Acta Biotechnol. 15 (1995) 1, 27–39 Akademie Verlag

# Colonisation of Seedling Roots by 2,4-D Degrading Bacteria: A Plant-Microbial Model

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## Summary

The development of model plant-microbial associations between Gram negative soil microbes capable of degrading phenoxyacetate herbicides, such as 2,4-D and 2,4-D methyl ester, and the crops canola and wheat was described. Both an *Acinetobacter baumannii* pJP4 transconjugant and *Alcaligenes eutrophus* JMP134 colonised non-parasitically on the roots of sterilised seedlings in a hydroponic system. Laser scanning confocal microscopy has shown that colonisation occurred both on the root surface and deeper inside the mucilage layer or inside some surface root cells. When 2,4-D was added to the hydroponic medium supporting the growth of those seedlings colonised by 2,4-D degrading bacteria, the gas chromatographic analysis showed a rapid decrease in the concentration of this herbicide. These bacteria colonising the root system were shown to be responsible for the degradation of 2,4-D. Plants inoculated with the 2,4-D degrading microbes were subsequently found to be less susceptible to damage by the herbicide in such hydroponic systems.

## Introduction

The persistence of agricultural and industrial chemicals in the environment has undesirable consequences, both economic and social. The problems include the contamination of ground and surface water, unwanted phytotoxicity by herbicides to crops and unacceptable residue levels in produce such as meat and grains. Herbicides such as atrazine are widely used for selective pre-emergent and post-emergent weed control in Australia in crops such as maize and sorghum. Due to its relative persistence, there is concern that sensitive crops such as sunflower may be damaged where rotations are becoming more frequent or where recycled water is used for irrigation [1]. Although the application of microbes theoretically offers the prospect of the selective degradation of such pollutants, to date there are only a few examples of successful bacterial inoculations of soil to degrade contaminants or to act as plant bio-protectors. NAGY *et al.* [2] describe the use of a thiocarbamate degrading strain of *Rhodococcus* to protect maize against residual EPTC in the soil.

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In part, the lack of data on successful biological remediation results from the fact that soils act as biological buffers resisting attempts to induce changes in native microbial populations [3]. Likely reasons for this resistance to change include competition for available nutrients between inoculated and native microbes.
Nonetheless, changes in the microbial populations in soil ecosystems can be achieved where microbial growth occurs in association with plants, an example being the growth of nitrogen-fixing *Rhizobium* in leguminous root nodules. Also, chemically induced nodular structures (*para*-nodules) on wheat roots colonised by diazotrophic bacteria have been formed under controlled laboratory conditions [4]. This indicated that forced associations between plants and microbes are possible, with potential benefits to both the growth of the plant and the inoculated microbe.
In this paper it is demonstrated that a bacterial isolate capable of degrading 2,4-D is not able to specifically colonise the induced nodular structures of either canola or wheat. However, extensive colonisation at the surface of the root indicates that a plant-microbial rhizoplane association capable of 2,4-D degradation might be possible.

## Materials and Methods

### *Isolation of 2,4-D Degrading Bacteria*

An enrichment culture technique was used to isolate the 2,4-D degrading microorganisms. Soil from a mixed cropping farm in northern New South Wales, Australia, was treated three times at fortnightly intervals with 2,4-D to a level of 1,000 μg/g of soil. A 2 g sample of soil was then used as an inoculum for a basal minimal nutrient (BMN) broth containing 1.6 g/l $K_2HPO_4$, 0.4 g/l $KH_2PO_4$, 0.2g/l $MgSO_4 \times 7\ H_2O$, 0.1g/l NaCl, 0.025g/l $CaCl_2$, 0.5g/l $NH_4NO_3$ (pH 6.8 – 7.0) and 2,4 D sodium salt at 1,000 μg/ml as a carbon source. This was repeated three times at fortnightly intervals with each re-inoculation using a small volume of the previous broth. The final screening method involved the use of an eosin/methylene blue acid indicating agar [5], from which single colonies that may degrade 2,4-D could be isolated.

### *Transfer of the Plasmid pJP134 by Conjugation*

A commercially available strain of *Alcaligenes eutrophus* JMP134 encoding the genes for 2,4-D degradation was grown together on a nutrient agar plate (DIFCO$^R$) with the strain of *Acinetobacter baumannii* isolated from the soil. The agar was supplemented with 200 μg/ml 2,4-D sodium salt. After one week, the mixed culture was streaked onto a BMN plate containing 2,4-D sodium salt at 200 μg/ml as a carbon source and spectinomycin at 100 μg/ml as a selective antibiotic. The *Alcaligenes* strain was susceptible to the spectinomycin while the *Acinetobacter* was tolerant to 100 μg/ml of the antibiotic. The pJP4 plasmid encodes the genes for the 2,4-D degradation [6] as well as conferring resistance to $HgCl_2$ [7, 8]. In order to isolate the transconjugant, plates of nutrient agar containing 100 μg/ml spectinomycin and 10 μg/ml $HgCl_2$ were used.

### *Growth of Plants*

The seeds were surface sterilised with 0.5% mercuric chloride in water under vacuum. Wheat (*Triticum aestivum*, cv. MISKLE) was exposed for three minutes and canola (*Brassica napus*, cv. EUREKA) for 1.5 minutes. The seeds were then washed several times in sterile water prior to germination on nutrient agar plates in an incubator set at 30 °C. Small seedlings, free from visible contamination, were transferred aseptically to a 30 ml test-tube containing 15 ml of sterile nutrient solution (0.25 g/l $MgSO_4$, 0.2 g/l $CaCl_2$, 0.2 mg/l NaCl, 0.7 mg/l $FeCl_3$, 0.2 g/l $KNO_3$, 0.23 g/l $NH_4Cl$,

0.5 mg/l $ZnSO_4$, 5 µg/l $Na_2MoO_4 \times 2\ H_2O$, 25 mg/l $H_3BO_3$, 0.5 mg/l $MnSO_4$, 0.2 mg/l $CuSO_4$, 0.1 µg/l $CoCl_2$). A strip of filter paper was inserted into the tube before sterilisation to allow the suspension of the seedling just above the level of the nutrient solution with the roots immersed in the solution. The test-tube was then covered with a slightly larger sterile inverted tube to keep the system free from contaminants [9].

All handling of plants was carried out in a laminar flow cabinet. Both canola and wheat were grown in a growth cabinet set at 21 °C. Plant treatments involving the formation of nodular structures in an attempt to improve colonisation as with the wheat *Azospirillum* association, had the nutrient solution brought to 0.1 µg/ml 2,4-D when the plant shoots were 5 cm high.

Plant treatments requiring bacterial associations were inoculated with a few drops of a bacterial suspension in water one week later (app. $10^6$ organisms). The bacterial suspension was prepared by removing bacterial cells actively growing on agar plates containing 10 µg/ml $HgCl_2$ for the *Acinetobacter baumannii* pJP4 transconjugant and 40 µg/ml $HgCl_2$ for the *Alcaligenes eutrophus* and by mixing with 10 ml of sterilised water.

### *Counting Bacterial Numbers Associated with the Root System*

Simple bacterial counts of *Acinetobacter baumannii* colonising on the roots of wheat were conducted. These involved a gentle grinding of the root system with 10 ml of sterile water prior to several serial dilutions. The dilutions were then plated onto nutrient agar to count bacterial colonies and visualise any contamination.

### *Assaying the Degradation of the Herbicide in the Plant System*

After two weeks of incubation, the nutrient solution was removed, and the root system washed several times with sterile water. A freshly sterilised nutrient solution (15 ml) containing 1 µg/ml of 2,4-D was added. The washed plants were also transferred into tubes of freshly sterilised nutrient solution. This was done to ensure that the degradation of the herbicide was performed by the root-associated bacteria and not by the bacteria remaining on the surface of the glass tubes. The herbicide concentration in the nutrient solution was measured initially and at intervals up to 72 h later. Triplicate replications for each treatment and sampling time were done. Sampling involved the combination of the nutrient solutions of 2–4 plants for pesticide analysis. The standard deviations in the results are from the averages of the analyses in triplicate.

### *Extraction and Clean-up of 2,4-D and 2,4-Dichlorophenol Samples*

The extraction of 2,4-D was achieved using a MIXXOR$^R$ liquid/liquid extractor [10, 11]. If a clean-up of the sample was required, the volume of the extract was reduced to less than 2 ml in a rotary evaporator, and the sample was loaded onto a neutral alumina column (5% water w/w); three grams of alumina below one gram of anhydrous sodium sulphate. Hexane (10 ml) was passed through the column and discarded. The 2,4-D and 2,4-dichlorophenol (2,4-DCP) were then eluted with 30 ml of 0.5% potassium hydroxide in methanol.

### *Derivatisation of 2,4-D*

The methylation of the carboxylic acid group of 2,4-D was achieved by refluxing with the $BF_3$/methanol complex [10]. The presence of bacterial cells in the aqueous sample did not affect the extraction efficiency of 2,4-D. A recovery of 92% ± 2% was achieved for 2,4-D. 2,4-DCP was not affected by the methylation procedure, and a recovery of 85% ± 4% was achieved. The limit of the detection of 2,4-D in the aqueous phase using the reported extraction technique was 0.01 µg/ml and for 2,4-DCP was 0.02 µg/ml.

*Gas Chromatographic Analysis*

A HEWLETT PACKARD 5890 Series II gas chromatograph equipped with a $^{63}Ni$ electron capture detector was used to analyse the 2,4-D methyl ester and 2,4-DCP. A splitless injection by the autosampler of 1 µl of sample in hexane followed by an injection port purge at 1 min was used. A J & W SCIENTIFIC (DURABOND$^{R}$) DB17 fused silica capillary column, 30 m by 0.32 mm internal diameter, 0.25 µm film thickness, was fitted and a temperature programme (50 °C – 260 °C) was used. Helium was used as the carrier gas at a column head pressure of 24 kPa giving a linear carrier flow of 40 cm/s at mid-run temperature, with nitrogen as the detector make-up gas (25 ml/min). The injector temperature was 240 °C, and the detector temperature was 300 °C. 2,4-DCP eluted at around seven min and 2,4-D methyl ester at around 14 min with a total chromatographic analysis of 30 min. The chromatograms were recorded and integrated using a HEWLETT PACKARD computer and CHEMSTATION software. The identity of the analysis components was confirmed with a HEWLETT PACKARD model 5971 mass spectrometer interfaced with the gas chromatograph, using similar chromatographic conditions. The Hewlett Packard NBS54K.L. mass spectral software library was used for the spectrum analysis and the identification of the sample components.

*Fluroescent and Laser Scanning Confocal Microscopy*

One-cm lengths of the plant roots were stained in a solution of 0.1% acridine orange for 10 min. The root segments were washed five times with sterile water. The lengths of the root were then placed in a well of a microscope slide and covered with water and a coverslip. The sample was viewed with the fluorescent microscope to locate objects of interest before viewing the image on a computer screen provided by a BIORAD MRC 600 laser scanning confocal microscope. The routine observation of the bacterial colonisation on the root surface was carried out using an OLYMPUS BHA microscope with a UV light source. The staining of the plant samples with a fluorescent stain was similar to the method already described for confocal microscopy.

## Results and Discussion

*Isolation of Acinetobacter baumannii*

This microbe was isolated from a rich farming soil as described in the methods using several enrichments with 2,4-D. The bacterium was identified by the fatty acid analysis using gas chromatography at the Biological and Chemical Research Institute, NSW Agriculture. A HEWLETT PACKARD 5898A Microbial Identification System was used in conjunction with the TSBA fatty acid software library. A similarity index of 0.76 was obtained. Similarity indexes of 0.6 – 1.0 are considered to be excellent microbial fatty acid matches for bacterial species [12].

The bacterial strain isolated was a GRAM negative rod, with properties of rapid multiplication on nutrient agar and in nutrient broth. The culture was found to be resistant to tetracycline, ampicillin and spectinomycin, and sensitive to kanomycin and streptomycin. It readily utilised glucose, lactose, mannitol, sucrose and xylose as carbon sources. However, after several transfers on nutrient agar in the absence of 2,4-D, the microbe lost its 2,4-D degrading capability.

As a result of the good colonisation of the roots of seedlings found with the *Acinetobacter* isolate, it was decided to transfer the plasmid pJP4 to the isolate by conjugation with *Alcaligenes eutrophus* JMP134. Plates of nutrient agar containing 100 µg/ml spectinomycin and 10 µg/ml $HgCl_2$ had little growth of either *Alcaligenes* or the

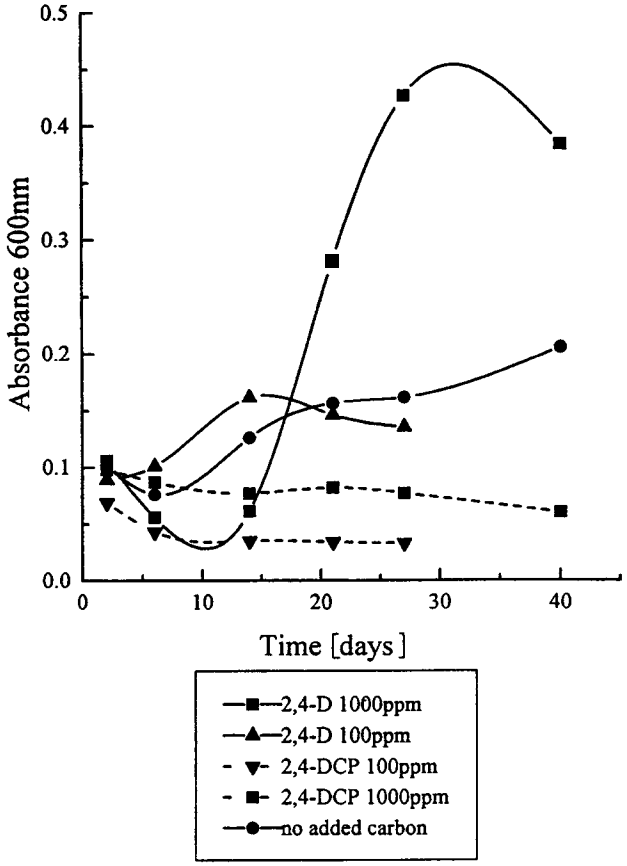


Fig. 1. Growth of the *Acinetobacter baumanii* pJP4 transconjugant on 2,4-D and 2,4-dichlorophenol as the souces of carbon substrate

*Acinetobacter baumannii* parent strain, while the *Acinetobacter baumannii* pJP4 tansconjugant showed rapid growth. The *Acinetobacter baumannii* transconjugant was then maintained on medium with 10 μg/ml $HgCl_2$ to favour the retention of cells containing the plasmid. The *Acinetobacter baumannii* pJP4 transconjugant was used in all subsequent studies.

Fig. 1 shows the transconjugant organism grew in a BMN broth with 1,000 μg/ml 2,4-D sodium salt as a sole source of carbon. Concentrations of 100 μg/ml 2,4-dichlorophenol were toxic to the transconjugant bacteria with no cell growth.

The degradation of 1 μg/ml of 2,4-D and 1 μg/ml 2,4-DCP in an enrichment medium (EM) [13] was monitored for up to 72 h (see Fig. 2). The broth was inoculated with one loop of bacteria from the selective agar plate containing spectinomycin and $HgCl_2$. A complete removal of 2,4-D was seen within 42 h after the inoculation with the bacteria. The degradation of 2,4-D appeared to be more rapid in the absence of glucose (Fig. 2), indicating that the transconjugant strain grew preferentially on glucose. No degradation or loss of 2,4-D from the system was seen in the control without bacteria or with the parent strain of *Acinetobacter*. The transconjugant removed 52% of the 2,4-DCP within

18 h with a complete removal within 48 h. The broth inoculated with the parent strain had a 20% loss of 2,4-DCP after 72 h. The slight loss may have been due to the volatilisation or binding of phenol to the glass.
The parent strain of *Acinetobacter* was capable of rapidly demethylating 2,4-D methyl ester, producing 2,4-D, and apparently using the methyl group as a carbon source for growth. The culture grew well in the BMN nutrient broth where the sole source of carbon was 1,000 μg/ml 2,4-D methyl ester. The broth contained no 2,4-D methyl ester after five days; however, 2,4-D remained at a concentration near 1,000 μg/ml in the same broth. A concentration of 10 μg/ml of 2,4-DCP was found to be toxic to the parent strain of *Acinetobacter*, with no growth occurring when glucose was supplied as a carbon source. This level of 2,4-DCP was not toxic to the *Acinetobacter baumannii* pJP4 transconjugant.

*Degradation of 2,4-D in the Plant Growth System*

When seedlings colonised by the transconjugant of *Acinetobacter baumannii* and also with *Alcaligenes eutrophus* were examined for their capacity to degrade 2,4-D, a complete breakdown of the herbicide was observed as shown in Tabs. 1 – 4.
The total amount of 2,4-D applied to the nutrient solution of each plant in all of the experiments was 15 μg. Treatments of the seedlings without associated bacteria (controls)

Tab. 1. 2,4-D degradation by *Alcaligenes eutrophus* JMP134 associated with wheat seedlings

| | 2,4-D concentration shown as μg/ml remaining in the plant nutrient solution | | | |
|---|---|---|---|---|
| Treatment | 0 h | 10 h | 24 h | 48 h |
| Control plants | 0.94 ± 0.04 | 0.82 ± 0.13 | 0.82 ± 0.23 | 0.81 ± 0.03 |
| Plants with associated bacteria | 0.73 | 0.34 ± 0.23 | 0.02 ± 0.01 | 0.01 ± 0.01 |
| Plants with associated bacteria and 2,4-D pre-treatment | 0.84 | 0.20 ± 0.14 | 0.01 ± 0.02 | 0.01 |

had little if any 2,4-D degrading capacity was revealed over the period when the herbicide concentration was assayed. All the treatments with associated bacteria were shown to degrade nearly 100% of the applied 2,4-D within 12 h. *Alcaligenes eutrophus* associated with canola seedlings were responsible for a removal of 54% of the applied 2,4-D within 10 h. A pre-treatment of the seedlings with 2,4-D appeared to increase the rate of the degradation of the herbicide, with 75% of the 2,4-D being degraded within the same 10 h period. The results shown for wheat with associated *Alcaligenes* are the averages of two experiments conducted at separate times. Therefore each result is the average of six 2,4-D analyses for that particular sampling time. These data provide conclusive evidence that there is a significant difference between plants with associated bacteria and those without.

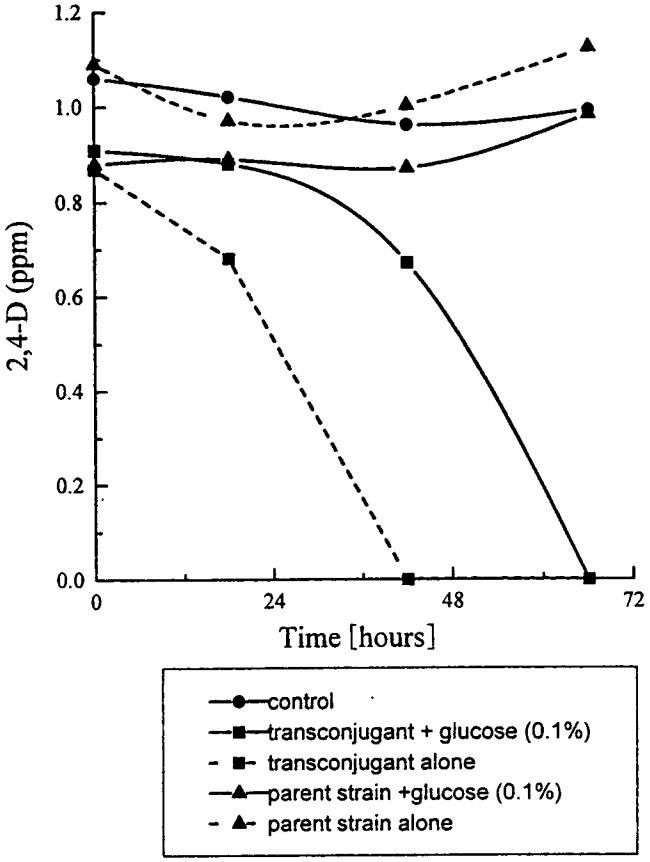


Fig. 2. Degradation of 2,4-D by the *Acinetobacter baumanii* PJP4 transconjugant and its parent strain

Tab. 2. 2,4-D degradation by *Alcaligenes eutrophus* JMP134 associated with wheat seedlings

| | 2,4-D concentration shown as µg/ml remaining in the plant nutrient solution | | |
|---|---|---|---|
| Treatment | 0 h | 24 h | 48 h |
| Control plants | 0.70 ± 0.13 | 0.79 ± 0.19 | 0.76 ± 0.15 |
| Plants with associated bacteria | 0.78 ± 0.19 | 0.01 ± 0.02 | 0.01 ± 0.02 |

Averages and standard deviations ($\pm \partial^{n-1}$) are shown for two experiments each in triplicate.

Tab. 3. 2,4-D degradation by the *Acinetobacter baumannii* pJP4 transconjugant associated with wheat seedlings

| | 2,4-D concentration shown as μg/ml remaining in the plant nutrient solution | | | |
|---|---|---|---|---|
| Treatment | 0 h | 12 h | 24 h | 48 h |
| Control plants | 1.00 | 1.07 ± 0.01 | 0.83 ± 0.05 | 0.89 ± 0.03 |
| Plants with asociated bacteria | 1.00 | 0.01 ± 0.01 | 0.00 ± 0.01 | 0.00 ± 0.01 |

Standard deviations ( $\pm \partial^{n-1}$) are shown, calculated for triplicate analysis.

Tab. 4. 2,4-D degradation by the *Acinetobacter baumannii* pJP4 transconjugant associated with canola seedlings

| | 2,4-D concentration shown as μg/ml remaining in the plant nutrient solution | | |
|---|---|---|---|
| Treatment | 0 h | 24 h | 72 h |
| Control plants | 0.68 | 0.76 ± 0.03 | 0.71 ± 0.02 |
| Plants with asociated bacteria | 0.70 | 0.01 ± 0.02 | 0.01 ± 0.02 |

Standard deviations ( $\pm \partial^{n-1}$) are shown, calculated for triplicate analysis.

*The Plant Microbial Association and the Effect of the 2,4-D Pre-treatment on the Numbers of Colonising Bacteria*

The aim of the pre-treatment of the seedlings with 0.1 μg/ml of 2,4-D at the time of inoculation with bacteria was to attempt to increase the amount of bacteria colonising on the roots system of the seedlings. The formation of the nodular structures [4, 14] was a result of the pre-treatment, however, it cannot be concluded that these nodular structures were directly responsible for any increased colonisation seen with the *Acinetobacter* or *Alcaligenes* strains of bacteria used. From the results shown in Tab. 5 it is evident that an initial treatment of the seedlings with 0.1 μg/ml of 2,4-D appears to have enhanced the colonisation of the wheat seedling roots by the *Acinetobacter baumannii* transconjugant, a phenomenon also encountered in the *Azospirillum brasilense* and wheat association [14]. Almost 20 times greater numbers of colonising bacteria on pre-treated roots were observed. More analyses of numbers colonising the root system are required before it can be concluded that the pre-treatment with 2,4-D had a positive effect on the colonisation. Control plants (without bacterial inoculation) had no inoculated bacteria or obvious contaminants on the root surface. The formation of the nodular structures may not have directly assisted the numbers of bacteria colonising. However, the presence of a low concentration of 2,4-D may have provided some other stimulus for the bacterial colonisation, perhaps by damaging the root system and allowing a greater flow of nutrients from the roots to the rhizoplane.

Tab. 5. Viable *Acinetobacter baumannii* on wheat roots

| Treatment | Viable bacterial cells per root system | Average |
|---|---|---|
| Control plants | 0, 0, 0, 0 | 0 |
| Plants inoculated with *Acinetobacter baumannii* | $1.7 \times 10^5$, $4.0 \times 10^5$ | $2.9 \times 10^5$ |
| Plants pre-treated with 2,4 D and inoculated with *Acinetobacter baumanii* | $4.2 \times 10^6$, $6.8 \times 10^6$ | $5.5 \times 10^6$ |

*Acinetobacter baumannii* was able to colonise the root surface and possibly within some surface root cells of both canola and wheat. The observation of numerous root samples by fluorescent microscopy has shown that the majority of colonisation was either on the surface of the root or possibly inside the surface root cells. Using the laser scanning co-focal microscope, detailed three-dimensional observations of the colonisation of the bacteria on and within the root could be obtained, without the need to make thin sections of the sample. Plate 1 is a computer created image of the bacteria colonising the root surface of canola. The object was then scanned at different vertical planes. Plate 2 is the same section of the root scanned 5 µm deeper in the root cell. It appears from this that the bacteria on this section of the root are within the surface root cells. Control plants without bacterial inoculation showed no colonisation on the root system, allowing us to conclude that the bacteria seen by the confocal microscope were *Acinetobacter baumannii*.

*Alcaligenes eutrophus* also colonised the root systems of canola and wheat. However, the colonisation by these bacteria was less extensive than with *Acinetobacter baumannii*. Bacterial counts were not conducted. Despite the smaller numbers of this organism, there were still sufficient present to effectively degrade 2,4-D, as shown in Tabs. 1 and 2.

*Physical Observations of Canola Protected against 2,4-D by Associated Alcaligenes*

Tab. 6 shows the comparison between canola plants treated with *Alcaligenes* and those without and the effect of 2,4-D in the nutrient solution. From the results, it can be seen that the treatment of canola with 0.1 µg/ml of 2,4-D was damaging to the plants, with only 29% of the plants surviving after two weeks. A low dry weight of the plants from this treatment was also obtained. Treatments with inoculated *Alcaligenes eutrophus* had a considerably higher rate of survival with 68% of plants surviving. Some plant phytotoxicity was still found, indicating that the extent of the colonisation with the bacteria may not have been regarded as successful for all the replicates. The high standard deviation of dry weights also supports this.

*General Discussion*

The use of both fluorescent strains and the laser scanning confocal microscope showed significant colonisation by bacteria on the seedling roots. Although bacterial numbers

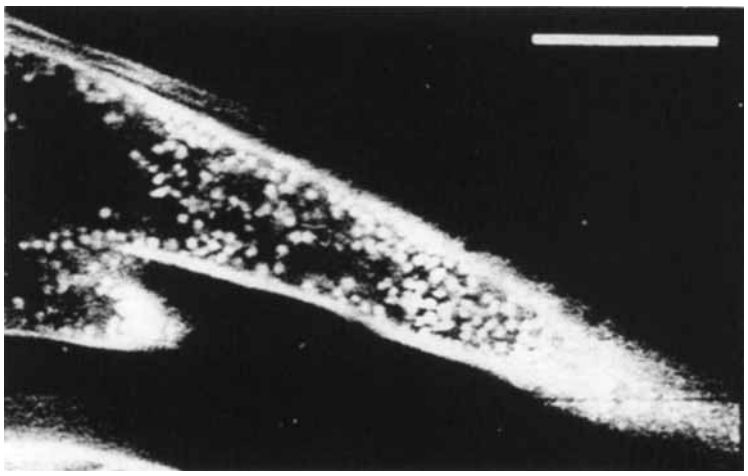

Plate 1. Confocal micrograph of canola root cells colonised by *Acinetobacter baumanii* Scale bar = 25 microns

could not be accurately determined using microscopy, their presence and distribution was determined. One of the advantages of having a rhizosphere association lies in the fact that plants have the ability to probe the soil matrix in search of nutrients and water, and in so doing, may help distribute the bacteria in the soil. These studies with a single bacterial population are a preliminary stage in testing such systems in soil where bacteria are subject to competition. Combining materials and surfaces in soils, the impact of predation and parasitism are some of the factors that may limit the extrapolation of such pesticide-degrading metabolic activity in axenic cultures to the soil [15]. The use of the plant as a vector for these cultures may, however, overcome some of these problems associated with more complex soil systems.

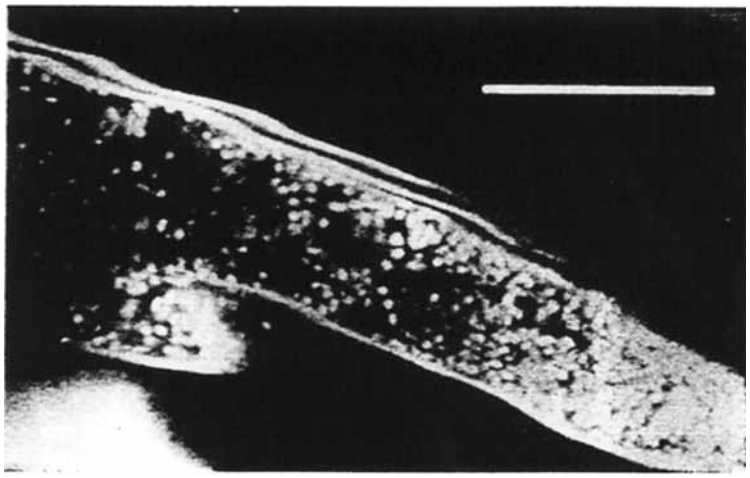

Plate 2. Confocal micrograph of canola root cells colonised by *Acinetobacter baumanii* Scale bar = 30 microns

Tab. 6. Physical effects of the 2,4-D treatment on canola seedlings demonstrating the bio-protecting effect of *Alcaligenes eutrophus* JMP134

| Treatment | Plant replicates | Surviving plants [%] | Average dry weight of seedlings [mg ± sd.] |
|---|---|---|---|
| Control plants: No addition of 2,4-D | 25 | 100% | 10.4 ± 1.4 |
| Control plants with associated *A. eutrophus*: No addition of 2,4-D | 23 | 100% | 10.8 ± 1.2 |
| Treatment: 0.1 µg/ml 2,4-D applied to plants without *A. eutrophus* | 24 | 29% | 5.3 ± 2.4 |
| Treatment: 0.1 µg/ml 2,4-D applied to plants with associated *A. eutrophus* | 25 | 68% | 8.8 ± 4.0 |

A demonstration of the plant-microbial association also performs as a plant bio-protector for the canola seedlings has been shown in this study. Experiments to demonstrate similar effects in the more complex environment of the soil are yet to be conducted. Compounds such as 1,8-naphthalic acid anhydride derivatives have been used for some years as chemical protectors to diminish the phytotoxic effect of residual thiocarbamates. The use of *Rhodococcus* NI86/21 for the degradation of a range of thiocarbamate EPTC, butylate and vernolate as a plant bio-protector in the growth of maize has also been investigated [2, 16]. Little or no data was given for these studies on the mechanisms of the bacterial colonisation of the rhizosphere.

Plant-microbial rhizosphere associations may allow the growth of increased numbers of pesticide degrading microbes in soil. JACOBSEN *et al.* [17] demonstrated the difference in the numbers of an inoculated strain of a 2,4-D degrading *Pseudomonad* applied to two soils, one containing 5 ppm 2,4-D, the other 500 ppm 2,4-D. The soil with 500 ppm 2,4-D had ten thousand times the number of the inoculated microbe than the soil containing 5 ppm after 44 days, indicating the importance of growth substrates. This attempt to amend bacterial numbers in soils with a relatively low level of pollutants, as it is common in agriculture, may be unsuccessful unless there are some factors stimulating the growth of the microbe. Plant-microbial associations, similar to those demonstrated here, may provide this stimulus particularly if there is some specificity between the microbe and the plant. This remains to be tested in future work using soil.

The formation of *para*-nodular structures so critical for nitrogen fixation in the wheat and *Azospirillum* association had little or no influence on the rate of degradation of 2,4-D by the bacteria used in this study. However, the specific colonisation of the *para*-nodular structures of wheat by specific strains of *Azospirillum brasilense* may be a useful tool in the future, if plasmids encoding pollutant-degrading genes can be transferred to these microbes. An example would be the plasmid pJP4 from *Alcaligines eutrophus*

JMP134 or the 77-kb plasmid responsible for the N-dealkylation of triazine herbicides and EPTC degradation found in *Rhodococcus* TE1 [18]. In related work, the pJP4 plasmid was previously transferred into *Rhizobium trifolii* [10], where it was able to provide a bio-protecting effect for subterranean clover and white clover plants exposed to 2,4-D.

## Conclusion

The aim of this research was to obtain a laboratory model of a plant-microbial association demonstrating a potential for use in agriculture to effect soil remediation and pollutant degradation. While the residues of 2,4-D in soil are unlikely to be a problem, we chose 2,4-D as a model compound as microbes capable of degrading it are readily available. The model was capable of degrading 2,4-D and it has been shown that microbes colonising on the root surface were responsible for this. The plant-microbial rhizoplane association was also shown to have a plant bio-protecting effect. Further research is required to determine the stability of the association in the soil, its possible use in high level pollutant situations where bioremediation may be required and to develop other pesticide-degrading capabilities.

## Acknowledgements

The authors wish to thank Dr. Guy COX of the Electron Microscope Unit at the University of Sydney for assistance with the operation of the Confocal Microscope.
This research was supported by a scholarship from the Grains Research and Development Corporation of Australia and by grants from the Rural Industries Research and Development Corporation.

Received 28 April 1994

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