Project No. 80-02
(Continuation of Project No. 79-S1)

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Project: Tree and Crop Research
Frost Epidemiology & Injury Control

Objectives:

1. To investigate epidemiology of colonization of almond leaves and flowers with ice nucleation bacteria, including assessment of sources of inoculum and species and population of ice nucleation active (INA) bacteria predominating on leaves.

2. To investigate cultural practices which favor establishment of INA bacteria on almond leaves and therefore increase frost sensitivity.

3. To evaluate possible methods of application of antagonistic bacteria for use as biological control agents of frost injury.

4. To determine most effective bactericides, antagonistic bacteria and INA bacteria inhibitors to control frost injury, and environmental parameters which influence their effectiveness.

Progress: Frost damage was significantly reduced in preliminary field trials in 1979 using either bactericides or bacterial ice nucleation inhibitors. Evaluations were made of three different bactericides and three bacterial nucleation inhibitors which, unlike bactericides, appear to nearly immediately reduce the frost sensitivity of the plants to which they are applied. Although no natural frost occurred in the plot area (minimum air temperature of 31 degrees F), encouraging results were obtained from laboratory analysis of the numbers of bacteria active in ice nucleation (primarily found to be strains of the blast organism *Pseudomonas syringae*), the actual numbers of ice nuclei contributed by these bacteria on leaves and flowers, as well as the frost sensitivity of detached flowering or fruiting spurs of two almond varieties. Unsprayed trees were found to be colonized with very high populations of *P. syringae*. Peak bacterial populations were found during the first week of April, reaching over ten million bacterial cells per fruit spur. All three bactericides significantly reduced both the numbers of bacteria on leaves and flowers and the number of ice nuclei; reductions in frost damage were significantly correlated with the extent of reduction of bacterial populations. Copper containing fungicides registered for use on almond appear very promising as potential frost control agents based on initial field trials. Three different experimental bacterial ice nucleation inhibitors significantly reduced the number of ice nuclei on leaves and flowers of almond without actually killing the bacteria.

These results suggest that control of frost injury to almond may be controlled by use of either bactericides, some of which are already registered for use on almond or bacterial ice nucleation inhibitors which, although not registered for use on almond, may be granted exempt status and thus made available for use in the near future.

Another avenue of frost control which should be investigated involves the use of antagonistic bacteria to prevent establishment of ice nucleation active bacteria on almond, forming a basis for biological control of frost injury. Antagonistic bacteria were isolated from almond in 1979 and screened for effectiveness in greenhouse and controlled environmental chamber tests and will be evaluated in 1980 field tests, as well as chemicals which gave good control during 1979.

Plans:

1. To evaluate new bactericides and rates and frequency of application of existing bactericides in control of frost injury under field conditions.

2. To examine weeds and natural plants in the area of almond orchards as possible sources of ice nucleation active bacteria.

3. To 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.

4. To examine the normal bacterial microflora on leaves and select bacteria which produce compounds inhibitory to ice nucleation active bacteria.

    A. Evaluate these antagonists for effectiveness in reducing frost injury to plants grown in the greenhouse or artificially forced to bloom and frozen in controlled environmental chambers.

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

5. In field trials evaluate chemicals shown to reduce the nucleation activity of bacteria in laboratory tests.

    A. Determine the most effective rates of these compounds in reducing frost injury and in eliminating ice nuclei from leaf surfaces.

    B. Determine the optimal time before a frost for the application of these chemicals.

    C. Determine the presistence of reductions in frost sensitivity of almond following application of nucleation inhibitors.

Almond Industry Participation $5,100

A second plot was established in Fresno County just east of the city of Fresno. Dormant applications of Kocide 101 as well as a maintenance spray of Kocide starting at first bloom were evaluated as well as a mixture of streptomycin and oxytetracycline. Na_2CO_3 and a mixture of Hyamine 2389 and Na_3PO_4 were also applied as nucleation inhibitors just prior to an anticipated frost during March. Samples were taken from experimental trees in both plot areas on a weekly basis to evaluate both the bacterial populations of various kinds on the leaves and flowers as well as directly determining the numbers of ice nuclei associated with these plant parts, as well as the frost sensitivity of the plants treated with either bactericides or bacterial nucleation inhibitors. Bacterial populations were measured by detaching leaves and flowers, washing the bacteria from the leaves and flowers in sterile buffer solution followed by dilution planting of the leaf washings onto suitable agar medium which was then followed by enumeration of the bacterial colonies on this medium, subsequently followed by a procedure known as a replica freezing technique which allows determination of that subset of the bacterial population which is active in ice nucleation. The numbers of ice nuclei associated with these plant materials were also determined by concentrating by centrifugation an aliquot of the leaf washing and determination of the ice nucleus concentration by a procedure known as the droplet freezing assay, in which a solution is divided into a large number of small droplets which are frozen at a given temperature, typically at -5 C. The numbers of ice nuclei associated with these droplets can be determined by a defined function from the number of droplets which remain unfrozen. A second technique involved freezing of large numbers of discs of leaf or flower material which were submerged in small droplets of water and cooled to various temperatures to determine the nucleation point of the plant tissue. Since no natural frost was observed during 1979, supplemental data on the frost sensitivity of these plant parts was determined by detaching fruit spurs, including leaves, flowers and young nutlets, bringing them to the laboratory and freezing in a controlled environmental chamber at given temperatures, typically 25 F.

IV. Results

Monitoring of the bacterial populations on unsprayed control trees throughout the 1980 frost season allowed us to determine the importance of various ice nucleation active bacteria in their role as determining frost sensitivity. Total numbers of bacterial populations increased from approximately 10^4 bacteria/g fresh wt. of leaf and flower material on about March 5 to in excess of 1 million and approximately 10 million bacteria/g fresh weight by mid-April falling again to about 2 x 10^5 bacteria/g by mid-May. Thus the seasonal fluctuation in bacterial populations active in nucleation seemed to reach a maximum at the time of maximum frost sensitivity of these plants. The populations of ice nuclei associated with the leaf material in unsprayed control trees also paralleled the increase and decrease in numbers of bacteria on these leaf surfaces, increasing from less than 5 nuclei/g fresh weight early in March to in excess of 1000 nuclei/g fresh weight in mid-April, again falling to less than 100 nuclei/g fresh weight in mid-May. All bactericides effectively reduced populations active in ice nucleation. Kocide 101 was nearly as effective as a mixture of Streptomycin and oxytetracycline in reducing bacterial populations. Total numbers of bacteria present on leaves sprayed either with the Kocide or the Streptomycin/oxytetracycline mixture were typically less than about 10^6 cells/gm fresh weight throughout the growing season. More importantly, bacterial populations active in ice nucleation were lower than about 10^3 bacteria/gm fresh weight at all times during the growing season and thus were found to be more than one thousand-fold lower than unsprayed control trees. The numbers of ice nuclei associated with leaf surfaces was also reduced approximately one thousand-fold by application of either Kocide 101 or Streptomycin and terramycin. Three of 5 antagonistic bacteria successfully colonized almond flowers, leaves and nuts during late February, March and April. One antagonist comprised about 10% of the total bacteria present on treated trees. Populations of ice nucleation active bacteria were reduced on trees treated with antagonistic bacteria. Reductions in populations of ice nucleation active bacteria was directly related to the populations of antagonistic bacteria colonizing a tree at a given time. Neither of the three bacterial nucleation inhibitors reduced the populations of viable bacteria associated with leaf surfaces. Total bacterial populations ranged from in excess of 10^6 to nearly 10^7 bacteria/gm fresh weight throughout the growing season and the numbers of bacteria active in ice nucleation increased from 1000 cells/gm fresh weight early in March to in excess of 10^6 cells/gm fresh weight in mid-April, again falling to approximately 10^3 cells/gm fresh weight by mid May. However, consistent with the mechanism by which these materials act, the numbers of nuclei associated with these bacterial populations were reduced. Nucleation inhibitors were applied only once during 1977 in mid-March at the Fresno plot; ice nuclei populations were reduced compared to unsprayed control trees for two weeks following the single application of these three nucleation inhibitors. Thus as expected, bacterial ice nucleation inhibitors did not affect the numbers of bacteria associated with plants but did reduce the numbers of nuclei associated with these plants and reduced the potential for damaging ice formation. Frost sensitivity of the treated plants was also evaluated during the same time as assays of bacterial populations. Injury was expressed as a fraction of fruiting spurs which sustained measurable frost injury at 24 - 25 F after exposure of 1 - 2 hours. Untreated control spurs typically sustained maximum amounts of frost injury throughout the growing season, typically in excess of 80% of the spurs being injured. Kocide 101 and the Streptomycin/oxytetracycline mixture were consistently most effective at reducing the frost sensitivity of these leaves. Frost damage was reduced to less than 30% injury at this temperature at many time points. A mixture of streptomycin and tetracycline were slightly more effective in reducing frost injury compared to Kocide at all times. Antagonistic bacteria (Figure 1), particularly isolate B7 and T7-3, significantly reduced frost injury compared to untreated control trees. Dormant applications of Kocide increased frost control when compared with maintenance applications of Kocide applied starting at bloom only. The mixture of streptomycin and terramycin again was slightly more effective than Kocide in reducing frost injury at the Fresno plot (Figure 2).

V. Discussion

The 1980 data indicate that Pseudomonas syringae is very effective at colonizing the leaves and flowers of almond. Because of this, frost sensitivity of almond can easily be understood. Several different control methods appear to be very effective in reducing the numbers of bacterial ice nuclei on leaf surfaces including both bactericides and antagonistic bacteria as well as bacterial ice nucleation inhibitors. Because frost sensitivity was reduced in the presence of either of these two types of materials, bacterial ice nuclei appeared to account for most if not all of the nuclei associated with the leaf surfaces of this plant.

Because of the extremely high populations of primarily P. syringae on leaves and flowers, control methods must be very effective at reducing either the numbers of these bacteria and/or the nucleation activity of these bacteria on leaves to achieve significant frost control. The extent of frost control will be dependent on, and is directly correlated with, the redutions of bacterial populations. It appears however, that commonly available bactericides when applied on an altered schedule compared with standard disease control, can offer significant frost control. Significant bacterial populations measured even early in March prior to significant bud growth, indicated that dormant applications of bactericides may be effective at reducing initial inoculum which may be important in determining large populations later in the season. For this reason, additional dormant applications will be evaluated in 1981 trials. Similarly, combinations of Streptomycin/oxytetracycline, Kocide or other bactericides will be evaluated in conjunction with bacterial nucleation inhibitors in an attempt to prolong the effect seen by those nucleation inhibitors. Preliminary tests outside of the plot area indicate that lower rates of Kocide than the maximum rate used in this plot area also significantly reduced bacterial populations. Further work will be initiated in 1981 to determine effective rates of application of bactericides. In addition, other bacterial nucleation inhibitors and other experimental and commercially available bactericides will also be evaluated.

Successful reductions of frost injury to almond using antagonistic bacteria also offer the promise of a very economical and self sustaining control measure for frost injury. Although bactericides are very effective at preventing bacterial growth on treated plant tissue, the rapid growth of almond during its period of maximum frost hazard necessitates the frequent application of these materials to provide adequate frost control. However, 3 of 5 antagonistic bacteria tested in 1980 were found to colonize almond tissue sufficiently to prevent colonization of their tissue with P. syringae. Because these antagonistic bacteria grow on leaf surfaces they continue to colonize the plant surface as they grow - eliminating the need for subsequent applications of either bacteria or chemicals. Laboratory work during 1980 has also determined that the primary mechanisms of antagonism by non-ice nucleation active antagonists is due to nutrient and/or site competition with ice nucleation active bacteria. Therefore, selection procedures have been devised that have allowed selection of even more effective antagonists, as indicated by greenhouse tests, than those evaluated during 1980. These will be tested in the field during 1981. Similarly, because P. syringae is particularly adept at colonizing almond trees, usually comprising over 90% of the total bacteria found on these plants, an antagonist will be developed from P. syringae. This strain will have come originally from almond but will no longer be active in ice nucleation, and will be evaluated in the field for control of frost injury.

VI. Publications

None during 1979.

Figure 1 and 2 Frost sensitivity of fruiting spurs of almond detached and frozen at -4 to -5 C at various dates during 1980 from the Turlaré and Fresno plots respectively.

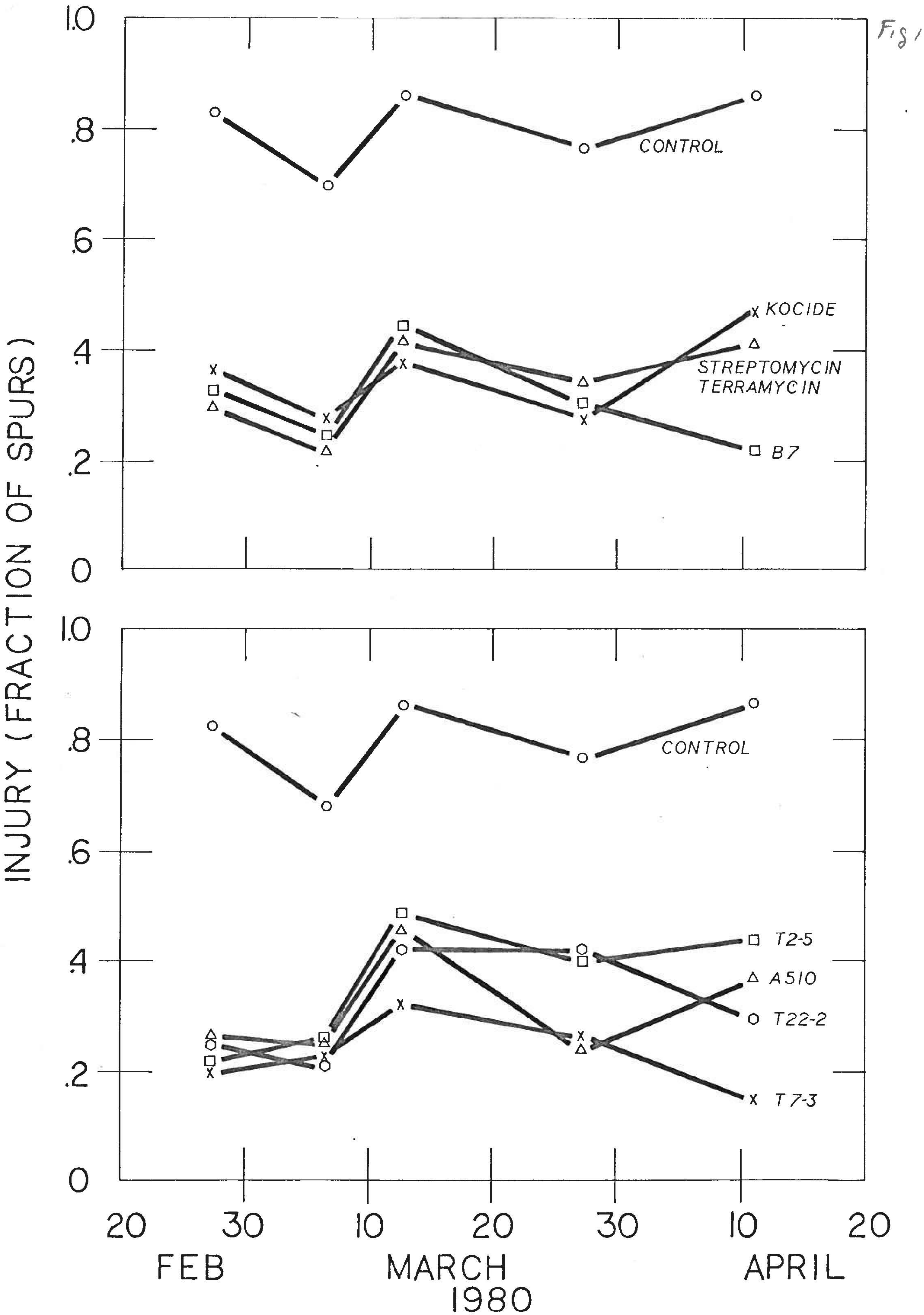
Fig 1
1.0
.8
.6
.4
.2
0
CONTROL
KOCIDE
STREPTOMYCIN
TERRAMYCIN
B7
INJURY (FRACTION OF SPURS)
CONTROL
T2-5
A510
T22-2
T7-3
20
30
10
20
30
10
20
FEB
MARCH
APRIL
1980

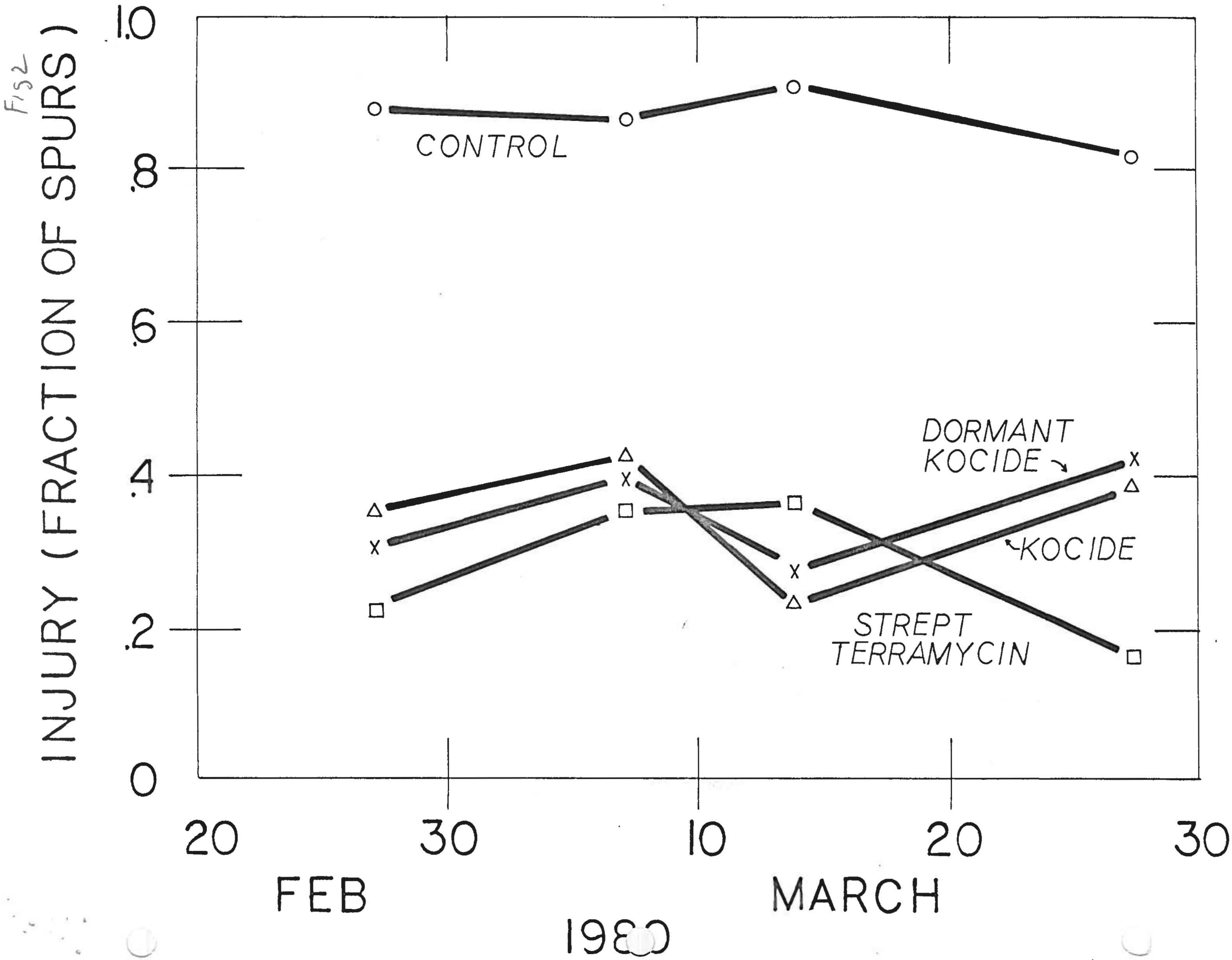

Fig 2
INJURY (FRACTION OF SPURS)
1.0
.8
.6
.4
.2
0
CONTROL
DORMANT
KOCIDE
KOCIDE
STREPT
TERRAMYCIN
20
30
10
20
30
FEB
MARCH
1980