ALMOND BOARD OF CALIFONRIA

1985 ANNUAL REPORT

Project Number: 85-J7

PROJECT:

Epidemiology and Control of Frost Injury to Almond Caused by Ice Nucleation Active Bacteria

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OBJECTIVES DURING 1985:

The basis for avoidance of frost injury by avoidance of bacterial ice nucleation for protection of almond flowers agains frost injury and the preliminary field data on several possible methods of control have shown the utility of control of frost injury using this concept. To further evaluate and improve methods of frost control, several broad questions were investigated:

1. Evaluate possible methods and timing of application of antagonistic bacteria for use as biological control agents of frost injury.

2. Determine the most effective bactericides, antagonistic bacteria on ice nucleation inhibitors to control frost injury, and the environmental parameters which influence their effectiveness.

3. Evaluate rates and frequency of application of existing bactericides in control of frost injury under field conditions.

4. Quantitate the number of ice nucleation active bacteria of all kinds on leaves of almond as a function of time during the period of frost hazard to these plants. Emphasis will be placed on the number, species, and location of ice nucleation active bacteria and ice nuclei on dormant tissues.

5. Determine seasonal and treatment effects on the supercooling point of almond flowers and nuts.

6. Determine the numbers, sources and activity of ice nuclei in dormant and pink bud stage almond tissue.

7. Determine the level of copper sensitivity among ice nucleation active bacteria on almond.

8. Determine factors influencing differential bacterial populations and thus differential frost sensitivity of different almond orchards of different vegetation management.

9. Evaluate antagonistic bacteria for effectiveness in reducing frost injury to plants grown in the greenhouse or almond twigs artificially forced to bloom and frozen in controlled environmental chambers.

10. Evaluate most effective antagonists from step 9in field trials by spraying young leaves and flowers of almond with these bacteria.

11. Evaluate integrated control of frost injury by application of bactericides with bacterial antagonists resistant to a given bactericide.

12. Evaluate in field trials, chemicals shown to reduce the nucleation activity of bacteria in laboratory tests.

13. Determine the most effective rates of nucleation inhibiting compounds in reducing frost injury and in eliminating ice nuclei from leaf surfaces.

14. Determine the optimal time before a frost for the application of nucleation inhibiting chemicals.

15. Determine the persistence of reductions in frost sensitivity of almond following application of nucleation inhibitors.

16. Determine the additive effects of undertree sprinkling with control of bacterial ice nuclei for control of frost injury under field conditions.

17. Determine the distribution of freezing temperatures (supercooling points) of treated and untreated almond flowers and fruit to predict effects of frost control in terms of degrees of protection.

18. Determine the numbers and activity of ice nucleation active bacteria and dead bacterial cells on dormant almond tissue. Test the effects of nucleation inhibiting chemicals on reducing the supercooling point of dormant tissues.

19. Determine the tolerance of a collection of ice nucleation active bacteria from various almond growing areas to copper sulfate and chelated forms of copper. Determine the effectiveness of addition of copper chelators on the bactericidal effects of fixed copper fungicides.

20. Survey several different commercial almond orchards with different surrounding crops for populations of ice nucleation active bacteria and tissue supercooling point.

INTERPRETIVE SUMMARY.

Extensive field trials were established at five different locations around California during the winter and spring of 1985. The largest field trial which included fifteen treatments was established in Modesto, California. Field trials including twenty-two different treatments were established at other locations including the west side field station at the University of California, Chico, California, near Fresno, California, and Snelling, California. Temperatures in the spring of 1985 did not drop below 31 F (-0.5 C) at any location. Therefore no frost damage was observed in any of our plot areas. Populations of ice nucleation active bacteria, entirely the species Pseudomonas syringae, were much lower during the spring of 1985 than in previous years. Population size of ice nucleation active bacteria did not exceed approximately 1,000 cells per gram fresh weight in most plot areas during 1984. The populations of ice nucleation active bacteria were from 100 to 1000 fold lower than in previous years. The populations of ice nucleating bacteria at a ochard near Snelling, California were significantly higher than at other locations. The lower population size of ice nucleation active bacteria during 1985 was presumably due to the below normal rainfall and infrequency of rainfall occurring during the early spring of 1985.

Significant reductions in population size of ice nucleation active bacteria, ice nuclei found on treated almond, and the supercooling point of detached almond fruiting spurs were measured in all treatments for which copper containing fungicides were applied. A mixture of Kocide 101 with other dithiocarbamate fungicides generally were superior to copper fungicides alone in controlling bacterial populations and ice nuclei on plants. Some reduction in bacterial populations were conferred by applications of Maneb alone. The supercooling point of untreated almond tissues generally ranged from -1.5 C to -4 C in different locations. The supercooling point of almond tissues treated with copper based fungicides and certain antibiotics such as a mixture of streptomycin and Terramycin were reduced from 1 C to 3 C compared to untreated trees. The greatest reduction in supercooling point of treated tissues occurred approximately ten days following the initiation of treatment of trees with bactericides.

Non-ice nucleation active bacteria applied to almond at 10 percent bloom colonized almond tissue and reduced both the numbers of ice nucleation active bacteria and the numbers of ice nuclei and the supercooling point of almond tissues. Naturally occuring non-ice nucleation active strains of Pseudomonas syringae isolated from almond trees near Modesto, California were highly effective both in colonizing almond tissue and in reducing the freezing temperature of almond plant parts.

A large collection of ice nucleation active bacteria form almond trees collected throughout the almond growing regions of California have been made. These collections of ice nucleation active bacteria have been tested for their sensitivity to copper ions. Preliminary data indicates that highly significant differences in resistance to copper ions exist among these bacterial strains. Some strains of bacteria are resistant to in excess of 16 parts per million of free copper ions in culture media and in distilled water in laboratory assays. Copper sensitive strains are killed less than approximately 0.5 parts per million of free copper ion. The concentration of free copper ions on plants treated with copper sprays is apparently less than one part per million. Therefore preliminary data indicates that copper tolerant strains of *Pseudomonas syringae* will not be readily controlled by standard applications of copper fungicides. Preliminary results with greenhouse experiments indicate that the viability of copper-tolerant of *P. syringae* strains is higher than those of copper sensitive strains of bean plants treated with copper fungicides with and without bis-dithiocarbamate fungicides. Further work is planned to ellucidate whether bis-dithiocarbamate fungicides will increase the soluability of copper ions on almond leaf surfaces to an extent such that all copper resistant bacterial strains are killed.

EXPERIMENTAL PROCEDURES

Nearly all of the experimental procedures used in this study during 1985 were those similar to those reported in 1982 through 1984. An article in the Journal for Horticultural Science 109: 48-53 also details these procedures. Bactericides and bis-dithiocarbamate fungicides applied to orchards at Snelling, California were applied with a speed sprayer at a rate of approximately 60 gallons per acre. Other applications were made with handgun applications at a rate in excess of 100 gallons per acre (to runoff) using a commerically available surfactant (Triton CS-7).

RESULTS

Ice nucleation active bacteria were detected on all samples of untreated fruiting spurs of almond during the spring of 1985. However, the population size of ice nucleation active bacteria from orchards throughout California were generally from 100 to more than 1000 fold lower than in the previous three years. Only approximately 1000 bacteria per gram of almond tissue were observed on trees in several plot areas during the period of maximum frost hazard (Figure 1 and Table 3). The population size of ice nucleation active bacteria on untreated almond at a commercial orchard near Snelling, California was significantly higher than in other areas sampled (Table 1). The populations of ice nucleation active bacteria were generally much lower during 1985 because of the low frequency of rainfall during this period. Comparison of the frequencies of rainfall events during 1985 with that of the previous four years indicated that the population size of ice nucleation active bacteria is generally correlated with the occurrence of rain and not necessarily with the amount of rain. The population size of ice nucleation active bacteria on untreated almond during 1985 did not increase rapidly after first bloom as in previous years due to the low frequency of rainfall. Population size of ice nucleation active bacteria increased from only approximately 100 cells per gram fresh weight to about 1000 cells per gram fresh weight in most orchards. The numbers of ice nuclei active at -5 also paralleled the population of ice nucleation active bacteria. Only Pseudomonas syrigae was found to be active in ice nucleation on almond during 1985. No other bacterial species were detected with ice nucleation activity.

The population size of ice nucleation active bacteria was reduced approximately 100 fold by all copper based fungicide applications (Figures 2-4 and Table 3). Because of the low population size of ice nucleation active bacteria on treated trees, and the fact that Kocide 101 alone reduced the population size of ice nucleation active bactive approximately 100 fold, any improvements in control of ice nucleation active bactive bacteria by additives to copper based fungicides were difficult to demonstrate in these trials. The population of ice nucleation active bacteria on trees treated with Kocide 101 dormant as well as at three intervals starting at pink bud (Figure 2) were generally slightly lower than those on trees in which Kocide 101 was started only at pink bud (Figure 4). Similarly, the population size of ice nucleation active bacteria on almond trees treated at pink bud and at two subsequent times with a mixture of Kocide 101 and Maneb were reduced 100 to 1000 fold compared to untreated trees and were generally fairly low at all dates (Figure 3). As has been frequently observed, applications of copper based fungicides (either Kocide 101 alone or Kocide 101 in combination bis-dithiocarbamates) reduced the population size of ice nucleation active bacteria on almond trees significantly compared to those on trees treated at pink bud and at subsequent dates at the same intervals with a mixture of streptomycin and Terramycin (Figure 5). Copper based fungicides used alone or with bis-dithiocarbamate fungicides reduced the population size of ice nucleation active bacteria approximately 10 times better than did a mixture of steptomycin and Terramycin. The high incidence of sunny days during 1985 could likely account for the breakdown of steptomycin and Terramycin on trees between treatments. Copper based fungicides, not being photolabile, were likely not broken down between application dates. Such photolability of streptomycin and Terramycin will be of significance in sunny years. For the first time, some bactericidal activity of Maneb was observed, particularly in the early spring of 1985. While it cannot be totally ruled out that some residual copper fungicide remained in spray tanks and therefore was chelated by Maneb prior to application, some literature reports indicate Maneb alone, or more likely contaminants in commercial preparations of Maneb have bactericidal activity. The population size of ice nucleation active bacteria was reduced on almond trees treated with Maneb at pink bud and at two subsequent dates compared to untreated trees (Figure 6). The population size of ice nucleation active bacteria had increased by late April in these trials however and may have reflected the low concentration of bactericidal compounds in Maneb tank mixes.

Bis-dithiocarbamate fungicides generally improved the control of ice nucleation active bacteria on almond trees treated in a commercial orchard near Snelling, California (Table 1). The high variability of ice nucleation active bacteria among trees did not allow significant separation of mean population sizes of ice nucleation active bacteria treated with Kocide 101 or with one of several different bis-dithiocarbamate fungicides (Table 1). However, the population size of ice nucleation active bacteria on trees treated with Kocide 101 or with Kocide 101 in any combination with bis-dithiocarbamate fungicides were significantly lower than on untreated trees, however. Numerically, Kocide in combination with Ziram was superior to Kocide containing Maneb when applied to tank mixes with sprayer applications (Table 1). As described earlier, however, these differences were not statistically different and need to be verified by subsequent field trials.

Several different bis-dithiocarbamate fungicides were evaluated under greenhouse conditions for their ability to enhance the bactericidal activity of Kocide 101. In these trials, Mancozeb, (Dithane M45) was superior in enhancing the bactericidal activity of Kocide 101 compared to Zyram (Table 2). It can also be observed that the copper sensitive P. syringae strain B728A was approximately 10 fold more sensitive to killing by copper based fungicides than the copper tolerant strain of P. syringae (glen 22) (Table 2). These greenhouse evaluations of the bactericidal activity of copper based fungicides were performed by treating greenhouse grown been plants with P. syringae strains, allowing them to grow to a maximum population size and then treating plants with field rates (1 pound per 100 gallons) of copper based fungicides. Thus these trials were done using an eradicant mode of these copper fungicides. Similar trials are underway in which the protective mode (treatment of plants with copper based fungicides prior to application of ice nucleation active bacteria) is being conducted.

Several non-ice nucleation active bacteria applied to almond trees at approximately 15 percent bloom colonized almond tissues during the entire period of frost hazard during 1985 (Figure 7-10). It was noteworthy, that the total population size of bacteria on almond trees was about 100 fold less than in previous years. The applied naturally occurring non-ice nucleation active bacteria comprised approximately the same high percentage of the total bacteria on almond trees (20%) as they had in previous years. That is; in previous years the total bacterial populations observed on almond trees was approximately 10^7 cells per gram fresh weight and the population size of applied antagonistic bacteria remained in excess of approximately 10^6 cells per gram fresh weight, thus accounting for in excess of 10 percent of the total bacteria present on almond tissues. During 1985, total bacterial populations on untreated trees remained at approximately 10^4 cells per gram fresh weight during much of the growing season until spring rains occurred during late March, and applied antagonistic bacteria were detected in excess of approximately 10^3 cells per gram fresh weight during the entire season, thus accounting for in excess of 10 percent of the total bacteria found on trees treated during 1985. The population size of ice nucleation active bacteria was reduced significantly on trees treated with one of any of four different naturally occurring non-ice nucleation active bacteria during 1985 (Figure 7-10). The population size of ice nucleation active bacteria on almond trees treated with naturally occurring non-ice nucleation active strains of P. fluorescence, (strain) A526 (Figure 7) and strain A506 (Figure 8)) was reduced more than 100 fold during the first month after application compared to untreated trees. Population size of ice nucleation active bacteria began to increase during late spring on trees treated with either strain A506 or A526. In was noteworthy that the population size of ice nucleation active bacteria on trees treated with naturally occurring non-ice nucleation active strains of Pseudomonas syringae were reduced significantly compared to those treated with strains of

Pseudomonas fluorescence during 1985 (Figures 9 and 10). Naturally occurring non-ice nucleation active strains of Pseudomonas syringae appear to be superior to strains of Pseudomonas fluorescens in reducing the population size of ice nucleation active bacteria on almond tissues. Because Pseudomonas syringae itself is the primary ice nucleating species of bacteria on almond tissues, it may use sites and grow under conditions identical to those of non-ice nucleation active strains of Pseudomonas syringae applied to these trees.

The relationship between the numbers of ice nucleation active bacteria found on almond trees and the freezing temperature of individual fruiting spurs has been determined (Figure 11). A logarithmic relationship between the numbers of ice nucleating active bacteria on plant part and the freezing temperature of that plant part has been found. For example, the freezing temperature of a plant part dropped approximately one degree Celsius by reducing the population size of ice nucleating active bacteria on that plant part by 100 fold (Figure 11). The reductions in the supercooling point of almond tissues observed during field trials are consistent with the observed relationship between population size of ice nucleation active bacteria and freezing temperature of that plant part. For example, non-ice nucleation active bacteria reduced the freezing temperature of almond spurs by from 1 C to an excess of 2 C compared to untreated control tissues (Figures 12 and 13). Similarly bactericides including Kocide 101 and streptomycin also reduce the supercooling point of almond tissues from in excess of 1 C to more than 2 C. It was generally observed that the supercooling point of treated almond tissues differed most greatly from that of untreated tissues at approximately two to three weeks following the initiation of treatment of plant tissues. That is, the population size of ice nucleation active bacteria following initiation of blossoming in the spring increases rapidly on untreated trees whereas the population size remains steady or decreases on plants treated with antagonistic bacteria or bactericides. Therefore the greatest difference in population size of ice nucleation active bacteria generally occurs from one to three weeks following initiation of treatment and therefore this is reflected in the difference in the supercooling point observed on these tissues (Figure 12 and 13).

Ice nucleation active strains of Pseudomonas syringae were collected from 29 different almond orchards throughout California during 1985. These bacterial strains were examined for their sensitivity to copper ions in culture media containing a range of different concentrations of copper ions. P. syringae strains were observed that had a range of copper sensitivities (from being killed by less than 0.5 parts per million copper ion in culture to surviving in excess of 16 parts per million copper ions). Randomly isolated Pseudomonas syringae colonies exhibit a bimodal distribution of copper sensitivity. Approximately two thirds of all Pseudomonas syringae strains isolated randomly from almond orchards exhibit a relatively high sensitivity to copper ions. However, approximately one third of 600 Pseudomonas syringae strains assayed are resistant to in excess of five parts per million of copper ion and there would be expected to escape killing by standard applications of copper based fungicides when bis-dithiocarbamate were not tank mixed or applied or oversprayed to trees receiving copper based sprays.

DISCUSSION

Work during 1985 indicates that the frost sensitivity of almond should be significantly reduced under natural field frost conditions. It was unfortunate that field frosts were not observed during 1985, but significant measurements were made of the sensitivity of almond tissues under laboratory conditions. It was noteworthy that although 1985 was an anomolous year in terms of rainfall patterns and therefore of populations of ice nucleation active bacteria, treatments that had performed well under different environmental conditions continued to perform well under these unique weather conditions. That is, frost control procedures using antagonistic microorganisms and bactericides which had been effective on trees under conditions of high rainfall again performed well during periods of infrequent rainfall. These results themselves were highly significant in that they give higher expectations to a general set of treatments which should lead to reductions in ice nucleation active bacteria and therefore frost damage under all conditions. That is, treatments to control ice nucleation active bacteria will not need to be designed to match weather conditions which themselves are unpredictable. Copper based fungicides continue to exhibit good control of ice nucleating bacteria and reduce the supercooling point of almond tissues. Since these materials are registered for use on almond, they would appear to be the most important immediate agents for recommendation for frost control. Bis-dithiocarbamates continue to exhibit activity in enhancing the supercooling point of plant tissues, but the lower population size of ice nucleating bacteria during 1985 made it difficult to unambigiously determine their effect under field conditions because of the otherwise good control of ice nucleating bacteria by copper based fungicides alone. The high incidence of copper resistant bacteria observed under field conditions throughout California would appear to justify continued emphasis on additives to copper based fungicides to control such resistant strains. Many strains of Pseudomonas syringae collected during 1985 have yet to be assayed for copper sensitivity under laboratory conditions and additional studies are underway to determine the behavior of such strains in the presence of copper fungicides on the leaves of plants. Similarly much data was collected during 1985 relating to the effect of nearby crops on the population size of ice nucleating bacteria and in particular on the population size of copper resistant bacteria on almond trees. This data is not yet fully colated and will be summarized later during 1986. Further emphasis on the role of copper resistant bacteria in almond frost damage and disease epidemiology will be emphasized as well as to study the importance of epiphytic populations of ice nucleating bacteria in causing blast of almond.

Fig 1

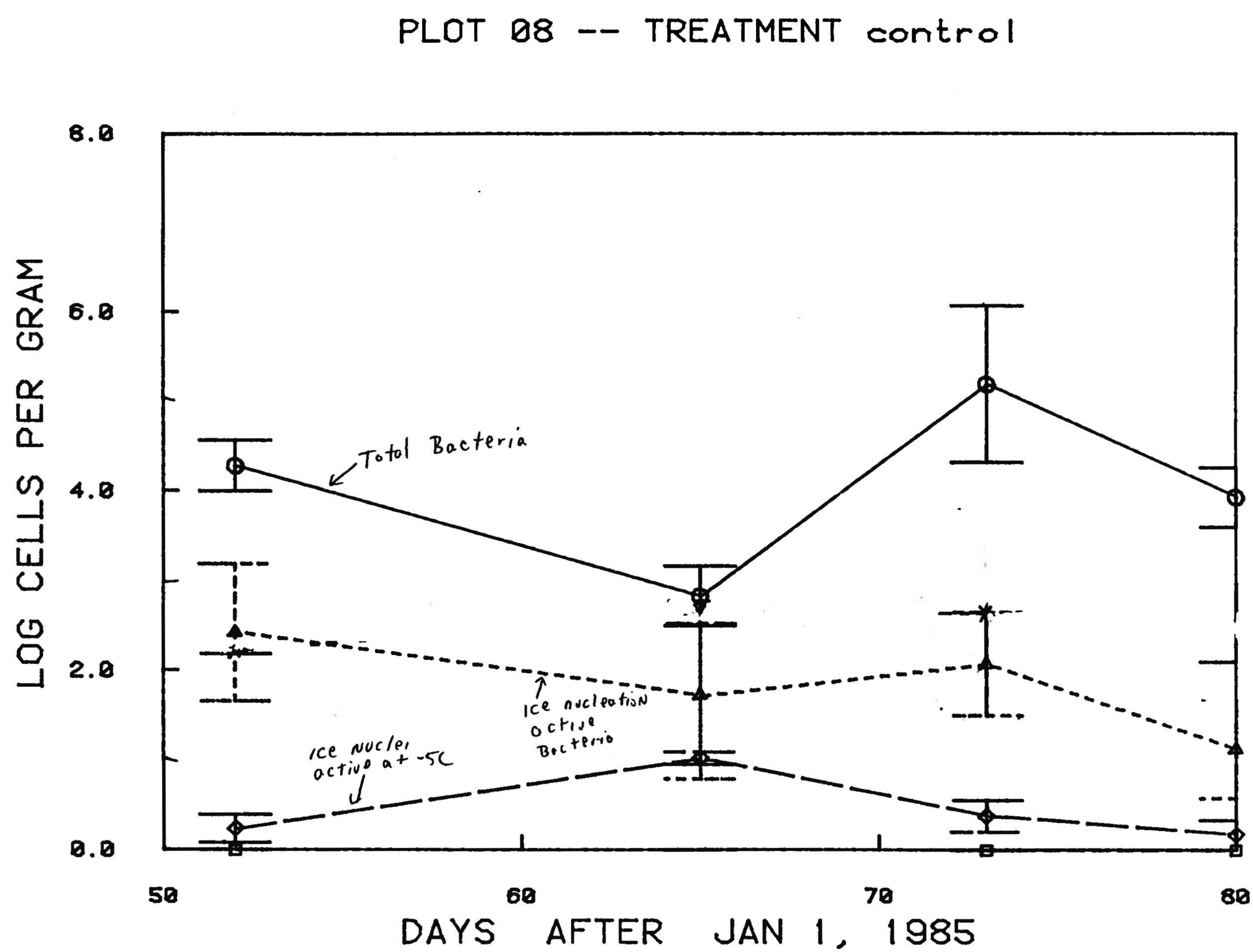

PLOT 08 -- TREATMENT control
LOG CELLS PER GRAM
8.0
6.0
4.0
2.0
0.0
50
60
70
80
DAYS AFTER JAN 1, 1985
Total Bacteria
Ice nucleation active Bacteria
ice nuclei active at -5C

Fig 2

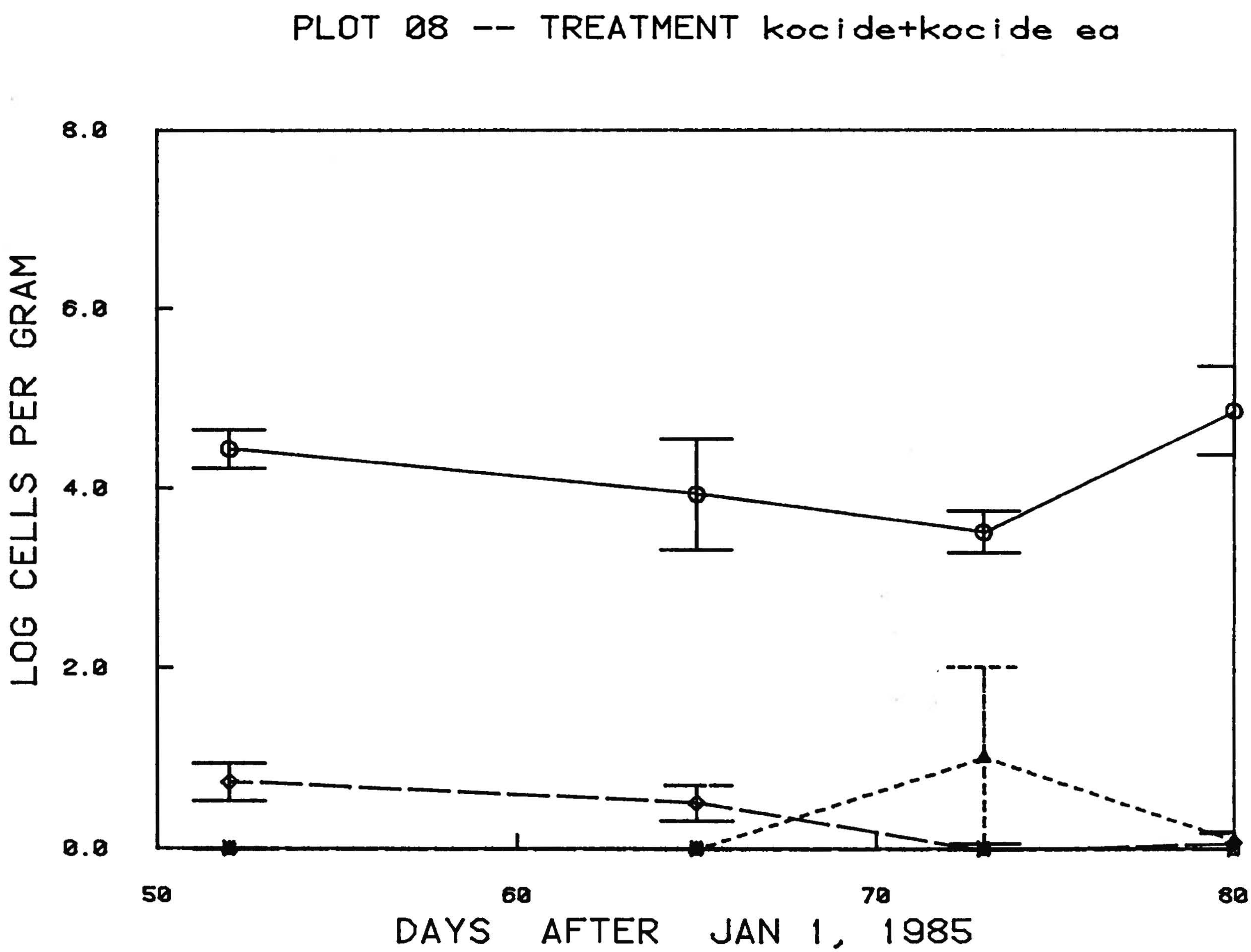

PLOT 08 -- TREATMENT kocide+kocide ea
LOG CELLS PER GRAM
8.0
6.0
4.0
2.0
0.0
50
60
70
80
DAYS AFTER JAN 1, 1985

Fig 3

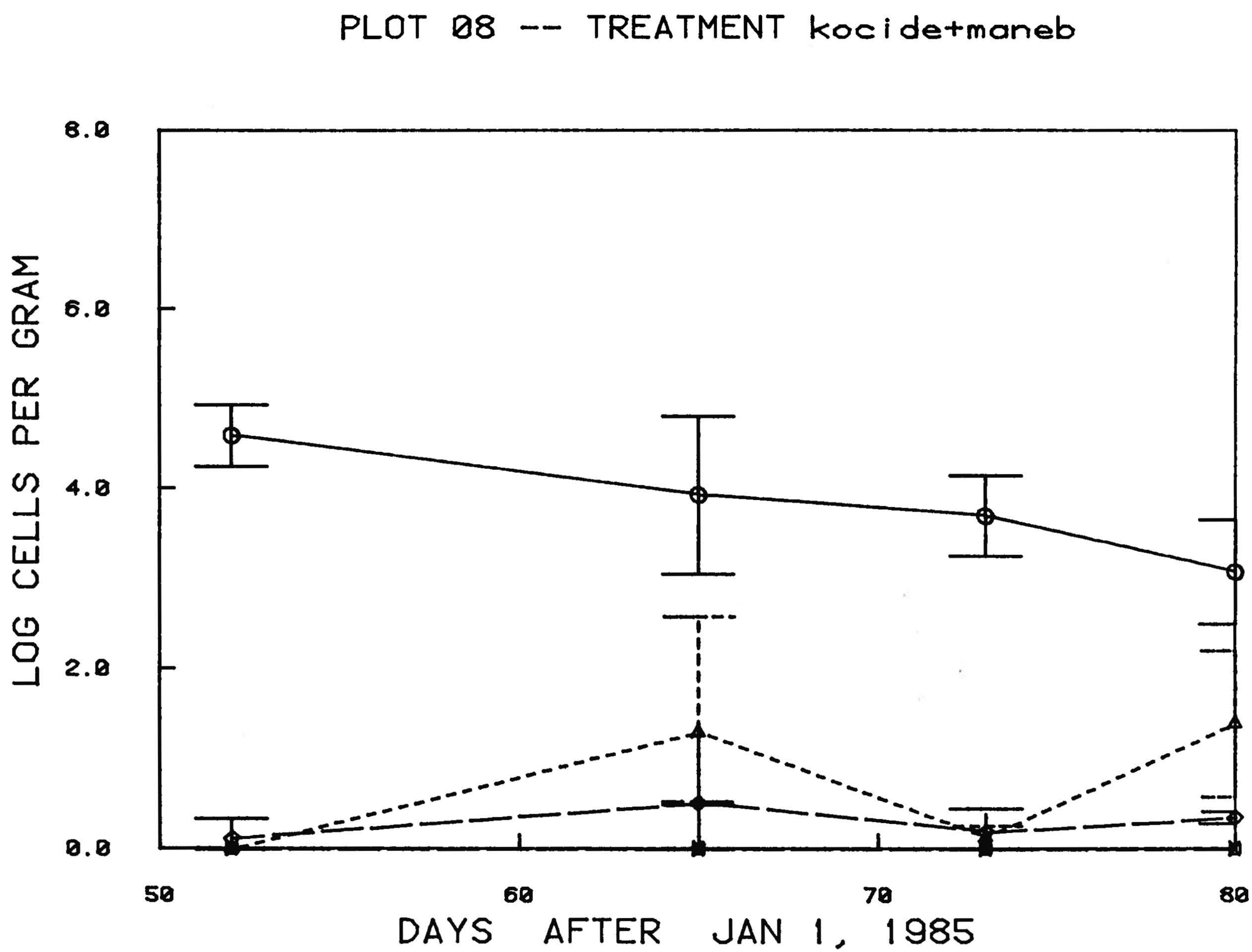

PLOT 08 -- TREATMENT kocide+maneb
LOG CELLS PER GRAM
8.0
6.0
4.0
2.0
0.0
50
60
70
80
DAYS AFTER JAN 1, 1985

Fig 4

PLOT 08 -- TREATMENT kocide early onl

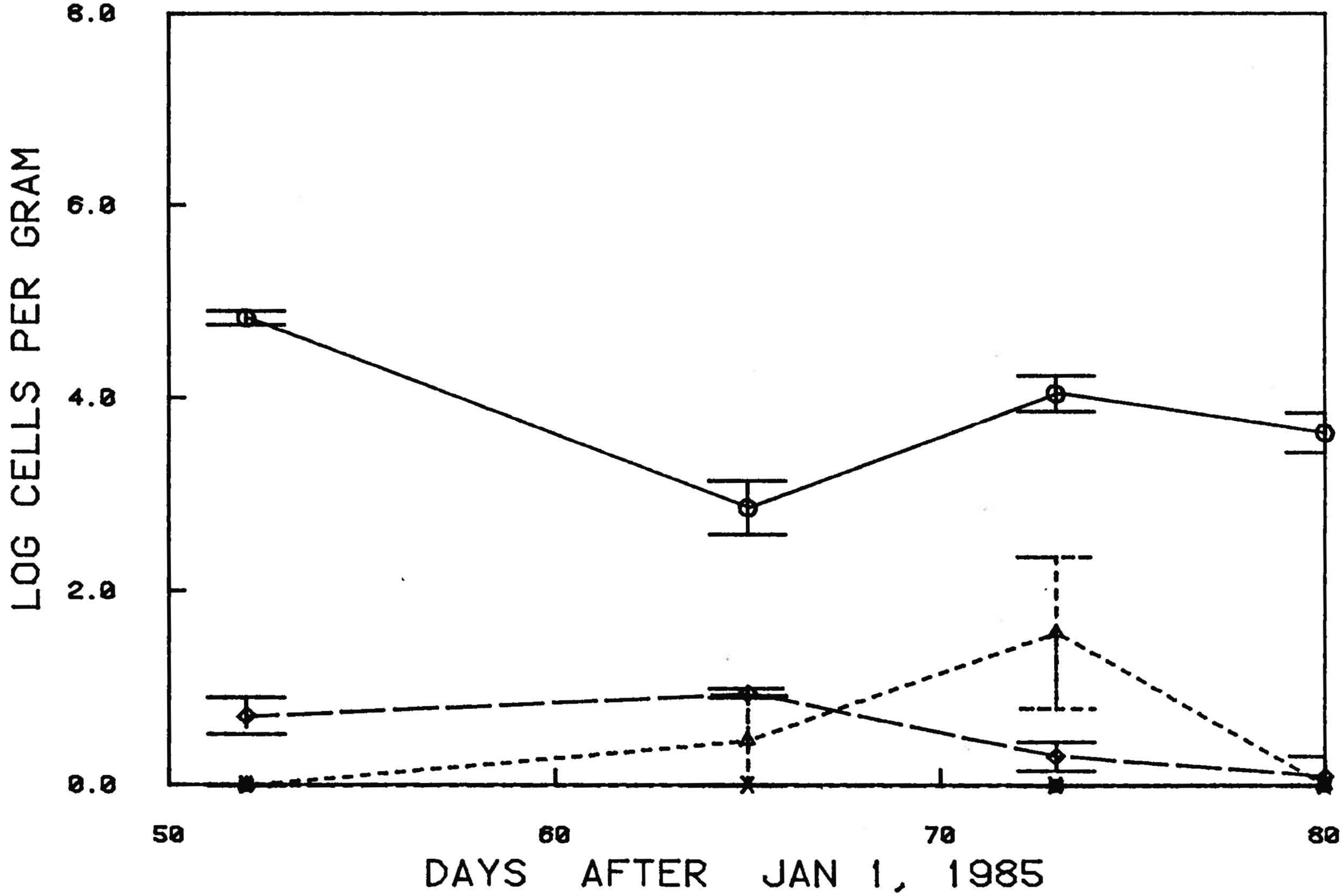
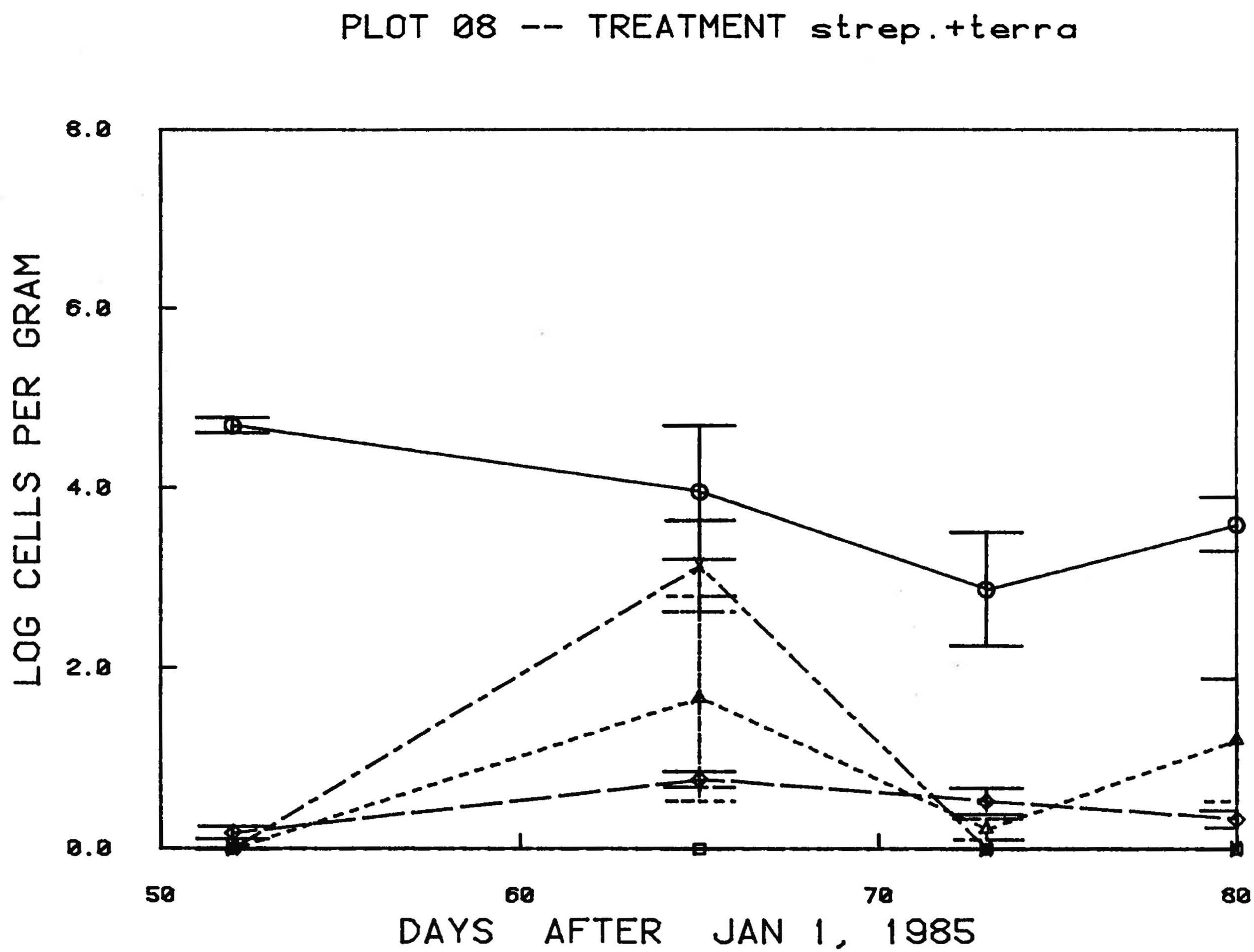
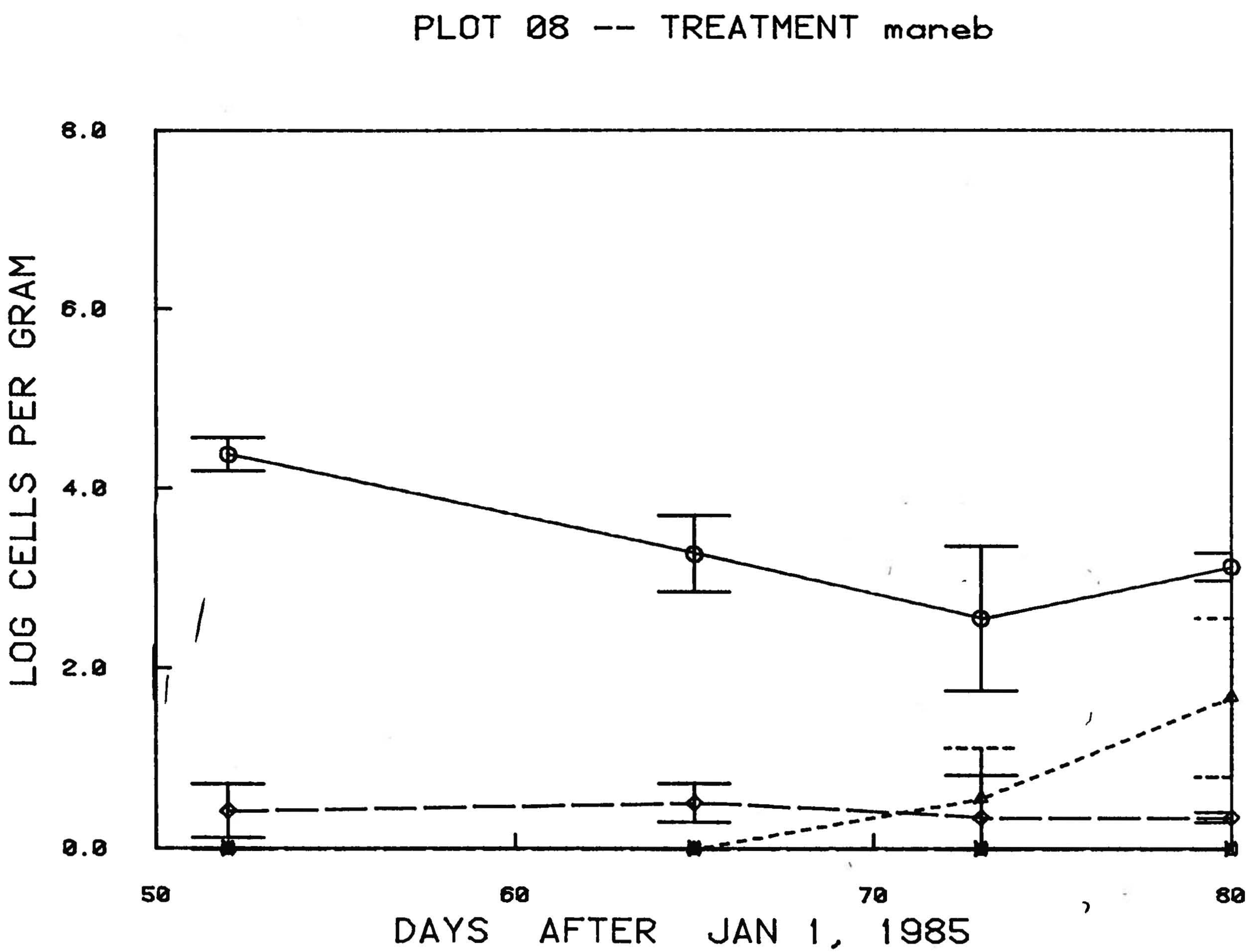


Fig 5



PLOT Ø8 -- TREATMENT strep.+terra
LOG CELLS PER GRAM
8.Ø
6.Ø
4.Ø
2.Ø
Ø.Ø
5Ø
6Ø
7Ø
8Ø
DAYS AFTER JAN 1, 1985

Fig 6



PLOT 08 -- TREATMENT maneb
LOG CELLS PER GRAM
8.0
6.0
4.0
2.0
0.0
50
60
70
80
DAYS AFTER JAN 1, 1985

Fig 7

PLOT 08 -- TREATMENT A526

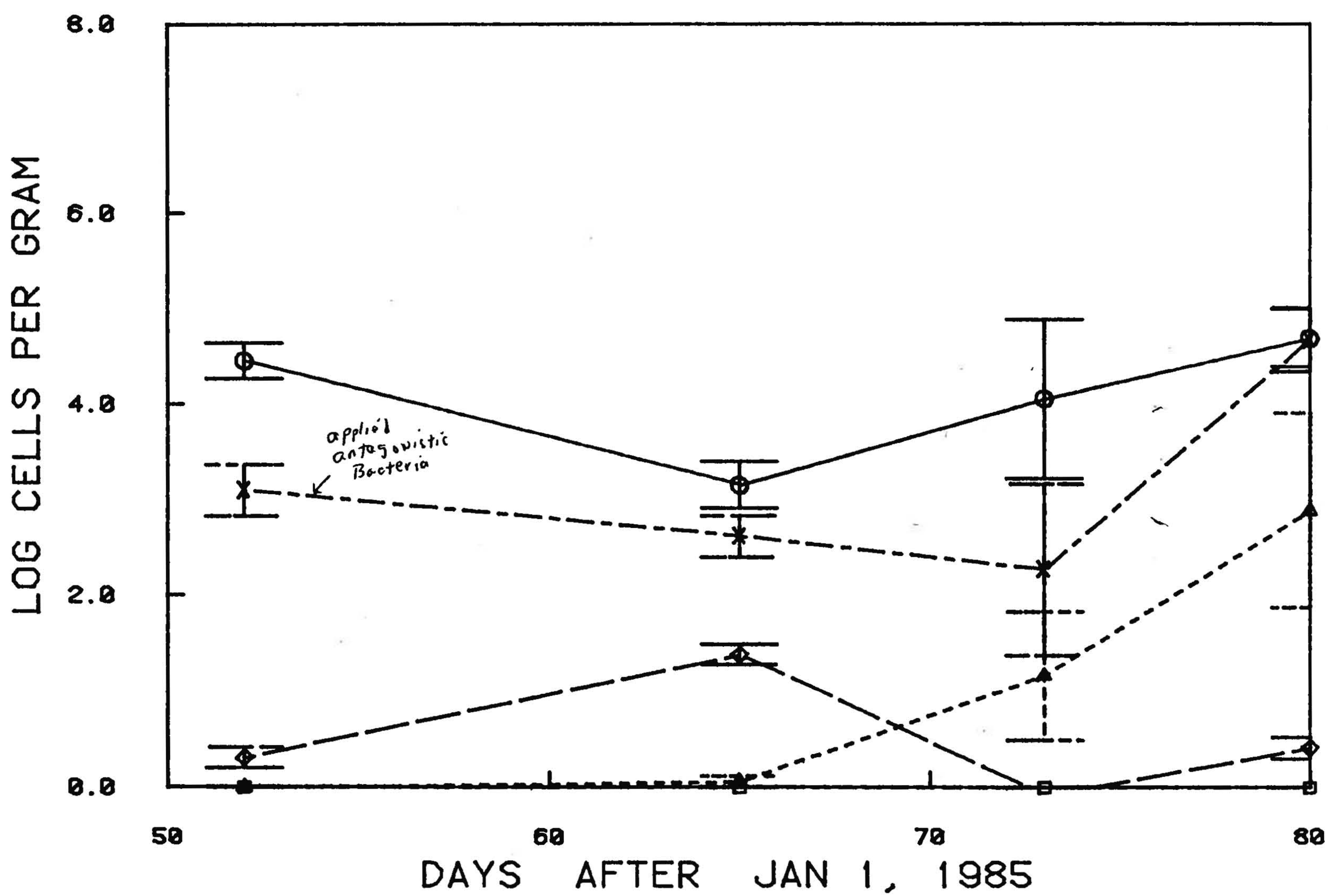
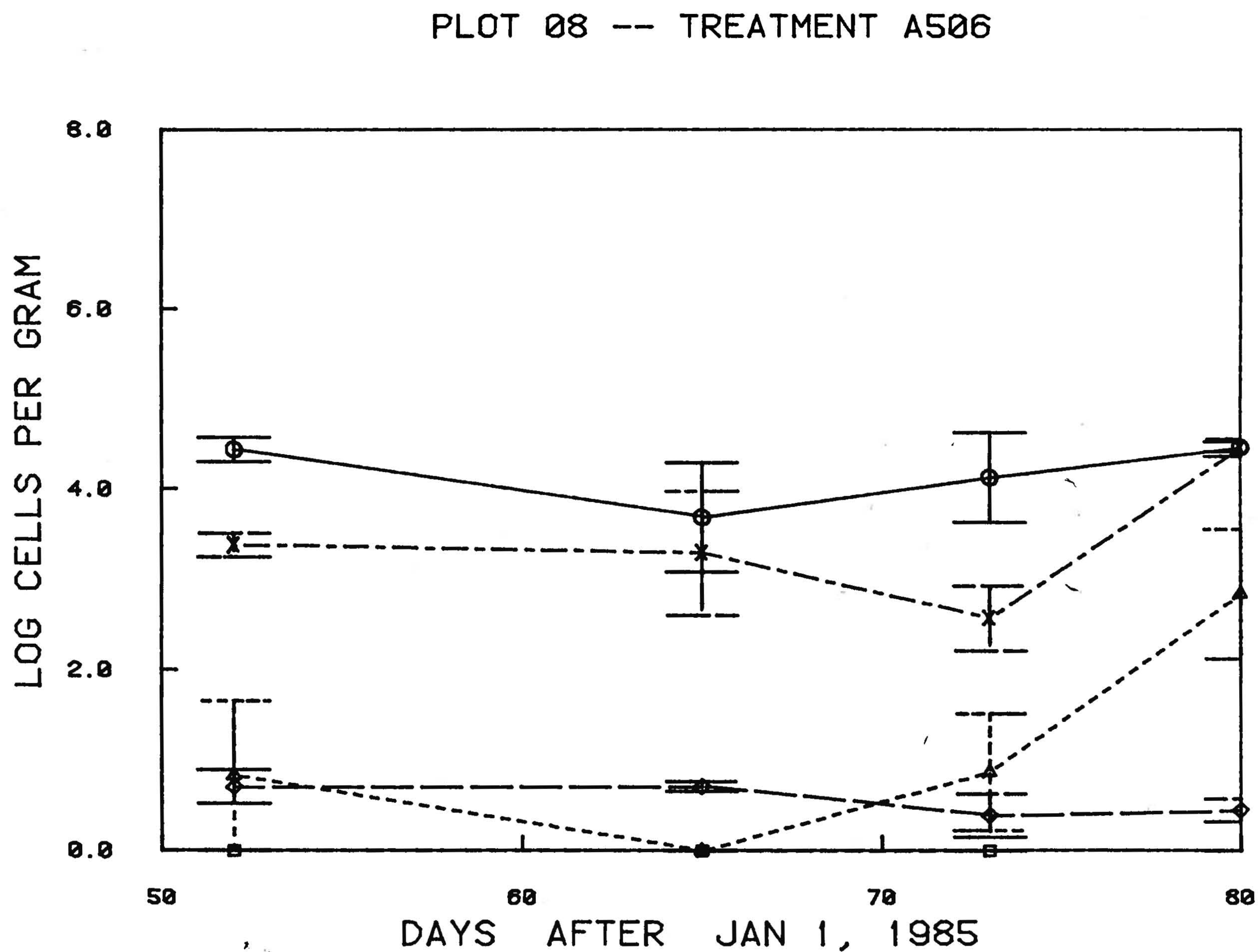


Fig 8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LOG CELLS PER GRAM
8.0
6.0
4.0
2.0
0.0
50
60
70
80
DAYS AFTER JAN 1, 1985

Fig 9

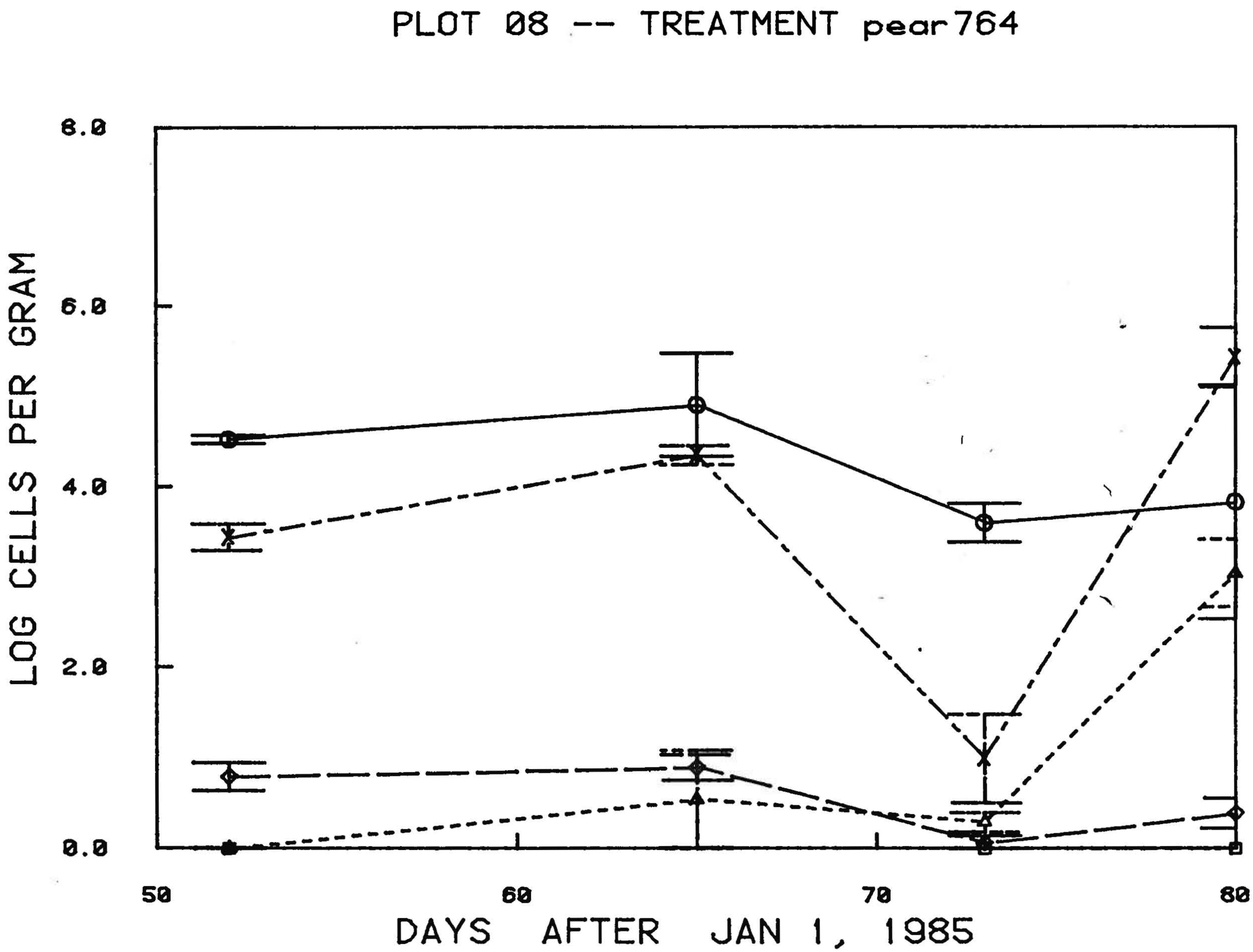
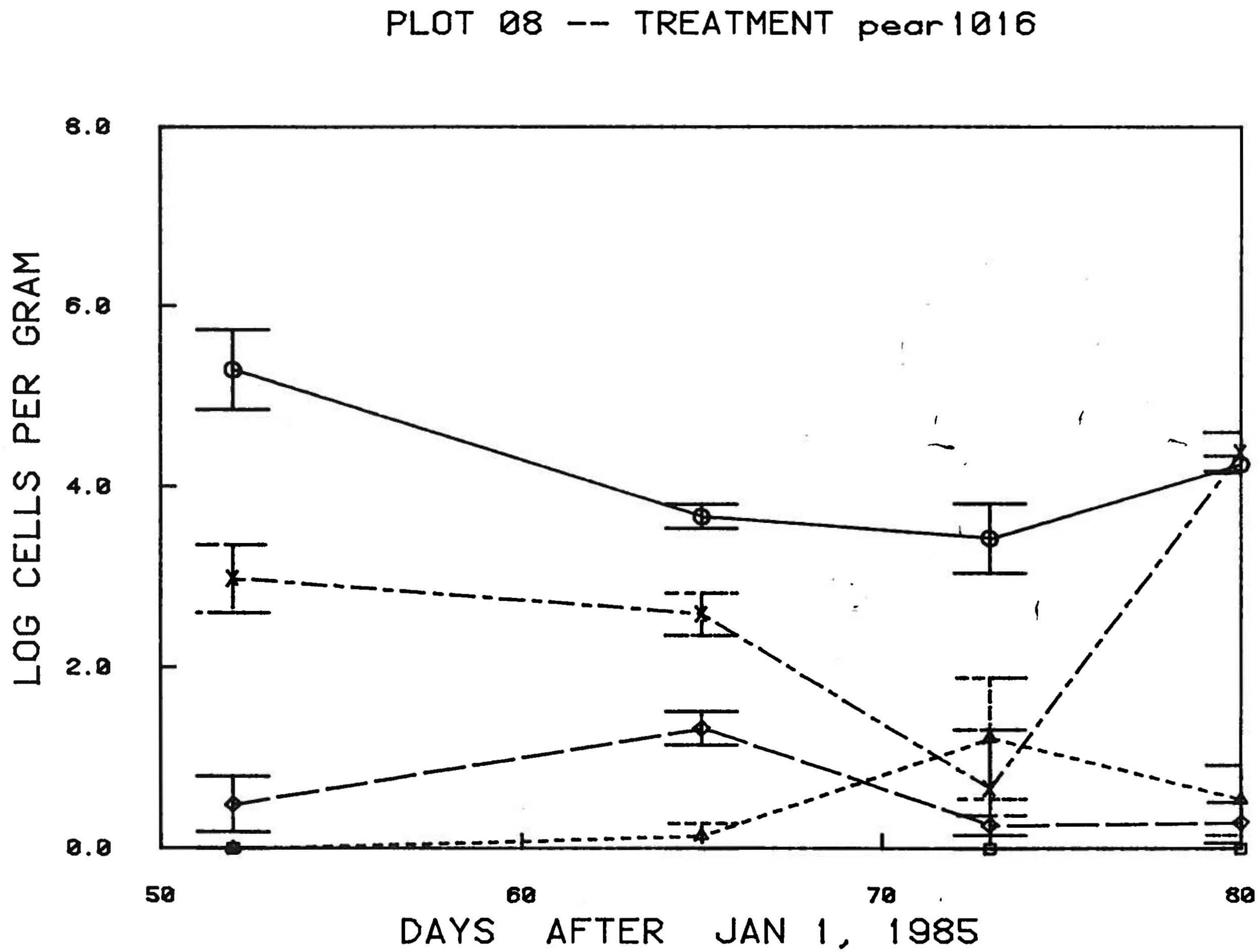
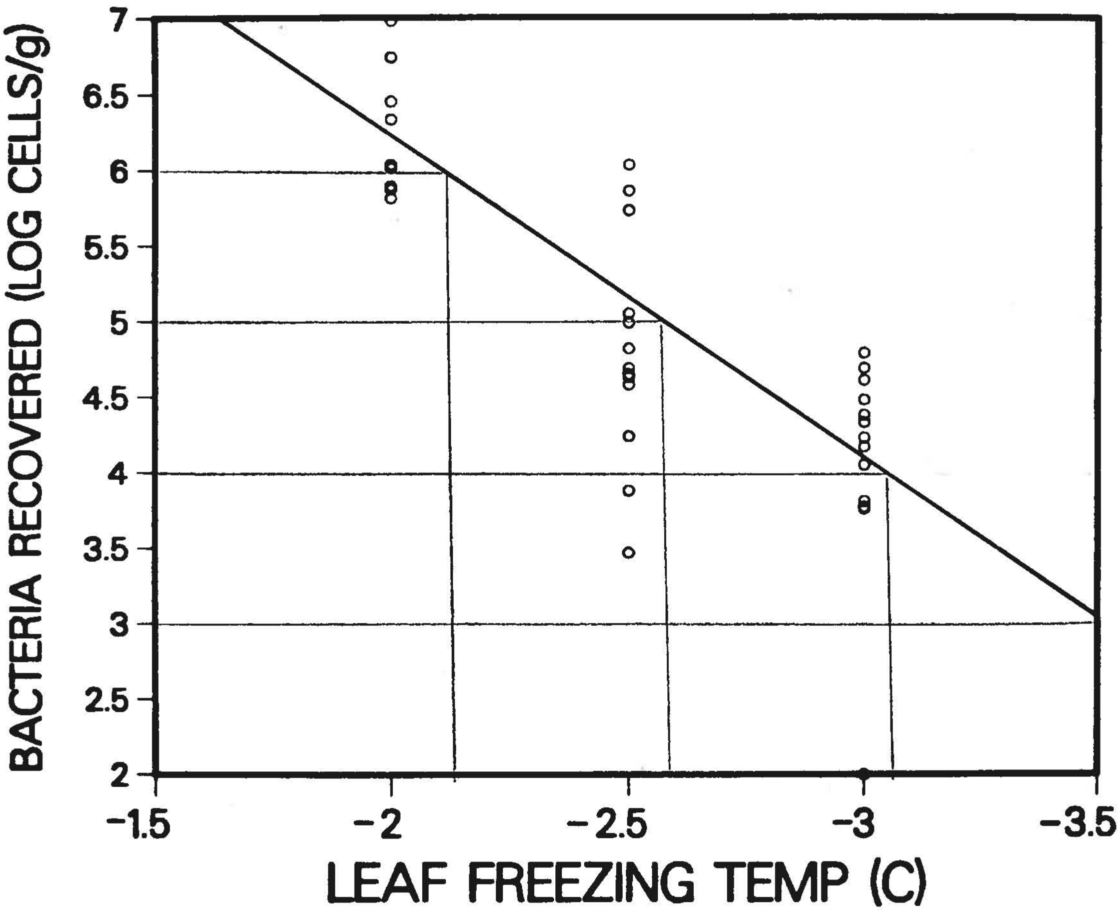

PLOT 08 -- TREATMENT pear764
LOG CELLS PER GRAM
8.0
6.0
4.0
2.0
0.0
50
60
70
80
DAYS AFTER JAN 1, 1985

Fig 10



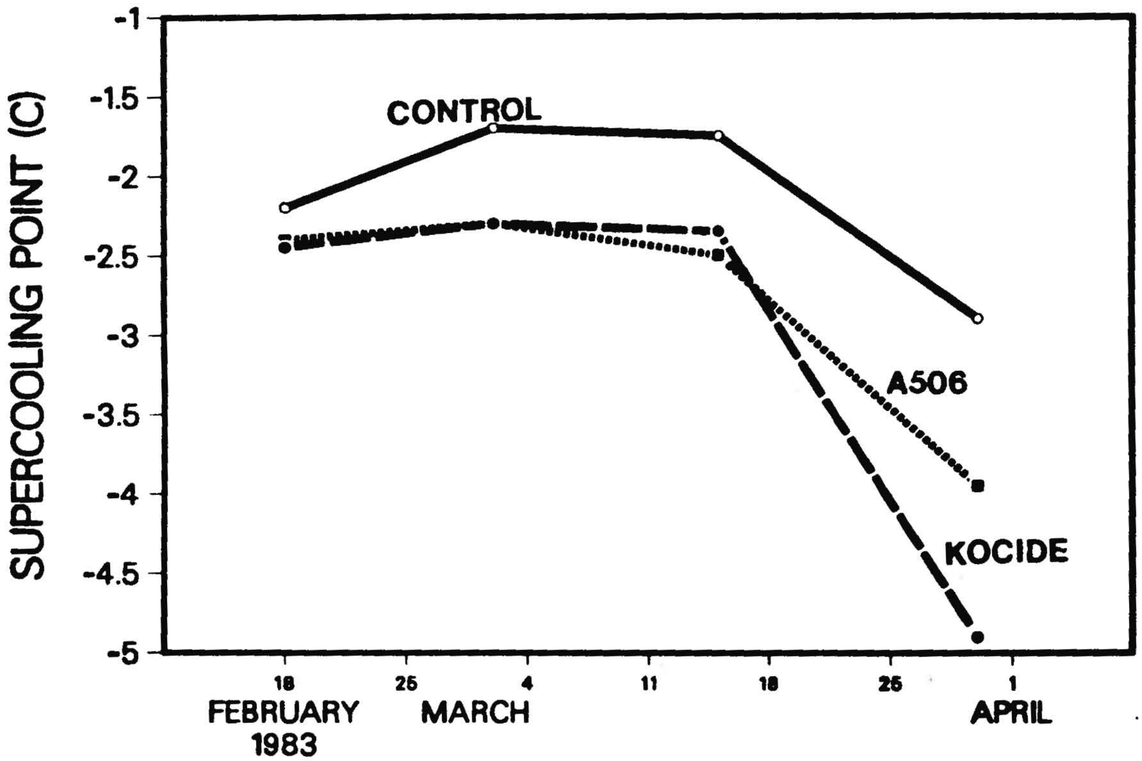
PLOT 08 -- TREATMENT pear1016
LOG CELLS PER GRAM
0.0
2.0
4.0
6.0
8.0
50
60
70
80
DAYS AFTER JAN 1, 1985

Fig 11



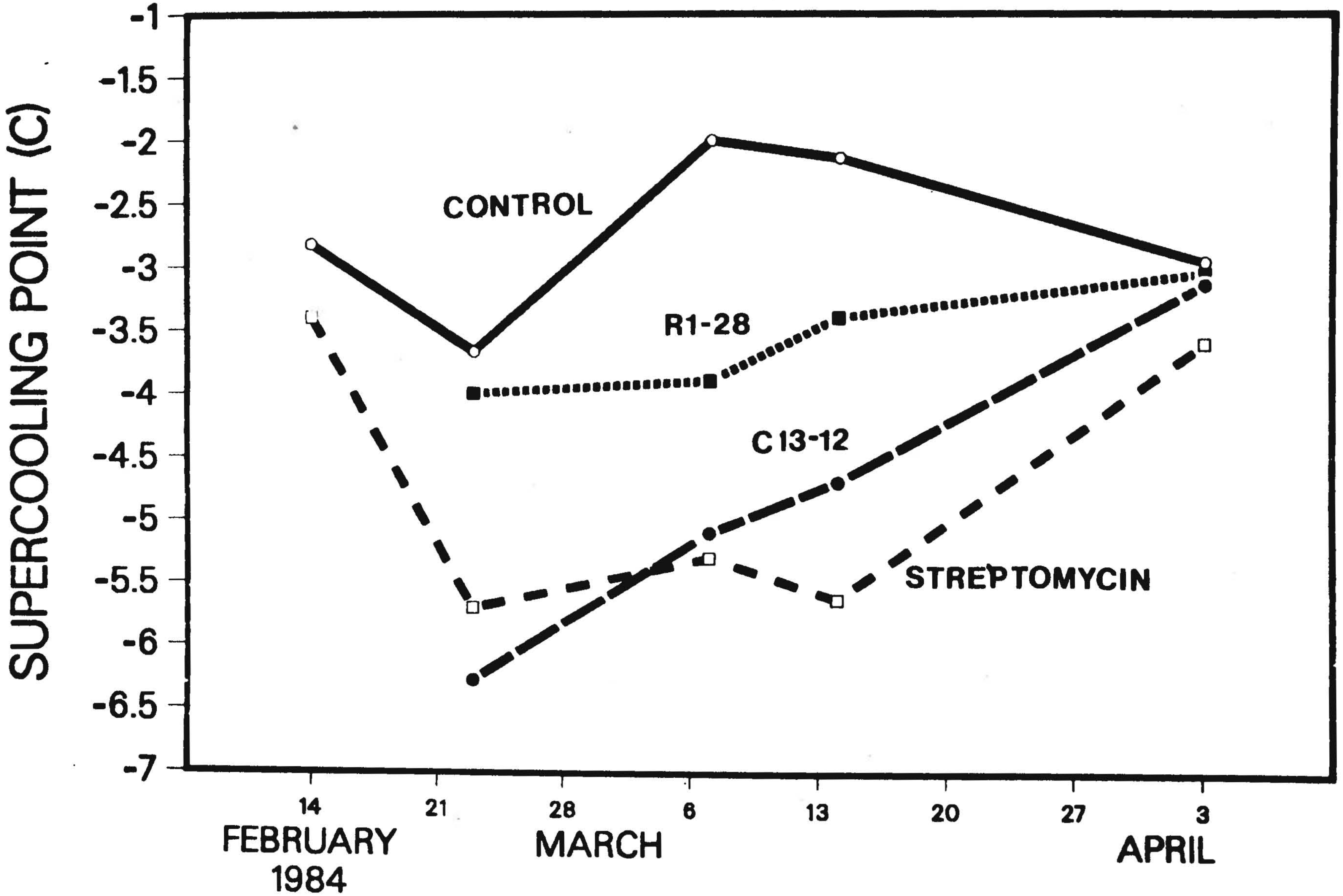
BACTERIA RECOVERED (LOG CELLS/g)
7
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
-1.5
-2
-2.5
-3
-3.5
LEAF FREEZING TEMP (C)

Fig 12



THESE GRAPHS GOOD

Fig 13



SUPERCOOLING POINT (C)
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
-5
-5.5
-6
-6.5
-7
CONTROL
R1-28
C 13-12
STREPTOMYCIN
14
21
28
6
13
20
27
3
FEBRUARY
1984
MARCH
APRIL

Table 1

Bacterial populations and number of ice nuclei on almond treated with Kocide 101 with and without Maneb and ziram -- March 20, 1985

Treatment	Bacterial Populations (log cfu/g fr. wt) Total	Ice Nucleation Active	Ice Nuclei (log nuclei/g) -5°C	-9°C
Control	5.16	4.47	1.63	2.17
Kocide + Maneb	4.49	2.02	-0.01	0.23
Kocide + Ziram	4.57	0.29	0.00	-0.18
Kocide + Ziram + Maneb	4.22	0.52	0.00	-0.10
Kocide only	3.88	1.33	-0.27	-0.19
F (treatment)	2.86	5.03	5.27	7.74
LSD 5%	0.98	2.25	1.01	1.09

Table 2

Pseudomonas syringae populations remaining after treatment of plants with Copper compounds and bis-dithio-carbamate fungicides

Chemical Treatment	Bacteria Recovered (log (cells/g fr. wt.)) Strain Glenn 22	Strain B728a
H_2O	6.86 a	6.85 a
Kocide 101	6.67 ab	6.59 ab
Kocide + Ziram	6.57 abc	6.45 abc
Kocide + Dithane M22	6.16 bc	5.13 ef
Kocide + Dithane M45	5.44 de	4.55 f
Basic Copper Sulfate	5.34 de	5.92 cd

Means followed by the same letter do not differ ($P<.05$) by Duncan's multiple range test.

Table 3. Populations of total and ice nucleation active bacteria on almond spurs collected on March 3, 1985 from Fresno, CA

Treatment	Bacteria Recovered (log cells/g fr. wt.) Total	Ice nucleation active
Kocide 101 + Maneb	3.94	0.74 A
Kocide 101	3.24	0.55 A
Control	4.39	2.44 A
F (treatment)	1.50	3.00
LSD 5%	1.46	1.85