Research article

New Biotechnologies for Sustaining Greener Agriculture

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Abstract Sustainable agriculture must face the challenge of sufficient productivity to feed and clothe up to 10 billion people this century. As a result, strictly organic methods, while environmentally welcome, can be no more than a partial solution because more land is needed than is available. However, recent development in a range of biotechnologies may provide safer environmental protection together with improving yields. For example, plant growth-promoting (PGP) microorganisms in Vietnam have been shown in field studies to offer reduced needs for seed, chemical fertilisers, pesticides, water and harvest yield losses. Interestingly, one PGP inoculant called BioGro was designed using validation of microbial strains from farmers' results; but these very same species are now being characterised in laboratories world-wide as PGP strains. Their laboratory analysis has shown similar traits as observed by farmers, such as extended root structure, nutrient mobilisation, biocontrol for pathogens and insecticidal activity. Despite less nutrient and pesticide needs and thus reduced environmental impacts, agrochemicals will still be needed for sufficient productivity. Research on tools for environmental risk management by farmers such as immunological ELISA analysis can also contribute here. Rapid 5-minute tests for monitoring both food and environment for pesticides can provide better stewardship of such chemicals, helping to ensure a 'greener future'.

Keywords nitrogen fixation, PGPR, safer pesticides, immunogold tests, risk management

INTRODUCTION

In this paper, two recent areas of biotechnology developments now being applied will be described. These are based on original research findings made in our group at the University of Sydney, in Vietnam and in China since around 2000 or earlier, leading to the application of these findings and their launching in a commercial format. More than 10 years has been required for this result, given the need for robust field research and extensive quality control of all outputs.

The two areas that will be described cover plant growth promoting microbes used to achieve nutrient efficient rice production and the development of rapid test kits for measuring pesticides used in crop production. Both of these outcomes are directed towards achieving more sustainable agriculture, by reducing environmental impacts and improving food safety.

MICROBIAL BIOFILMS AND PGPR RESOINSES ON RICE YIELDS

Microbial biofilms play an important role in agriculture and in food and fibre production. Whilst some microbial effects on crop plants are deleterious, it is increasingly recognised that biofilms may have strong beneficial effects in agriculture. Furthermore, realising these potential benefits is likely to play an increasing role in achieving sustainable solutions regarding improved crop yields and in human and environmental health. Such gains may be essential in the face of the increasing global population and climate change.

Plant growth promotion (PGP) for crops by microorganisms has been recognised for some time in biological nitrogen fixation (BNF) in legumes, shown in Germany in the late 19th century in the face of opposition by Justus Von Liebig. More subtle are the beneficial effects of the PGP microbes in cereals and other crops. These involve a complex of phytohormonal effects, nutrient mobilisation, BNF and biocontrol that benefit plant growth and overall yield (Kennedy et al. 2008). Essentially, it is possible to grow crops such as rice inoculated with microbes forming biofilms on the surfaces of their roots that allow significantly reduced input of chemical fertilisers, seed, pesticides and water, as well as reduced harvest losses.

In this paper we describe a successful biofertiliser, BioGro - now a commercial product in Vietnam invented by Professor Nguyen Than Hien; it consists of four microbial strains in peat (*Pseudomonas fluorescens*, *Bacillus* spp. and a soil yeast, *Candida tropicalis*) inoculated to the rhizosphere of rice seedlings (Nguyen et al., 2003; Phan et al., 2009, 2011). Remarkably, BioGro contained unidentified strains selected empirically on the basis of their effectiveness for rice farmers; later, these were identified in Australia to include species now recognised around the world in research laboratories as having a strong genetic basis for beneficial effects as inoculant biofertilisers (Kennedy et al., 2008).

As was the case about a century ago with legumes and the rhizobia that form N_2-fixing nodules on their roots, obtaining an effective outcome from applying these biofilm-forming organisms to crops in the field is challenging. It is important that inoculants of high quality in terms of particular microbial strains in sufficient numbers (ca. 10^7 -10^8 cells per g of inert carrier). Furthermore, convincing farmers of the beneficial effects and the scale of economic benefits possible, limiting their risk, is also important. An efficient supply chain that includes quality control and delivery of economic and environmental benefits from biofertilisers to farmers is also essential.

A strategy for simultaneously achieving environment-friendly and economic benefits developed in a World Bank Development Marketplace project *Sustaining nitrogen-efficient rice production* (DM2008), was developed. Recent unpublished research indicates the role of cyclic lipopeptides and phenazines produced by *Pseudomonas* sp. CMR12a in root colonization, biofilm formation and biological control of soil borne plant pathogens, regulation of insect plant-beneficial pseudomonad and insecticidal activity of plant-associated pseudomonads: how they became insect pathogens, aiding plant salt tolerance by tissue specific regulation of sodium uptake, thus conferring plant growth promotion with yield increases from less fertiliser (Nguyen et al., 2003; Phan et al., 2009, 2011) in the application of such microbes to crops as biofertilisers like BioGro still needs to be determined.

However, the results of a participatory action process with the application of BioGro at village level in the Mekong Delta has led to significant increase in the profitability of rice farming (Fig. 1). Fig. 1 shows plots of yield ratio to profit ratio that farmers using BioGro replacing about 40% of urea application can obtain as greater yields and profits as a result of better tillering, lower N-fertiliser inputs, reduced pesticide inputs, improved water use efficiency and reduced harvest losses as machine harvesting could occur more promptly. The tendency to be in the top sector with ratios greater than 1.0 increased with experience, most of the values falling below 1.0 occurring in the summer-autumn crop of 2009 during the third crop before the annual flood in September when soil condition was acid sulphate and nutrients were low and soil toxicity greatest. Crops on the same plots twelve months later nearly all had ratios exceeding 1.0.

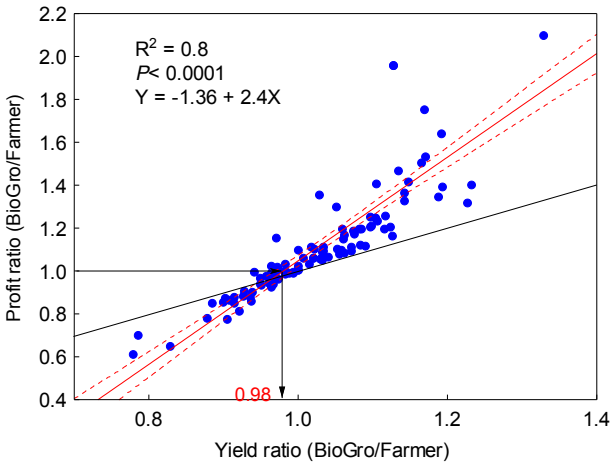


Fig. 1 Yield and profit ratios over four crops comparing farmers using 100 kg of BioGro reducing urea inputs to about 60% compared to normal farmer practice (100 kg urea-N per ha) on individual plots less than 1 ha are shown. T

An imperative here for success of this biotechnology shown in Fig. 1 with BioGro is ensuring the quality control of these inoculants; the correct strain species in the correct numbers must be present in commercial inoculant products. Using techniques similar to those employed to produce immunogold tests for herbicides described in more detail below, we have also generated similar immogold tests for beneficial PGPR microbes such as *Pseudomonas fluorescens*, one of the key components of BioGro (see Fig. 1).

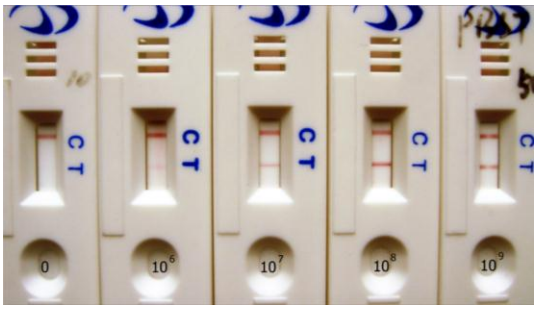


Fig. 2 Rapid immunogold quicktests for 1N (*Pseudomonas fluorescens*), a component of BioGro used in rice production

The test lines (T) show increasing concentrations of 1N (cfu's per mL). These devices are being applied to testing commercial peat cultures as part of their quality control, ensuring that specific strain cell numbers exceed 10^6-10^7 viable cells per g.

QUICK IMMUNOTESTS FOR HERBICIDES

In our research, three polyclonal antibodies against herbicides used in cotton growing, diuron, fluometuron and prometryn for the preparation of rapid lateral flow immunogold tests have recenly been developed. We will provide some details of the preparation of two of these rapid tests, for diuron and prometryn.

1. Diuron

A rapid immunotest for diuron was developed using published techniques. Haptens were synthesised as indicated. The hapten was attached to the protein carriers of keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin(OVA) and horseradish peroxidase (HRP) using the active ester method. The conjugates of hapten 4C-KLH, hapten 6C-KLH, hapten 4C-BSA and hapten 6C-BSA were all used as immunogens.

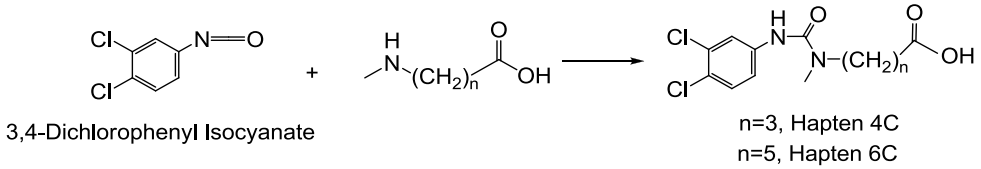


Fig. 3 Preparation of haptens for antibodies reactive with diuron

Sensitivity of the test could be controlled by varying the length of the coupling group used for conjugation to protein for injection into rabbits

A pair of male rabbits was immunized with each immunogen. Test results showed that rabbits immunized with the conjugate of hapten 4C-KLH produced the best specific antibody. To test the suitability of the antibodies, competitive indirect ELISA microtiter plates were coated with 100 µL per well of hapten 4C-OVA (0.1 µg mL^{-1} in 50 mM carbonate buffer, pH 9.6) overnight at 4 ^{o}C. The following day, the plates were washed with PBST and blocked with 200 µL per well of 0.5% milk powder diluted in PBS. 50µL per well of diuron and 50 µL per well of rabbit anti-diuron pAb (1 µg mL^{-1}, diluted in PBS) were added. Plates were shaken gently for one minute before incubated for 1 h at 37 ^{o}C. Then the plates were washed again with PBST, and 100 µL per well of 10000-fold diluted secondary antibody were added and incubated at 37 ^{o}C for 30min. After another washing step, 150 µL per well of substrate were added and incubated for 15 min. The enzyme reaction was stopped with 1.5 M H_2SO_4 and the plates were read at 450 nm (reference 650 nm). Having shown the suitability of the IgG, immnogold strip tests were prepared. Full details of their manufacture will be published elsewhere.

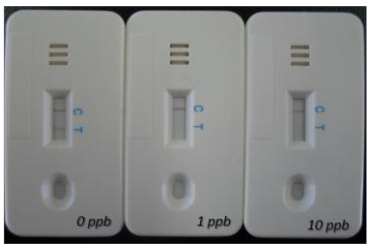
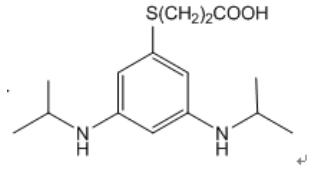


Fig. 4 Diuron Quick Test

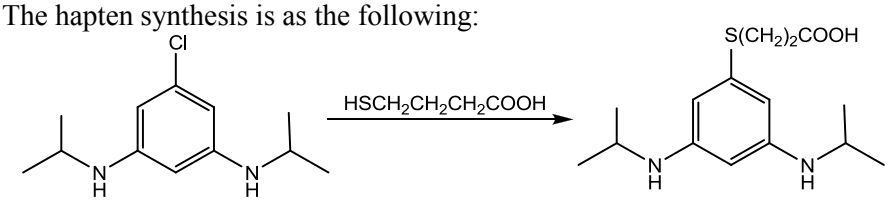
As test water samples contain diuron as a contaminant, the test line (T) disappears, The control line, containing non-specific antibody (C) indicates the antibody test is effective

2. Prometryn

The following is the chemical structure of the hapten for prometryn:



The hapten synthesis is as the following:



The hapten was attached to the protein carriers bovine serum albumin (BSA), using the N-hydroxysuccinimide active ester reaction Hapten-BSA was used as immunogen. The titre of the serum produced was 160,000, which was purified by protein A-sepharose-4B.

The optimization of a direct competitive ELISA for prometryn is ongoing, under the preliminary condition of ELISA, IC50 is 0.4 ppb.

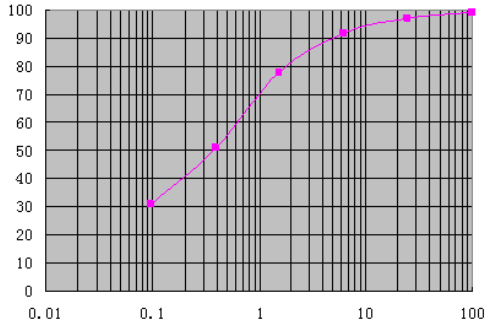


Fig. 5 The standard curve of direct competitive ELISA for prometryn

Immunogold tests for pesticides based on these ELISA results are currently in preparation

CONCLUSION

Farmers need many new tools to assist them to achieve sustainable agricultural practices. In this paper, we have described two areas where biotechnology has allowed advances that both help achieve higher crop yields for rice and also allow farmers to manage environmental risk regarding their application of agrochemicals. We plan to generate a large range of such rapid tests, including some for screening food products for aflatoxin produced by *Aspergillus flavus*. The availability of such rapid tests at a reasonable cost will provide tools supporting best management practices.

Organic production of crops is a worthwhile aim when consumers of farm products can afford to pay the higher costs. Organic production requires at least 50% more land than conventional agriculture so that only a small proportion of organic products can be expected without clearing more forests. With global climate change, this is unacceptable. But feeding the world sustainably will require most food to be grown using all farming tools available, leaving choices of the best production systems to farmers. Government agencies must support farmers to make the best choice and to undertake stewardship. Banning chemical products and farming practices such as land clearing is counterproductive. Far better for farmers to volunteer protection of the environment, using modern biotechnology such as that described in this article.

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