th hour. 1 = control; 2 = officinale treatment of the virus; 3 = officinale treatment of the host; 4 = zerumbet treatment of the host. Source is fig. 20.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	35.25	17.15	2.05	4.199
1 vs. 3	287.5	17.15	16.76	4.199
1 vs. 4	284.0	17.15	16.56	4.199
2 vs. 3	252.3	17.15	14.71	4.199
2 vs. 4	248.8	17.15	14.50	4.199
3 vs. 4	3.50	17.15	0.20	4.199

TUKEY ANALYSIS: Bio-assay of the "total infective centers" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 16th hour. 1 = control; 2 = officinale treatment of the virus; 3 = officinale treatment of the host; 4 = zerumbet treatment of the host. Source is fig. 20.

Comparison	Difference	SE	q	$q_{0.05,12,4}$
1 vs. 2	78.50	11.10	7.07	4.199
1 vs. 3	338.25	11.10	23.96	4.199
1 vs. 4	385.25	11.10	27.28	4.199
2 vs. 3	263.75	11.10	23.76	4.199
2 vs. 4	310.75	11.10	28.10	4.199
3 vs. 4	47.00	11.10	3.33	4.199

TABLE:7

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 14th hour. (fig. 21)

SOURCE	SS	d.f.	MS	F	p
Groups	0.00	1	0.00	0.00*	1.000
Error	29.5	6	4.917	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 16th hour. (fig. 21)

SOURCE	SS	d.f.	MS	F	p
Groups	2701.1	1	2701.125	59.2*	0.000...
Error	273.75	6	45.63	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 18th hour. (fig. 21)

SOURCE	SS	d.f.	MS	F	p
Groups	1431.1	1	1431.12	31.8*	0.001
Error	269.8	6	44.96	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 20th hour.(fig. 21)

SOURCE	SS	d.f.	MS	F	p
Groups	1225.1	1	1225.1	14.4*	0.009
Error	509.71	6	84.96	_	_

* p 0.05

TABLE:8

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 2nd hour. (fig. 22)

SOURCE	SS	d.f.	MS	F	p
Groups	0.57	1	0.57	8.58*	0.026
Error	0.40	6	0.07	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 8th hour. (fig. 22)

SOURCE	SS	d.f.	MS	F	p
Groups	105.13	1	105.13	10.9*	0.016
Error	57.75	6	9.62	_	_

* p 0.05

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 14th hour. (fig. 22)

SOURCE	SS	d.f.	MS	F	p
Groups	18.0	1	18.0	1.13*	0.329
Error	95.5	6	15.92	_	_

* p 0.01

ANALYSIS OF VARIANCE: Bio-assay of the "total infective center" i.e. the unadsorbed LPP-1 virus and the progeny LPP-1 virus after the spontaneous lysis of the infected Plectonema cells in the Bold's media at the 20th hour. (fig. 22)

SOURCE	SS	d.f.	MS	F	p
Groups	10.125	1	10.125	1.05*	0.345
Error	57.75	6	9.63	_	_

* p 0.01

TABLE:9 Assay of LPP-1 virus replication in the Plectonema cells that are treated with the Bold's medium, i.e. control.

INCUBATION TIME (HOURS)	NO. OF PETRI PLATES INOCULATED	PER 1ML OF VIRUS NO OF PLAQUES IN EACH PLATE	AVERAGE NO OF PLAQUES
0	1	155	169.75
	2	184	
	3	169	
	4	171	
1	1	169	177.25
	2	171	
	3	196	
	4	173	
2	1	151	169.5
	2	178	
	3	169	
	4	180	
3	1	175	177.50
	2	181	
	3	190	
	4	164	
4	1	184	184.75
	2	182	
	3	186	
	4	187	
5	1	185	176.75
	2	170	
	3	160	
	4	192	
6	1	170	188.50
	2	187	
	3	198	
	4	199	
7	1	158	181.00
	2	192	
	3	176	
	4	198	
8	1	163	177.25
	2	166	
	3	188	
	4	192	

	1	181	
9	2	174	183.75
	3	185	
	4	195	
	1	155	
10	2	191	174.75
	3	189	
	4	164	
	1	154	
11	2	179	178.50
	3	197	
	4	184	
	1	195	
12	2	177	172.75
	3	160	
	4	159	
	1	162	
13	2	194	176.00
	3	159	
	4	189	
	1	181	
14	2	164	169.25
	3	158	
	4	174	
	1	162	
15	2	183	177.25
	3	176	
	4	188	
	1	160	
16	2	177	168.25
	3	148	
	4	188	
	1	171	
17	2	193	175.75
	3	198	
	4	141	
	1	161	
18	2	194	179.75
	3	166	
	4	198	
	1	174	
19	2	172	180.25

	3	181	
	4	194	
	1	192	
20	2	188	180.25
	3	162	
	4	179	
	1	199	
21	2	163	177.25
	3	176	
	4	171	
	1	156	
22	2	186	173.75
	3	195	
	4	158	
	1	153	
23	2	158	174.00
	3	203	
	4	182	
	1	179	
24	2	176	173.75
	3	155	
	4	185	

TABLE:10 Assay of LPP-1 virus replication in the Plectonema cells that are treated with the Zingiber officinale extract.

INCUBATION TIME (HOURS)	NO. OF PETRI PLATES INOCULATED	PER 1ML OF VIRUS NO OF PLAQUES IN EACH PLATE	AVERAGE NO OF PLAQUES
	1	179	
0	2	170	174.00
	3	167	
	4	180	
	1	184	
1	2	173	177.50
	3	183	
	4	170	
	1	123	
2	2	146	136.75
	3	142	
	4	136	
	1	96	
3	2	82	100.50
	3	126	
	4	98	
	1	78	
4	2	77	78.25
	3	82	
	4	76	
	1	32	
5	2	47	47.25
	3	51	
	4	59	
	1	40	
6	2	53	48.25
	3	48	
	4	52	
	1	16	
7	2	11	17.75
	3	21	
	4	23	
	1	19	
8	2	9	16.25
	3	20	
	4	17	

	1	16	
9	2	21	19.50
	3	22	
	4	19	
	1	17	
10	2	16	18.25
	3	24	
	4	16	
	1	27	
11	2	11	17.50
	3	17	
	4	15	
	1	14	
12	2	15	15.25
	3	19	
	4	13	
	1	18	
13	2	15	16.00
	3	20	
	4	11	
	1	11	
14	2	20	16.75
	3	17	
	4	19	
	1	19	
15	2	25	23.50
	3	22	
	4	28	
	1	19	
16	2	16	21.50
	3	32	
	4	19	
	1	24	
17	2	28	24.75
	3	22	
	4	25	
	1	12	
18	2	15	17.25
	3	11	
	4	31	
	1	14	
19	2	14	32.25

	3	49	
	4	52	
	1	19	
20	2	14	19.25
	3	21	
	4	23	
	1	14	
21	2	18	15.50
	3	15	
	4	15	
	1	9	
22	2	15	13.00
	3	13	
	4	15	
	1	14	
23	2	9	13.25
	3	17	
	4	13	
	1	7	
24	2	17	10.50
	3	7	
	4	11	

TABLE:11 Assay of LPP-1 virus replication in the Plectonema cells that are treated with the Zingiber zerumbet extract.

INCUBATION TIME (HOURS)	NO. OF PETRI PLATES INOCULATED	PER 1ML OF VIRUS NO OF PLAQUES IN EACH PLATE	AVERAGE NO OF PLAQUES
	1	149	
0	2	162	164.75
	3	194	
	4	154	
	1	125	
1	2	200	182.75
	3	199	
	4	207	
	1	89	
2	2	127	130.50
	3	158	
	4	148	
	1	24	
3	2	36	31.25
	3	19	
	4	46	
	1	7	
4	2	13	11.00
	3	13	
	4	11	
	1	4	
5	2	3	3.25
	3	3	
	4	3	
	1	1	
6	2	0	0.50
	3	1	
	4	0	
	1	-	
7	2	-	-
	3	-	
	4	-	
	1	-	
8	2	-	-
	3	-	
	4	-	

	1	-	
9	2	-	-
	3	-	
	4	-	
	1	-	
10	2	-	-
	3	-	
	4	-	
	1	-	
11	2	-	-
	3	-	
	4	-	
	1	-	
12	2	-	-
	3	-	
	4	-	
	1	-	
13	2	-	-
	3	-	
	4	-	
	1	-	
14	2	-	-
	3	-	
	4	-	
	1	-	
15	2	-	-
	3	-	
	4	-	
	1	-	
16	2	-	-
	3	-	
	4	-	
	1	-	
17	2	-	-
	3	-	
	4	-	
	1	-	
18	2	-	-
	3	-	
	4	-	
	1	-	
19	2	-	-

	3	-	
	4	-	
	1	-	
20	2	-	-
	3	-	
	4	-	
	1	-	
21	2	-	-
	3	-	
	4	-	
	1	-	
22	2	-	-
	3	-	
	4	-	
	1	-	
23	2	-	-
	3	-	
	4	-	
	1	-	
24	2	-	-
	3	-	
	4	-	

TABLE:12 Assay of the unadsorbed LPP-1 virus replication in Plectonema cells that are treated with the Bold's medium.

INCUBATION TIME (MINUTES)	NO. OF PETRI PLATES INOCULATED	PER 0.5ML OF VIRUS NO OF PLAQUES IN EACH PLATE	AVERAGE NO OF PLAQUES
	1	310	
	2	312	
	3	284	
0	4	299	298.00
	5	279	
	6	321	
	7	316	
	8	263	
	1	91	
15	2	99	105.25
	3	120	
	4	111	
	1	73	
30	2	76	74.25
	3	76	
	4	72	
	1	49	
45	2	41	53.75
	3	51	
	4	74	
	1	38	
60	2	43	45.00
	3	51	
	4	48	
	1	34	
75	2	17	29.25
	3	39	
	4	27	
	1	13	
90	2	16	15.75
	3	17	
	4	17	
	1	8	
105	2	10	11.25
	3	13	
	4	14	

	1	11	
120	2	4	6.75
	3	7	
	4	5	

TABLE:13 Assay of the unadsorbed LPP-1 virus replication in Plectonema cells that are treated with the Zingiber officinale extract.

INCUBATION TIME (MINUTES)	NO. OF PETRI PLATES INOCULATED	PER 0.5ML OF VIRUS NO OF PLAQUES IN EACH PLATE	AVERAGE NO OF PLAQUES
	1	310	
	2	312	
	3	284	
0	4	299	298.00
	5	279	
	6	321	
	7	316	
	8	263	
	1	156	
15	2	289	204.50
	3	188	
	4	185	
	1	139	
30	2	186	190.25
	3	224	
	4	212	
	1	131	
45	2	119	138.00
	3	157	
	4	145	
	1	94	
60	2	90	92.75
	3	91	
	4	96	
	1	86	
75	2	81	94.00
	3	111	
	4	98	
	1	53	
90	2	37	59.00
	3	72	
	4	74	
	1	48	
105	2	59	58.25
	3	57	
	4	69	

	1	12	
120	2	18	28.25
	3	39	
	4	44	

TABLE:14 Assay of the unadsorbed LPP-1 virus replication in Plectonema cells that are treated with the Zingiber zerumbet extract.

INCUBATION TIME (MINUTES)	NO. OF PETRI PLATES INOCULATED	PER 0.5ML OF VIRUS NO OF PLAQUES IN EACH PLATE	AVERAGE NO OF PLAQUES
	1	310	
	2	312	
	3	284	
0	4	299	298.00
	5	279	
	6	321	
	7	316	
	8	263	
	1	281	
15	2	278	272.25
	3	286	
	4	244	
	1	226	
30	2	297	276.50
	3	310	
	4	273	
	1	231	
45	2	218	265.25
	3	262	
	4	350	
	1	277	
60	2	243	252.00
	3	232	
	4	256	
	1	330	
75	2	298	289.75
	3	297	
	4	234	
	1	309	
90	2	214	281.00
	3	293	
	4	308	
	1	288	
105	2	234	266.00
	3	206	
	4	336	

	1	282	
120	2	244	260.25
	3	253	
	4	262	

TABLE:15 Assay of the unadsorbed LPP-1 virus replication in Plectonema cells after the treatment of the virus particles with the *Zingiber officinale* extract.

INCUBATION TIME (MINUTES)	NO. OF PETRI PLATES INOCULATED	PER 0.5ML OF VIRUS NO OF PLAQUES IN EACH PLATE	AVERAGE NO OF PLAQUES
	1	310	
	2	312	
	3	284	
0	4	299	298.00
	5	279	
	6	321	
	7	316	
	8	263	
	1	222	
15	2	229	195.00
	3	164	
	4	165	
	1	134	
30	2	103	125.75
	3	133	
	4	133	
	1	107	
45	2	105	95.75
	3	73	
	4	98	
	1	60	
60	2	53	62.75
	3	71	
	4	67	
	1	20	
75	2	50	33.50
	3	26	
	4	38	
	1	37	
90	2	31	42.25
	3	62	
	4	39	
	1	29	
105	2	27	32.75
	3	36	
	4	39	

	1	11	
120	2	12	11.75
	3	16	
	4	8	

TABLE:16 % Adsorption of LPP-1 virus on the Plectonema cells that are treated with the Bold's medium.

INCUBATION TIME (MINUTES)	NO. OF PETRI PLATES INOCULATED	% ADSORPTION	AVERAGE
0	1	0	0
	2	0	
	3	0	
	4	0	
15	1	77.7	74.2
	2	75.7	
	3	70.6	
	4	72.8	
30	1	82.1	81.8
	2	81.3	
	3	81.3	
	4	82.3	
45	1	88.0	86.8
	2	89.9	
	3	87.5	
	4	81.8	
60	1	90.7	89.0
	2	89.4	
	3	87.5	
	4	88.2	
75	1	91.7	92.8
	2	95.8	
	3	90.4	
	4	93.4	
90	1	96.8	96.1
	2	96.1	
	3	95.8	
	4	95.8	
105	1	98.0	97.2
	2	97.5	
	3	96.8	
	4	96.6	

	1	97.3	
120	2	99.0	98.4
	3	98.3	
	4	98.8	

TABLE:17 % Adsorption of LPP-1 virus on the Plectonema cells that are treated with the Zingiber officinale extract.

INCUBATION TIME (MINUTES)	NO. OF PETRI PLATES INOCULATED	% ADSORPTION	AVERAGE
0	1	0	0
	2	0	
	3	0	
	4	0	
15	1	61.7	49.8
	2	29.1	
	3	53.9	
	4	54.6	
30	1	65.9	53.3
	2	54.4	
	3	45.0	
	4	48.0	
45	1	67.9	66.2
	2	70.8	
	3	61.5	
	4	64.4	
60	1	76.9	77.2
	2	77.9	
	3	77.7	
	4	76.4	
75	1	78.9	77.0
	2	80.1	
	3	72.8	
	4	76.0	
90	1	87.0	85.5
	2	90.9	
	3	82.3	
	4	81.8	
105	1	88.2	85.7
	2	85.5	
	3	86.0	
	4	83.1	

	1	97.1	
120	2	95.6	93.1
	3	90.4	
	4	89.2	

TABLE:18 % Adsorption of LPP-1 virus on the Plectonema cells that are treated with the Zingiber zerumbet extract.

INCUBATION TIME (MINUTES)	NO. OF PETRI PLATES INOCULATED	% ADSORPTION	AVERAGE
0	1	0	0
	2	0	
	3	0	
	4	0	
15	1	31.0	33.2
	2	31.8	
	3	29.8	
	4	40.1	
30	1	44.5	32.1
	2	27.1	
	3	23.9	
	4	33.0	
45	1	43.3	34.9
	2	46.5	
	3	35.7	
	4	14.1	
60	1	32.0	38.2
	2	40.4	
	3	43.1	
	4	37.2	
75	1	19.0	28.9
	2	26.9	
	3	27.1	
	4	42.6	
90	1	24.2	31.1
	2	47.5	
	3	28.1	
	4	24.4	
105	1	29.3	34.7
	2	42.6	
	3	49.4	
	4	17.5	

	1	30.8	
120	2	40.1	36.1
	3	37.9	
	4	35.7	

TABLE:19 % Adsorption of LPP-1 virus that is treated with the Zingiber officinale extract.

INCUBATION TIME (MINUTES)	NO. OF PETRI PLATES INOCULATED	% ADSORPTION	AVERAGE
0	1	0	0
	2	0	
	3	0	
	4	0	
15	1	45.5	52.2
	2	43.8	
	3	59.8	
	4	59.5	
30	1	67.1	69.2
	2	74.7	
	3	67.4	
	4	67.4	
45	1	73.7	76.5
	2	74.2	
	3	82.1	
	4	76.0	
60	1	85.3	84.6
	2	87.0	
	3	82.6	
	4	83.6	
75	1	95.1	91.8
	2	87.7	
	3	93.6	
	4	90.7	
90	1	90.9	89.6
	2	92.4	
	3	84.8	
	4	90.4	
105	1	92.9	92.0
	2	93.4	
	3	91.2	
	4	90.4	

	1	97.3	
120	2	97.1	97.1
	3	96.1	
	4	98.0	

TABLE:20 Plaque counts on plates from F.G.T. and S.G.T. containing infected Plectonema cells that were treated with the Bold's medium.

TIME, HRS.	F.G.T.	S.G.T.
2	274	
4	256	
6	200	
7	238	88
8		11
9		16
10		69
11		93
12		170
13		223
14		291
16		392
18		379
20		396

TABLE:21 Plaque counts on plates from F.G.T. and S.G.T. containing infected Plectonema cells that were treated with the extract of Zingiber officinale **before** the adsorption of virus.

TIME, HRS.	F.G.T.	S.G.T.
2	242	
4	246	
6	194	
7	207	1
8		4
9		0
10		7
11		4
12		8
13		13
14		4
16		53
18		62
20		61

TABLE:22 Plaque counts on plates from F.G.T. and S.G.T. containing infected Plectonema cells that were treated with the extract of Zingiber zerumbet **before** the adsorption of virus.

TIME, HRS.	F.G.T.	S.G.T.
2	258	
4	275	
6	225	
7	259	3
8		9
9		4
10		6
11		8
12		5
13		5
14		7
16		6
18		8
20		10

TABLE:23 Plaque counts on plates from F.G.T. and S.G.T. containing infected Plectonema cells that were treated with the extract of Zingiber officinale **after** the adsorption of virus.

TIME, HRS.	F.G.T.	S.G.T.
2	217	
4	222	
6	246	
7	251	0
8		2
9		3
10		3
11		4
12		3
13		4
14		4
16		17
18		35
20		37

TABLE:24 Plaque counts on plates from F.G.T. and S.G.T. containing infected Plectonema cells that were treated with the extract of Zingiber zerumbet **after** the adsorption of virus.

TIME, HRS.	F.G.T.	S.G.T.
2	227	
4	252	
6	241	
7	268	0
8		2
9		2
10		3
11		4
12		3
13		4
14		4
16		4
18		7
20		7

TABLE:25 Plaque counts on plates from F.G.T. and S.G.T. containing Plectonema cells that were infected with the officinale treated virus.

TIME, HRS.	F.G.T.	S.G.T.
2	272	
4	245	
6	223	
7	282	1
8		10
9		16
10		16
11		53
12		133
13		172
14		256
16		317
18		334
20		342

APPROVAL SHEET

The thesis submitted by Ebby Paul Jido has been read and approved by Dr. Amrik S. Dhaliwal (chairman of the thesis committee), Dr. Fredrick Wezeman (chairman of the department of biology), Dr. Warren Jones, and Dr. Edward Palinscar. The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

9/12/91
DATE

Amrik S. Dhaliwal
Director's Signature