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Biological Control of Verticillium Wilt and Seedling Diseases of Cotton (II)

Cotton Research and Development Corporation Project DAN 96C

Final Report

Australian Cotton Research Institute
NSW Agriculture, Narrabri

Summary

Background

As part of the previous Cotton Research and Development Corporation (CRDC) Project DAN 70C, rhizosphere and endophytic microorganisms were isolated from several Australian cotton growing regions and a large culture collection was established at the Australian Cotton Research Institute (ACRI). The culture collection was tested in pot and field experiments for biocontrol of cotton seedling diseases and Verticillium wilt. Several isolates reduced disease incidence significantly. The culture collection and the results obtained through the project DAN 70C form the basis of the current project DAN 96C.

Objectives of the Project

The objectives of the project were (1) to evaluate potential biocontrol agents for seedling disease control, (2) to evaluate potential biocontrol agents for the control of Verticillium wilt of cotton and (3) to develop and compare strategies for the biocontrol of Verticillium wilt. It was also proposed that some preliminary work would be carried out to investigate the feasibility of biocontrol methods for the control of black root rot, Fusarium wilt and Galathera syndrome.

A major development in the course of the project was the shift in the emphasis from research on biocontrol of Verticillium wilt to that on biocontrol of Fusarium wilt. By the 1995-96 cotton growing season it was clear that the varietal resistance was working effectively against Verticillium wilt. Fusarium wilt, although largely confined to Queensland, was becoming a concern to the NSW and Australian cotton industry.

Field experiments were conducted in 1995-96 in Qneensland on a commercial cotton farm to test the potential of biocontrol against Fusarium wilt. The results from the trials were very encouraging. One of the biocontrol agents almost doubled the plant survival and reduced disease severity by 42% compared to the non-treated control. Based on these results, the CRDC decided to expand the biocontrol testing program and provided additional financial support.

Results of research towards objective 1 – biocontrol of seedling diseases

A significant aspect of this research component was the development of a new method for rapid screening bacterial inoculants in field. The method consists of two main features, first, an inoculum applicator fitted to a tractor driven planter and second, a two-stage field experiment strategy which allows both screening and detailed studies to be carried out in the same season. Using this technique we screened one hundred and eighty bacterial inoculants and tested the promising candidates for performance consistency. A *Pseudomonas* sp. strain performed consistently as well as industry standard fungicide. (In our culture collection at ACRI this strain is designated 101-2b). In the four field experiments conducted, this strain increased seedling survival in all the four trials. One of these experiments, which was conducted on a commercial cotton farm could not be harvested at maturity due to unforeseen practical difficulties.

Of the three experiments that were harvested at maturity, the strain 101-2b increased yield in two experiments. Results for the fungicide treatment were the same as those for strain 101-2b.

Results of research towards objectives 2 and 3 – biocontrol of Verticillium wilt

As mentioned above, the major focus of the research on biocontrol of vascular wilt diseases was shifted to Fusarium wilt. Research on biocontrol of Verticillium wilt was limited to field testing potential biocontrol agents that were identified through pot experiments in the previous project. No consistent and effective field biocontrol agents could be found in these screening tests.

Results of research towards biocontrol of Fusarium wilt

Through a field-based screening and selection program, a handful of effective bacterial biocontrol agents for managing Fusarium wilt have been identified. Of these, a strain of *Bacillus* sp. performed consistently well. (In our culture collection at ACRI this strain is designated EP1025). In 1997-98 season, strain EP1025 was tested on six different farms. The strain performed consistently well on three farms. Of the remaining three farms, disease severity was not adequate on two of the farms; on one farm we used a highly susceptible variety and the experiment was abandoned six weeks after planting.

Research into the mechanisms through which a biocontrol agent operates is essential for ensuring the robustness of, and enhancing disease control conferred by any biocontrol agent. Due to the urgent need to find a control measure for Fusarium wilt, we deferred work on biocontrol mechanisms to a future project. We focussed on testing the efficacy of strain EP1025 and integrating biocontrol with other disease management options. However, no other biocontrol options were available for managing the disease. For this reason we started researching on the utility of plant growth hormones, potassium, and systemic resistance inducers in controlling the disease.

Below are key findings of the work on biocontrol of Fusarium wilt.

- We found that 2-hydroxy benzoic acid (HBA), applied as sodium salt, was able to induce some degree of disease resistance in the cotton plant. A combination of biocontrol strain EP1025 and HBA provided greater degree of disease control than any of the two individual agents.

- Planting density of 20-25 plants m^{-1} resulted in higher plant stand later in the season.

- Higher concentrations of the inoculum of EP1025 elicited a greater degree of disease control.

- EP1025 worked effectively with Sicot 189 and Deltapearl, the two commonly used varieties on the Darling Downs.

Progress of Research Work

1. Objectives of the project and key outcomes

Objectives

- To evaluate potential biocontrol agents for seedling disease control.
- To evaluate potential biocontrol agents for the control of Verticillium wilt of cotton.
- To develop and compare strategies for the biocontrol of Verticillium wilt.
- Test feasibility of biocontrol of black root rot, Galathera syndrome and Fusarium wilt in preliminary experiments.

A major development in the course of the project was the shift in the emphasis from research on biocontrol of Verticillium wilt to that on biocontrol of Fusarium wilt. By the 1995-96 cotton growing season it was clear that the varietal resistance was working effectively against Verticillium wilt. Fusarium wilt, although largely confined to Queensland, was becoming a concern to the NSW and Australian cotton industry.

Key outcomes

- A technique for rapid screening of bacteria for selecting field-effective biocontrol agents has been developed.
- A Pseudomonas strain that works as effectively as industry standard chemical fungicides against seedling diseases has been identified.
- A Bacillus strain which reduces Fusarium wilt of cotton has been identified.
- The performance of the Bacillus strain is further enhanced by using a compound which induces systemic resistance in the plant.

2. Research activities

Screening and selection of biocontrol agents

Potential biocontrol agents are often selected on the basis of *in vitro* inhibition of the target pathogen, followed by growth chamber or glasshouse experiments, in the presence of host plant and target pathogen, which simulate field conditions. With the aim of delivering field-effective biocontrol agents faster, we took a different approach. We used field-based experiments for screening microorganisms and selecting biocontrol agents. The results were verified in bioassays.

We screened approximately 180 bacteria to select seedling disease biocontrol agents and 350 bacteria to select biocontrol agents for Fusarium wilt. For Fusarium wilt biocontrol agents our main focus was endophytic bacteria.

Testing the efficacy of biocontrol agents

Selected biocontrol agents were tested for their efficacy in further field tests. Efficacy experiments were carried out in multiple fields to ensure the inclusion of a range of edaphic factors, disease pressure and the naturally occurring genetic variation of the pathogen.

Selected seedling disease biocontrol agents were tested in four different fields. Fusarium wilt biocontrol agents were tested in six different fields.

In the efficacy testing experiments we also investigated the effect of the biocontrol agent on disease incidence and severity, plant growth, final yield and populations of microorganisms associated with the plant.

Integration of biocontrol methods with other disease control measures

We conducted experiments to test the potential of potassium, systemic resistance inducers and plant growth hormones in managing Fusarium wilt of cotton. We also investigated the interaction between these chemicals and biocontrol agents.

Population dynamics of inoculant bacteria and plant-associated microorganisms

In relation to seedling disease biocontrol, we investigated the population dynamics of inoculant bacteria and rhizosphere microorganisms using rifampicin resistant mutants of biocontrol agents. The purpose of this strand of experimentation was to determine the relationship between population levels of the biocontrol agent, other rhizosphere microorganisms and the degree of disease control.

Similar work on Fusarium biocontrol agents was severely limited due to the self-imposed ban on bringing samples from Fusarium infested areas into non-infested cotton growing areas.

Some samples from field experiments have been 'frozen' for analysis at a later date.

Our own experience and experience elsewhere in the world shows that antibiotic resistance is not a suitable marker for studying the ecology of endophytic bacteria (Kloepper, 1993). We conducted a limited amount of work, in collaboration with Cambia, to investigate the feasibility of using marker genes in for studying the ecology of biocontrol agents.

Mechanisms of biocontrol

Only preliminary work was carried out in this direction. Using transposons containing Gus maker, mutants deficient in antifungal compounds were generated.

3. Research methods

Methods for working with microorganisms

General microbiological methods were as per Gerhardt *et al.* (Methods for General and Molecular Bacteriology, 1994. American Society for Microbiology, Washington). Where necessary, modifications were made to suit the needs of the work. Endophytic microorganisms were used for developing biocontrol agents for Fusarium wilt.

Identification of bacteria and fungi was carried out using morphological tests, biochemical tests, carbon source utilisation tests and rDNA analysis.

Screening and selection of biocontrol agents

A new technique, which relies on field performance as opposed to laboratory and glasshouse assays, was developed for screening and selecting potential biocontrol agents (see Appendix 1). The results were then verified using bioassays.

Glasshouse and growth chamber experiments

Experiments were generally set up as randomised complete block designs. Experiments involving seedling disease control were usually conducted at temperature ranges of 17 – 23°C. No pot experiments involving *Fusarium* were conducted at the Australian Cotton Research Institute, Narrabri.

Field experiments

All field experiments were set up as randomised complete block designs. The number of replicates varied from 4 to 8. Screening experiments used small plots (8 to 15 m^2). In all the other experiments the plot size varied from 36 to 280 m2.

In some seedling disease experiments, the field soil was amended with *Rhizoctonia solani* to ensure disease pressure.

Inoculation treatments were applied either to seed or in furrow at planting. An inoculum applicator, specially built, was used for in furrow application of the inocula (see Appendix 1).

All Fusarium wilt experiments were conducted on commercial cotton farms in Queensland and NSW.

Plant growth measurements and a disease severity index were used as key criteria to measure the effectiveness of biocontrol methods. Data were collected for populations of microorganisms, disease incidence and severity, plant dry matter and final yield. In the case of small plot trials, and in some trials conducted on commercial farms, data for final yield could not be collected due to practical difficulties. In those cases, data for boll counts were recorded.

Statistical analysis

Results were analysed by ANOVA using Genstat 5 Release 3.2. REML analysis, taking the row and column effects into account.

4. Results and discussion

Research towards objective 1 – Biocontrol of cotton seedling diseases

A significant aspect of this research component was the development of a new method for rapid screening bacterial inoculants in field (Appendix 1). The method consists of two main features, first, an inoculum applicator fitted to a tractor driven planter and second, a two-stage field experiment strategy which allows both screening and detailed studies to be carried out in the same season. Using this techniques we screened one hundred and eighty bacterial inoculants and tested the promising candidates in further experiments for performance consistency.

Through screening experiments, a handful of bacteria were identified as potential biocontrol agents (Figure 1 and Appendix 1).

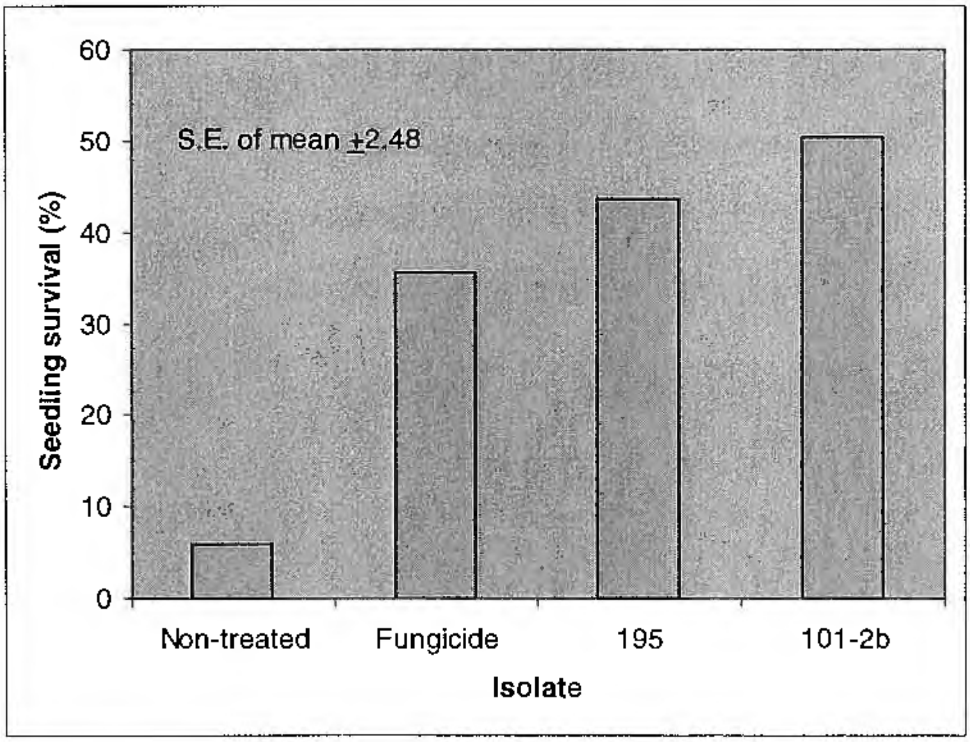

Figure 1. Field screening of bacterial isolates for identifying potential biocontrol agents. The experiment was conducted in a field amended with *Rhizoctonia solani* inoculum.

Further efficacy tests showed that a *Pseudomonas* strain, 101-2b, reduced seedling disease effectively. The degree of disease control conferred by 101-2b matched the degree of disease control provided by industry standard chemical fungicide (combination of apron and terrachlor) (Figures 2, 3, 4 and 5).

In the four field experiments conducted, this strain increased seedling survival in all the four trials. One of these experiments, which was conducted on a commercial cotton farm could not be harvested at maturity due to unforeseen practical difficulties. Of the three experiments that were harvested at maturity, the strain 101-2b increased yield in two experiments. Results for the fungicide treatment were the same as the ones for strain 101-2b.

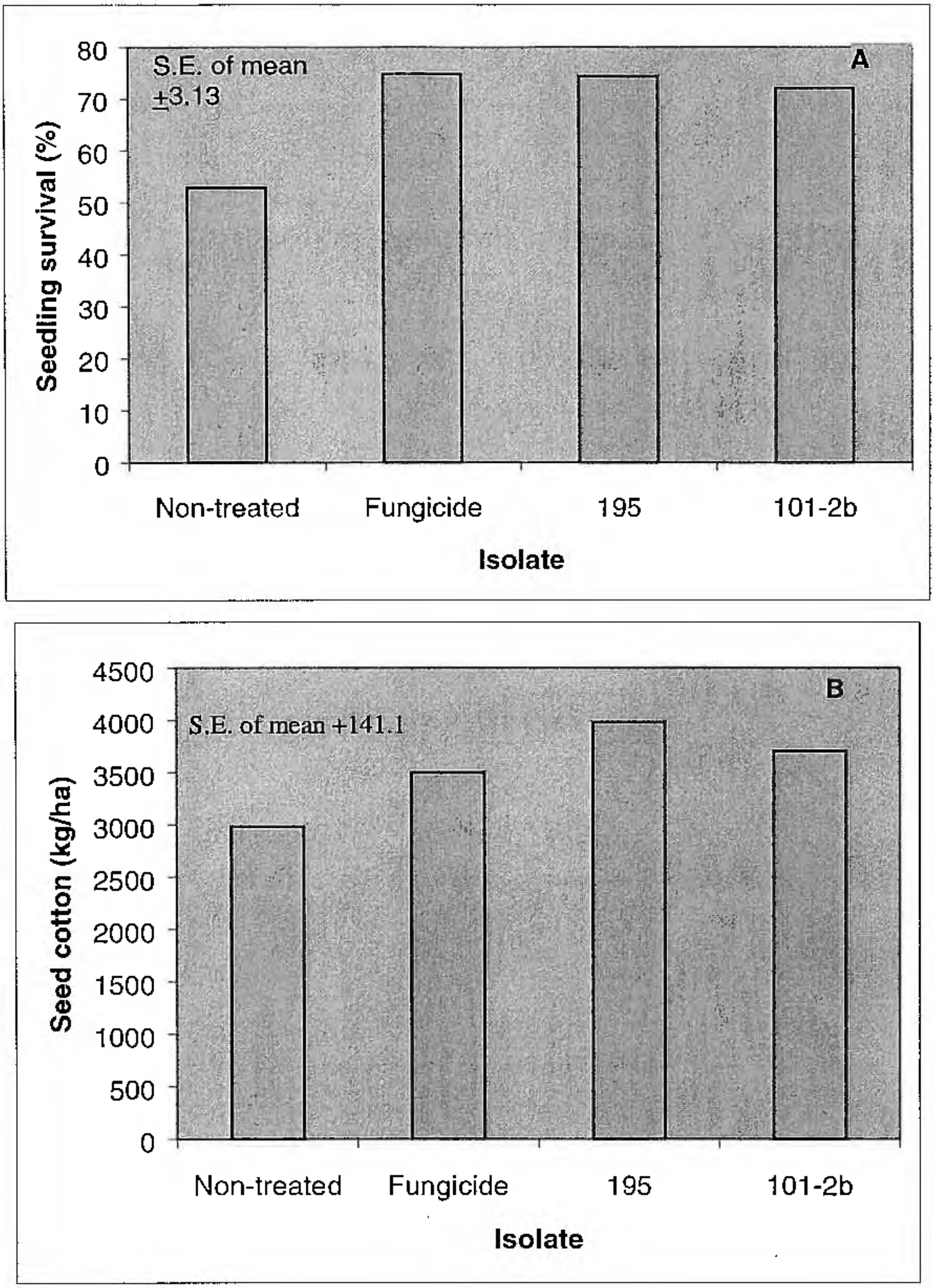

Figure 2. Effect of promising biocontrol agents on the survival of cotton seedlings at 6 weeks after planting. The experiment was conducted in Field 4, amended with Rhizoctonia solani inoculum. (A) Seedling survival and (B) Seed cotton yield.

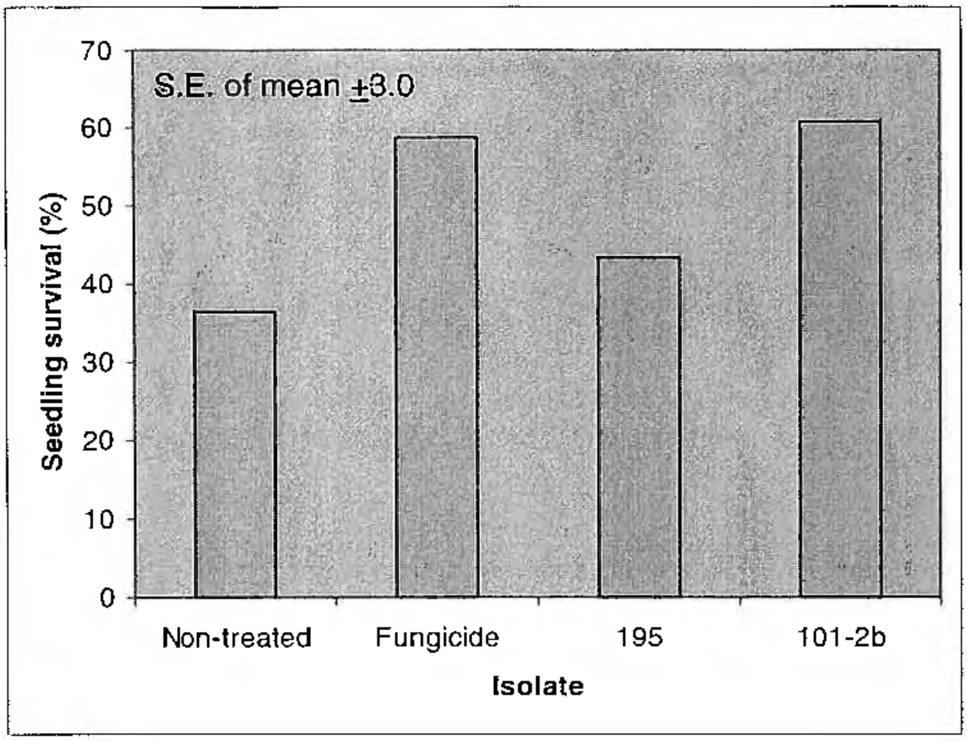

Figure 3. Effect of promising biocontrol agents on the survival of cotton seedlings at 6 weeks after planting. The experiment was conducted on a commercial cotton farm which was naturally infested with seedling pathogens. Final yield from the experiment could not be picked due to unforeseen circumstances.

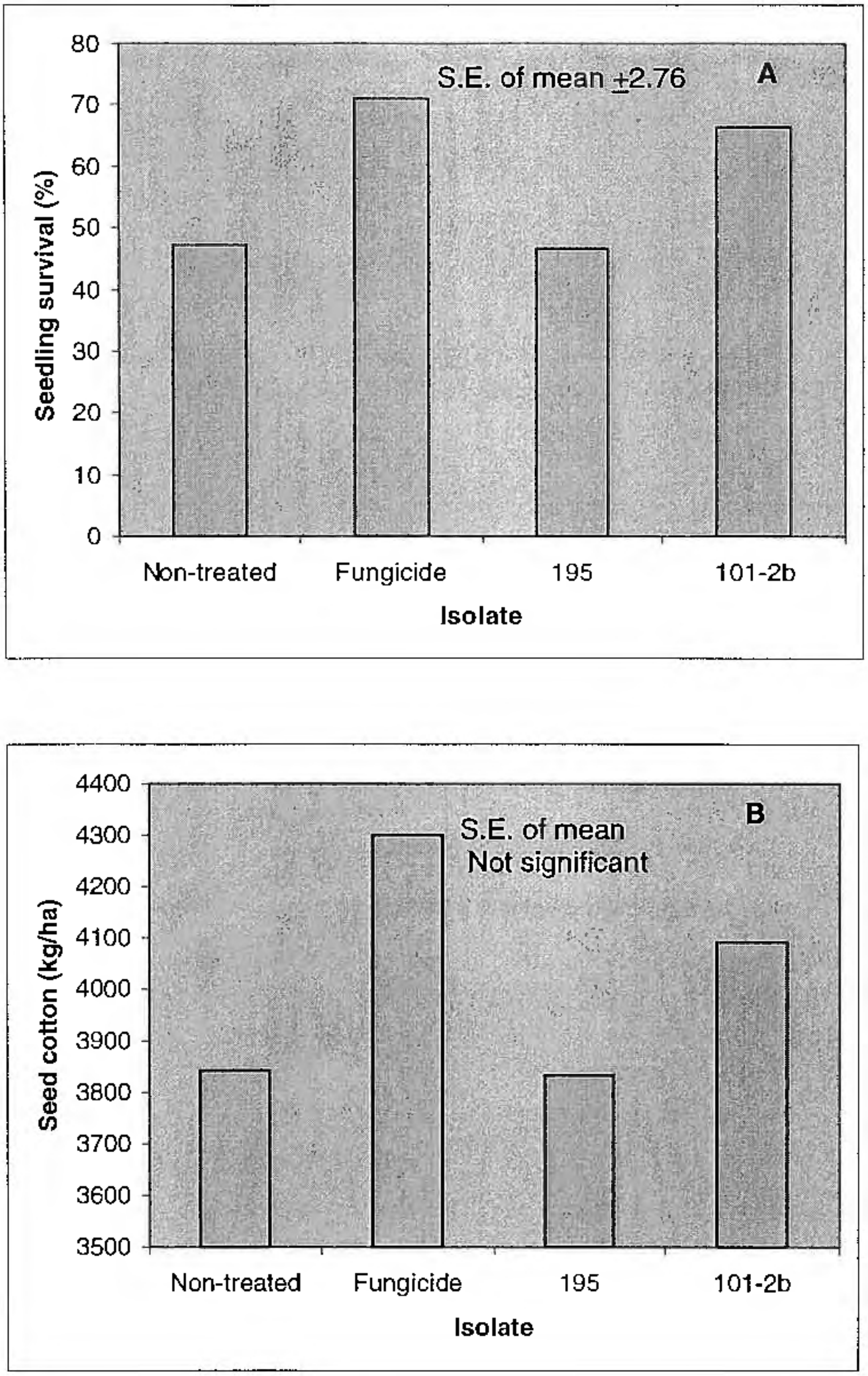

Figure 4. Effect of promising biocontrol agents on the survival of cotton seedlings at 6 weeks after planting. The experiment was conducted in Field 4. (A) Seedling survival and (B) Seed cotton yield.

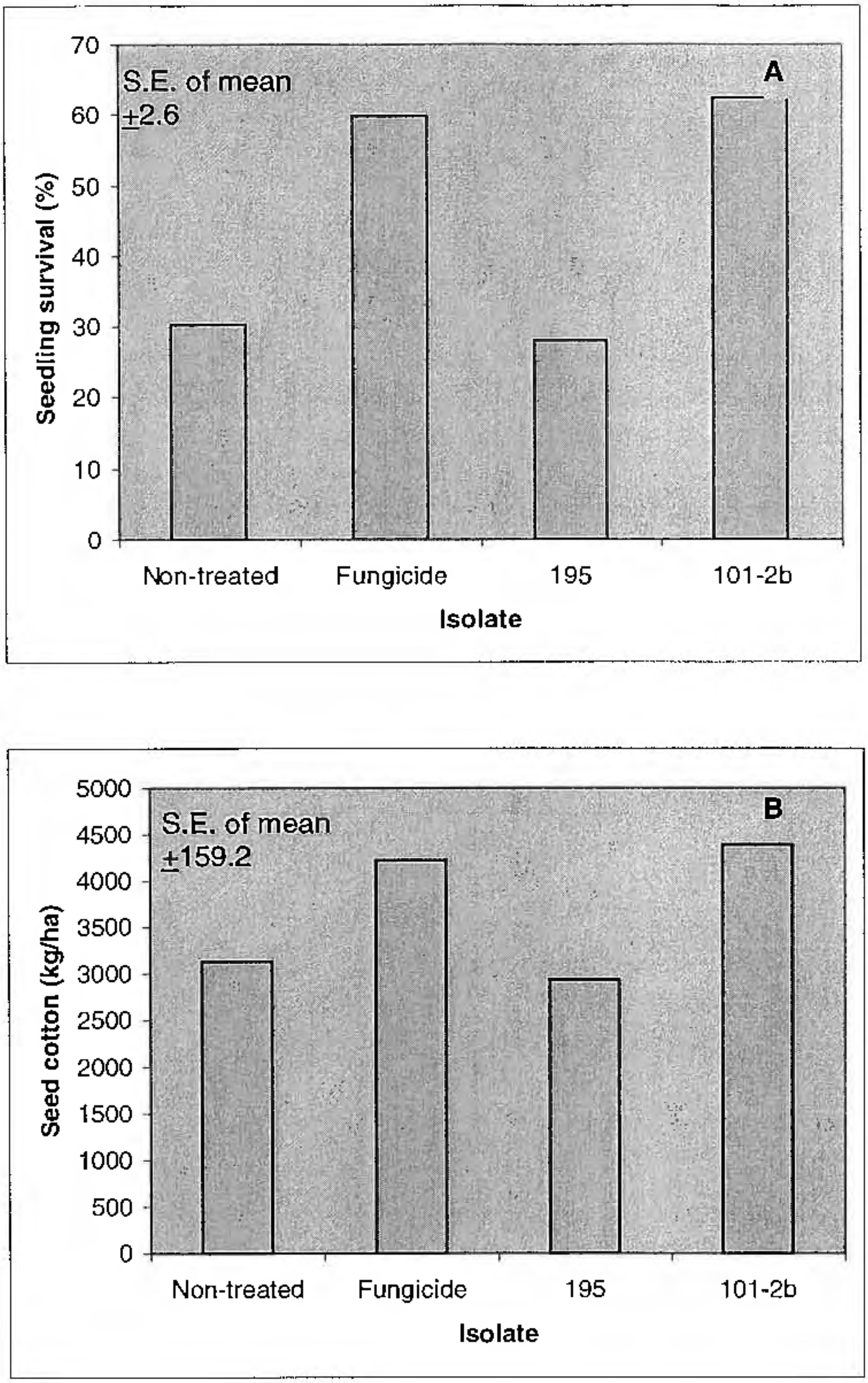

Figure 5. Effect of promising biocontrol agents on the survival of cotton seedlings at 6 weeks after planting. The experiment was conducted in field Old2. (A) Seedling survival and (B) Seed cotton yield.

Results of research towards objectives 2 and 3 – biocontrol of Verticillium wilt

As mentioned above, the major focus of the research on biocontrol of vascular wilt diseases was shifted to Fusarium wilt. Research on biocontrol of Verticillium wilt was limited to field testing potential biocontrol agents that were identified in pot experiments in the previous project. No consistent and field effective biocontrol agents could be found in these tests.

Research towards biocontrol of Fusarium wilt

Through a field-based screening and selection program, a handful of effective bacterial biocontrol agents for managing Fusarium wilt have been identified. Of these, a strain of Bacillus sp. (EP1025) performed consistently well.

In 1997-98 season, strain EP1025 was tested on six different farms. In-furrow application of liquid inoculum of this strain at planting reduced the impact of Fusarium wilt on three farms. Of the remaining three farms, disease severity was not adequate on two of the farms; on one farm we used highly susceptible variety and the experiment was abandoned six weeks after planting.

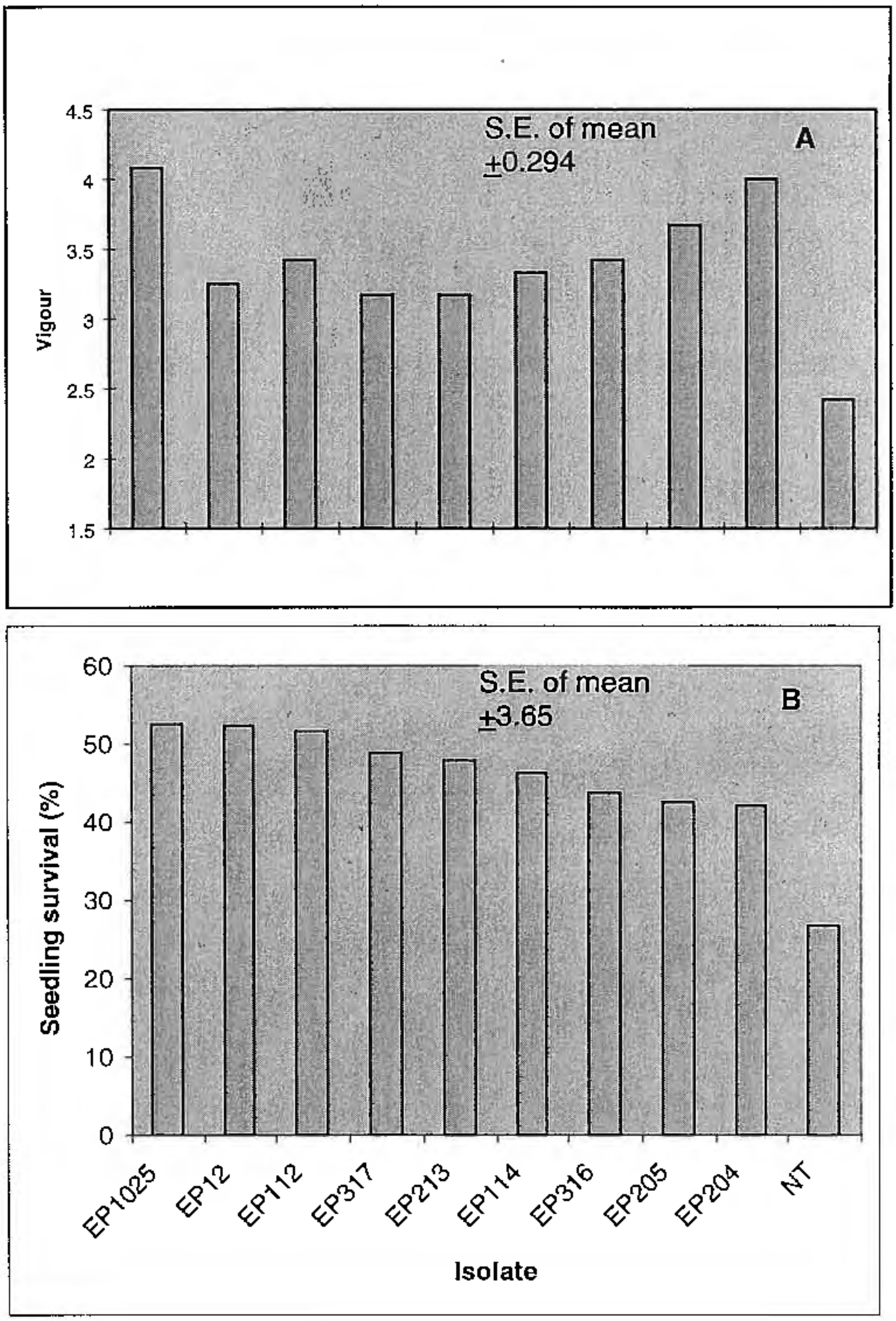

Figure 6. Screening of bacteria for the selection of biocontrol agents for Fusarium wilt. The experiment was conducted at Cowan, a commercial cotton farm in Queensland. (A) Plant vigour and (B). Seedling survival

Research into the mechanisms through which a biocontrol agent operates is essential for ensuring the robustness of, and enhancing disease control conferred by any biocontrol agent. Due to the urgent need to find a control measure for Fusarium wilt, we deferred work on biocontrol mechanisms to a future project. We focussed on testing the efficacy of strain EP1025 and integrating biocontrol with other disease management options. However, no other biocontrol options were available for managing the disease. For this reason we started researching on the utility of plant growth hormones, potassium, and systemic resistance inducers in controlling the disease.

We found that 2-hydroxy benzoic acid (HBA), applied as sodium salt, was able to induce some degree of disease resistance in cotton plant. A combination of biocontrol strain EP1025 and HBA provided greater degree of disease control than any of the two individual agents (Figure 7).

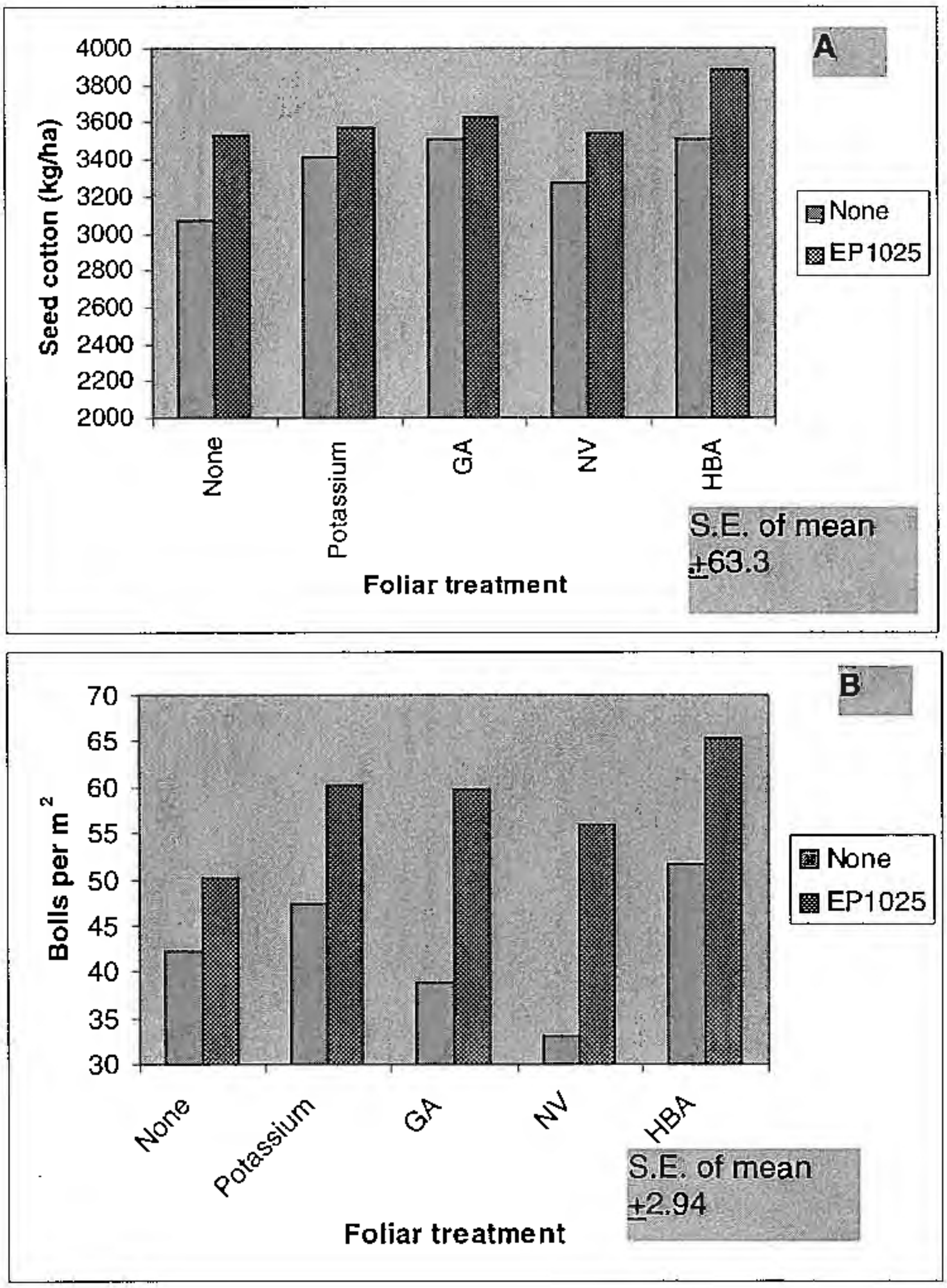

Figure 7. Interaction between EP1025 and, 2-hydroxy benzoic acid, gibberellic acid and potassium. A and B are two different farms. Final yields were not picked from the experiment on farm B. (GA-gibberellic acid, NV- a chemical systemic inducer supplied by Novartis, and HBA- 2-hydroxy benzoic acid).

In another experiment we examined the relationship between planting density (number of seed planted per metre) and plant survival (plants surviving per metre). Regression analysis of the data shows that planting density of 20 and 25 plants (m^{-1}) lead to higher plant stand later in the season (Figure 8). In-furrow application of EP1025 inoculum further enhanced plant survival (Figure 9).

In a separate experiment, we examined the relationship between the population levels of EP1025 in the inoculum and the degree of Fusarium wilt suppression. We tested the inoculum at three different levels- 10^4, 10^6 and 10^8 cfu/ml. All the three populations reduced the impact of disease significantly and increase seed cotton (Figure 10). Although the difference between the three inoculum levels were not significant, the seed cotton levels increased with increasing levels of EP1025 in the inoculum.

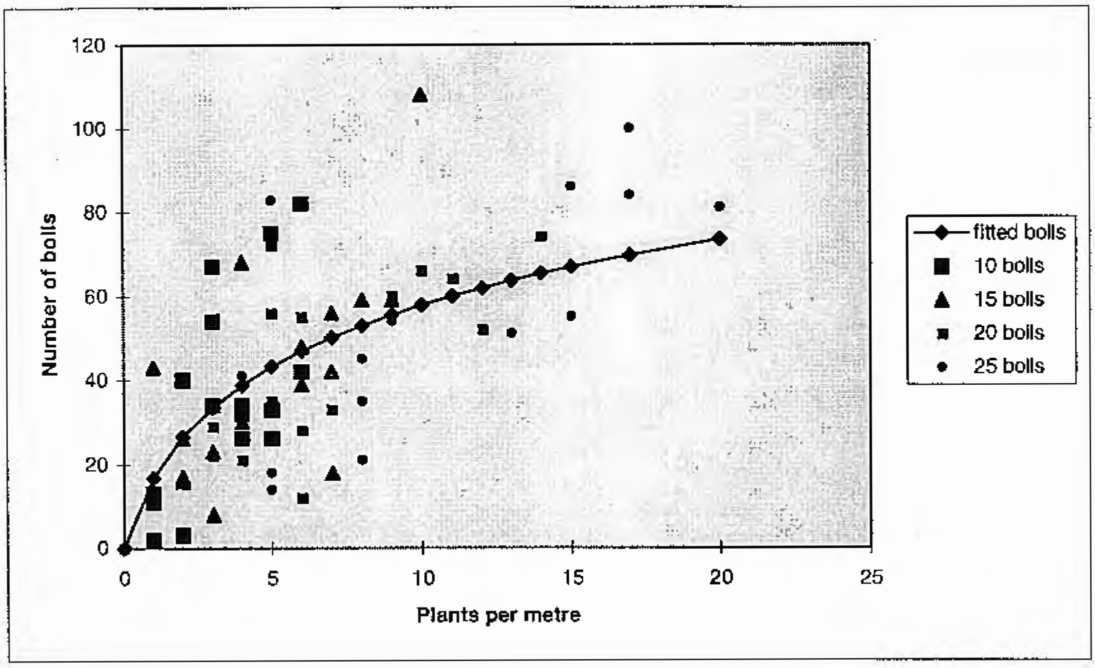

Figure 8. Relationship between planting density, plant survival and number of bolls in a Fusarium infested field.

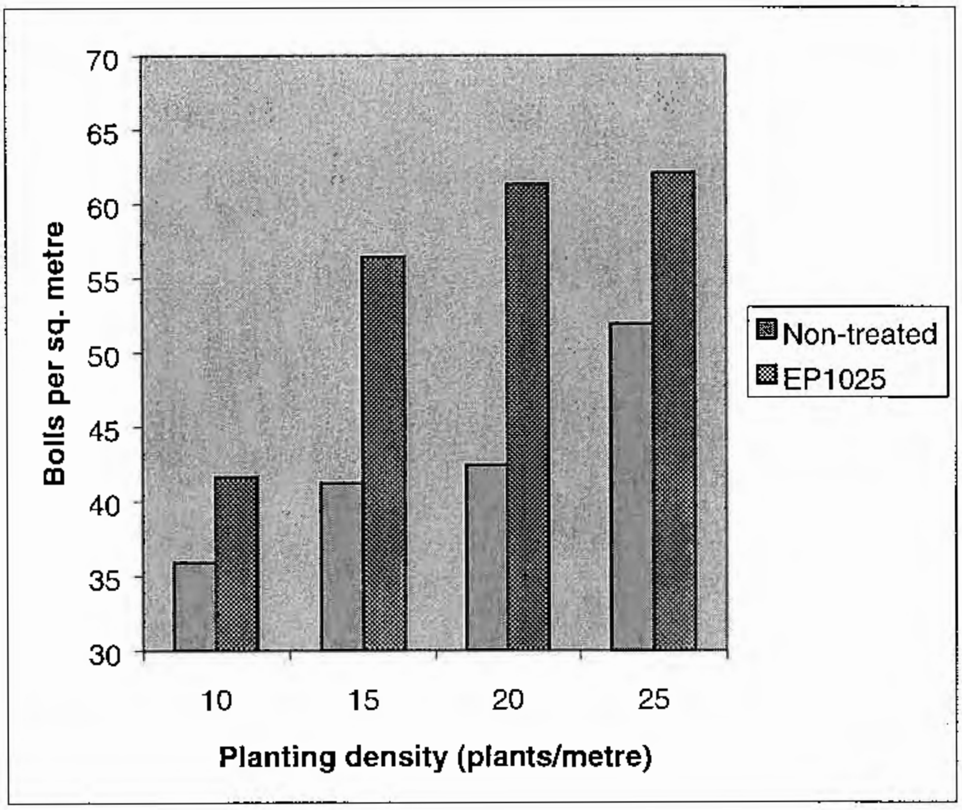

Figure 9. Effect of planting density on number of bolls per metre.

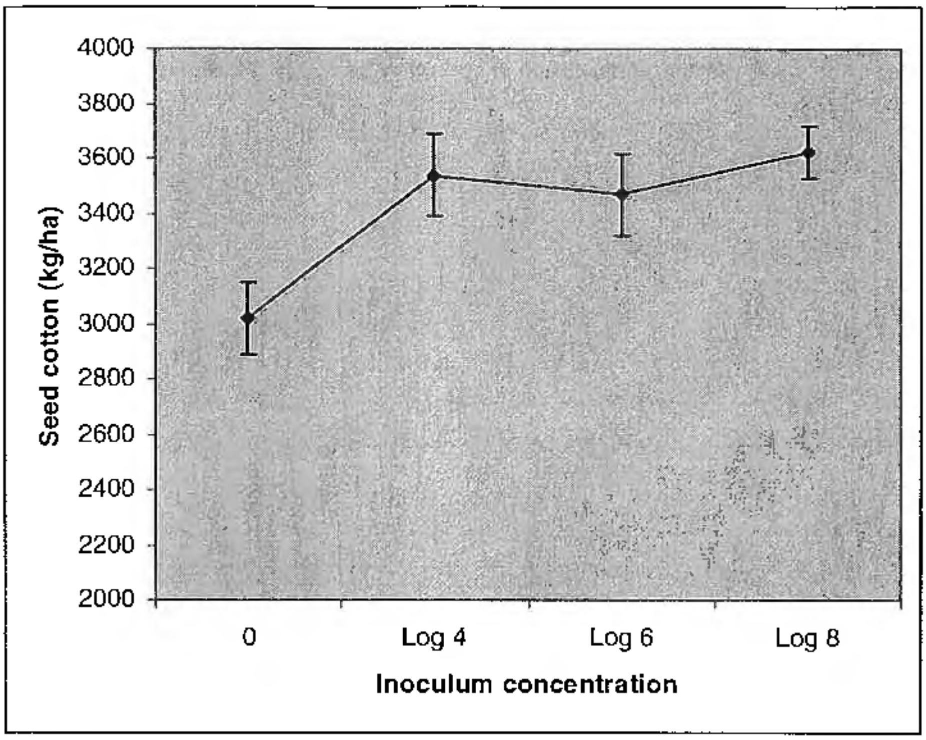

Figure 10. Effect of the concentration of EP1025 inoculum on seed cotton yield in a Fusarium infested field.

Appendix 1

A NEW TECHNIQUE FOR SCREENING BACTERIAL INOCULANTS IN THE FIELD

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Summary In this paper we describe a new method for screening large numbers of biocontrol agents in the field. The method consists of two main features, first, an inoculum applicator fitted to a tractor driven planter and second, a two-stage field experiment strategy which allows both screening and detailed studies to be carried out in the same season. Using this technique we have selected biocontrol agents that have provided similar control of seedling diseases as the industry standard fungicides.

Introduction

"Large-scale screening for new biological control agents is not a popular pastime for most researchers" (Kloepper, 1993). Potential biocontrol agents are often selected on the basis of *in vitro* inhibition of the pathogen, followed by growth chamber or glasshouse experiments, in the presence of host plant and target pathogen, which simulate field conditions. While this approach enables a researcher to select a manageable number of bacterial strains for further studies from a large culture collection, it has inherent limitations. Results based on microcosms and simulations may have little relevance to the complex ecological relationships which occur in the field (Schroth and Becker, 1990). Screening the isolates directly in the field, however, is labour intensive and time consuming due to (1) access to large enough field sites with uniform diseases levels, (2) equipment and/or methods to deliver large numbers of inocula in field plots rapidly and without cross contamination at planting. In this paper, we report a method for screening large numbers of biocontrol agents in field.

Materials and methods

The experiments were conducted at the Australian Cotton Research Institute, Narrabri, NSW, Australia (30° S 150° E).

In the first experiment, 100 bacterial strains were tested for their ability to control cotton seedling diseases. The field plots (9.8m long x 1 row) were planted using a 4-row mechanical cone seeder. The bacterial strains were applied as liquid inocula (diluted broth cultures, containing approximately log7 cfu/ml) using the inoculum applicator shown in Figure 1.

The 100 bacterial strains were divided into 25 groups (with 4 randomly chosen strains in each group). These 25 groups were then planted in a randomised complete block design with six replicates. Within each group, the inocula were randomly assigned to a single cone on the four-row planter. This set was then planted in all the six replicates before planting the next set of inocula. Between sets of inocula, the inoculum applicator was decontaminated using 70% alcohol followed by water.

Seedling disease control was assessed (%surviving seedlings) two weeks after planting. Bacterial strains which increased seedling survival compared to the non-treated control were selected for further testing in Experiment 2. This experiment was also planted as a randomised complete block with six replicates. Larger plots (9.8m x 4 rows) were used in this experiment. Since each bacterial treatment was applied in all four rows, unlike in Experiment 1, the treatments did not have to be grouped.

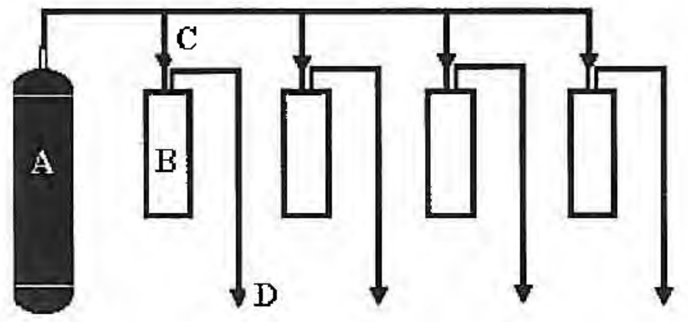

Figure 1. Schematic diagram of the inoculum applicator. (A) Compressed air cylinder (B) bottle filled with liquid inoculum of the bacterial strain, (C) air inlet into the bottle (D) inoculum outlet positioned near the seeding shoe of the planter.

In Experiment 2, seedling disease control, colonisation of cotton spermosphere and rhizosphere by inoculum bacteria and plant growth were assessed periodically. At the end of the season, plots were harvested mechanically to assess the effect of seed inoculation with bacterial strains on seed cotton yield and lint yield. However, only data on seedling disease control is presented in this paper.

This method enabled screening of a large number of bacteria and the selection of the best strains in the one season.

Results

In Experiment 1, fourteen bacterial strains reduced damping-off. All the bacterial strains were as effective, or more effective than, the fungicide (Figure 2A). In Experiment 2, only eleven of these bacterial strains reduced seedling damping-off significantly compared to the non-treated control (Fignre 2B). Further, only one bacterial strain, BCA 18, was as effective as the fungicide.

Discussion

Screening procedures for identifying potential biocontrol agents normally comprise *of in vitro* inhibition test of the target pathogen, and glasshouse or growth chamber assays for disease control on the host plant. Because of some inherent shortcomings in the screening procedures, some biocontrol agents may escape detection, or selected strains may subsequently fail in field tests. For this reason, screening of strains in the field is more desirable.

Variable disease pressure in a field can be a problem in conducting field screenings. Compact field sites are more likely to offer uniform disease pressure. For this reason, Single-row plots were used in Experiment 1.

However, field screening is labour intensive and time consuming. Hand planting is slow and tedious. When a mechanical planter is used, the equipment needs to be de-contaminated between plots. This frequent de-contamination slows down the planting operation enormously. The frequency of de-contamination can be reduced by planting the treatments sequentially rather than planting field plots sequentially. In other words, a given treatment is planted in all the replicates, the equipment is decontaminated and then next treatment is planted.

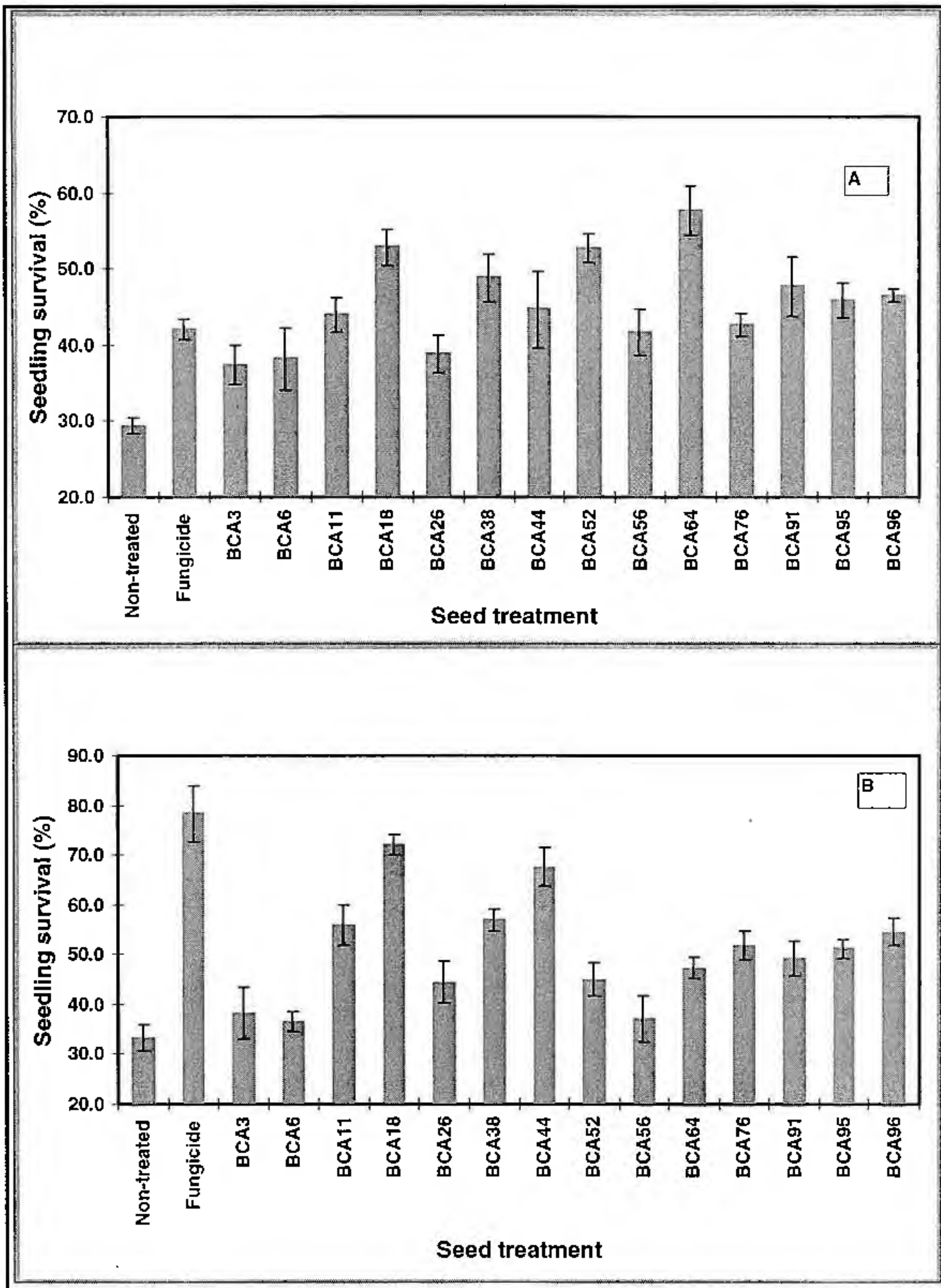

Figure 2. Seedling survival, expressed as percentage of the seed planted. (A) Experiment 1 and (B) Experiment 2. Error bars represent standard error of mean.

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We used a four-row planter to plant both the experiments. To be able to screen large number of strains, in Experiment 1 we used single-row plots (9.8m long) instead of four-row plots. This meant that for each run of the planter, four plots, each receiving different bacterial treatments were planted. For this reason, the treatments were divided into groups each containing four bacterial treatments.

For the purpose of screening, bacterial treatments are generally applied by soaking the planting seed in broth cultures, coating the seed with peat inocula or by applying liquid suspensions of bacteria on the seed before they are covered with soil. Liquid suspensions are easier to use in screening trials, if appropriate application equipment is available.

As the cotton hypocotyl emerges, it is susceptible to attack by damping-off pathogens. Therefore, the biocontrol agents have to colonise the emerging hypocotyl to protect it from the pathogens. When a bacterial inoculum is applied as a seed coat, initially it is resident only on the seed surface. Its subsequent spread on the hypocotyl is limited to small distances. The equipment used in this study has allowed the liquid inoculum to be distributed on to the seed and into the soil surrounding the seed including the covering soil.

Many factors (eg. temperatures) cannot be manipulated in a field screening trial, unlike in a glasshouse trial. Strains that failed in a given field screening may perform well in a different field screen conducted under different conditions, or soil with different properties. None the less, this indicates a potential lack of consistency which may prevent the use of the strain commercially.

The screening strategies presented in this paper have many benefits over traditional approaches and provide a faster and effective method for identifying biocontrol agents.

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